INTRAVASCULAR COAGULATION IN RENAL DISEASE

by

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SUMMARY

The role of intravascular coagulation in the pathogenesis and natural history of many human renal diseases has been studied with the use of advanced techniques in coagulation and fibrinolysis and the examination of renal biopsy material. Whenever possible, opportunity was taken to study patients daily for long periods often far beyond any period of hospitalisation. Information of most value was obtained from the use of Tanned Red Cell Haemagglutination Inhibition Immunoassay (T.R.C.H.I.I.) for the measurement of fibrin/fibrinogen degradation products (F.D.P.) in the serum and urine. Its routine application to urine samples in all long-term studies was particularly profitable.

In conditions such as acute ischaemic renal failure, the haemolytic uraemic syndrome, many forms of proliferative glomerulonephritis, and in rejecting renal homotransplants, coagulation data accurately reflected intra-renal events, which were evaluated by analysis of clinical details and histological, electron microscopical and in some cases immunofluorescent examination of renal biopsy material. Thus in acute ischaemic renal failure a novel hypothesis was proposed to explain the pathogenesis of the oliguria based on the findings, during the phase of oliguria, of significant abnormalities of coagulation and fibrinolysis, and electron microscopic evidence of glomerular coagulation. The

coagulation changes in the haemolytic uraemic syndrome were often more marked than in acute ischaemic renal failure and intrarenal thrombosis was more prominent and widespread. The prognosis in these cases seemed to depend on the degree of damage found in the kidney at the time of initial assessment, and whether the stimulus to coagulation was short-lived, persistent or recurrent. Anticoagulants did not influence the outcome.

The serial measurement of urinary F.D.P. excretion provided a reliable and sensitive index of disease activity, progression and natural history in proliferative forms of glomerulonephritis. Moreover, in certain instances of glomerulonephritis it was of diagnostic value. It also provided a useful monitor of the effects of certain anti-inflammatory drugs on some patients with glomerulonephritis as it was found that these drugs caused an acute reduction in urinary F.D.P. content. Similar observations were made on patients with renal homografts. In this situation elevated urinary F.D.P. concentrations were found in all episodes of clinical rejection. Furthermore, serial studies revealed other elevations which were considered to represent occult spontaneously reversible rejection.

The significance of these findings is discussed.

This Thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and, to the best of my knowledge and belief, the Thesis contains no material previously published or written by another person, except when due reference is made in the text of the Thesis.

Signed:

The work presented in this thesis is considered to advance medical knowledge and practice by:

1. Defining in detail many of the renal diseases in which intravascular coagulation plays a role;

2. Proposing a new approach to study the pathogenesis of acute renal failure;

3. Providing a method for studying the natural history of proliferative glomerulonephritis and renal homotransplant rejection, and

4. Providing a potential means whereby patients with proliferative glomerulonephritis and renal homotransplant rejection may be treated on a sound scientific basis.

During the past 20 years a fundamental alteration has taken place in the approach of clinicians and research workers to the study of renal disease. This has been brought about by the widespread acceptance of new techniques used for the investigation and management of patients with kidney disorders, and the subsequent stimulus given to the already growing subjects of renal physiology and pathology. The advent of dialysis served to concentrate patients with renal failure in confined areas, because the expense and logistics of providing treatment other than in single centres were too daunting to contemplate. For these same reasons attention has been redirected from the end-stages of renal disease to its beginnings and vast amounts of time have been spent in the exacting task of solving the problems of aetiology, pathogenesis and natural history of the diseases which may terminate in chronic renal failure. Despite advances along these lines there still remains much confusion, and it is becoming increasingly clear that as nephrology becomes more contracted as a specialty, significant new advance will not occur unless knowledge obtained from other disciplines, which are undergoing the same growth, can be instilled into the study of renal disease.

Percutaneous renal biopsy is now regarded as a simple and safe technique in the investigation of patients with diffuse renal disease, and its development in 1950 by Perez opened a new era of clinico-pathological correlation hitherto unavailable during life. With its use, definition and classification of many renal diseases has been

possible. Moreover, the application of electron microscopy and immuno-fluorescence microscopy while adding a degree of sophistication to renal pathology has added information of value in the interpretation of pathogenesis and natural history. On the other hand the results obtained using these specialized techniques have posed as many problems as have been answered. The work in this thesis is presented as an integrated study of the problems of two disciplines; that of nephrology and that of coagulation and fibrinolysis. Use has been made of advanced techniques in the study of renal biopsy material, coagulation and fibrinolysis and attempts have been made to determine the physio-logical and pathophysiological role of the coagulation-fibrinolytic process in renal diseases.

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Section I.

HISTORICAL DEVELOPMENT OF THE CONCEPT OF INTRAVASCULAR COAGULATION IN RENAL DISEASE

1.1 INTRAVASCULAR COAGULATION

Intravascular coagulation may be a disseminated or localised phenomenon and affects the structure and function of organs by causing obstruction to the microcirculation. It should be differentiated from deep venous thrombosis and the focal thrombosis which occurs in relation to atheroma in major arteries such as the coronaries, aorta, femorals and cerebrals, in that it simultaneously involves many vessels and the site of coagulation is at the microvascular level.

It is generally assumed, although not proven, that blood is maintained in a fluid state by a continuous and dynamic balance between coagulation and fibrinolysis, that is between the formation and removal of fibrin. Originally proposed by Nolf (1908) and renewed by Copley (1954) this hypothesis was extended by Astrup (1956). In its broadest form, it envisages the deposition of fibrin on to vascular endothelium as a continuous process which is matched by its continuing removal by the fibrinolytic system, in order to maintain vascular patency. Any upset in the delicate balance may lead on the one hand to excessive bleeding and on the other to excessive coagulation. While many arguments have been proposed for and against this theory, it remains attractive, even though Hjort (1966) admits that the coagulation-fibrinolytic equilibrium may be essentially designed for local rather than systemic action. The concept of disseminated or local intravascular coagulation may be regarded as a logical extension of this

equilibrium hypothesis for any shift in the balance towards coagulation may result in excessive fibrin formation with its consequences of fibrinolytic activation and tissue damage.

Intravascular coagulation is easier to describe than to define and involves much more than the simple formation of a thrombus. Even its description, however, is complicated by the many interplaying variables associated with its causation, component parts, manifestations and indeed its recognition. It is a biological process involving many chemical substances and physiological responses. "It begins with the entry of a procoagulant material or activity into the circulating blood; progresses to the stage of platelet aggregation and fibrin formation which may or may not result in thrombosis of capillaries, arterioles and venules of various organs; it is associated with activation of the fibrinolytic system, with dissolution of fibrinogen and fibrin and the release of fibrin split products into the serum, and is not complete until the haemostatic mechanism and vasomotor apparatus have returned to normal ...." (McKay and Müller-Berghaus, 1967).

Attention has recently been drawn to the concept of disseminated intravascular coagulation as an intermediary mechanism of disease (McKay, 1965; Hardaway, 1966). Following the observation of four cases with the clinical features of shock, a bleeding tendency and acute renal failure (Hardaway, McKay and Williams, 1954), interest turned to the pathophysiological mechanisms involved in the production

of this syndrome. Incompatible blood transfusion, septicaemia and pregnancy, the aetiological factors associated with the described cases were singled out for closer study in man and animals. As a result, there has been a gradual realisation that intravascular coagulation may play an integral part in the development of many human diseases. Moreover, a wider understanding of the coagulation-fibrinolytic mechanism in health and disease has been achieved. Of importance in this evolution has been the recognition of the part played by intravascular coagulation in the local and generalized Shwartzman reactions. Classically this reaction is produced in young rabbits by two intravenous injections of gram negative endotoxin spaced 24 hours apart, and leads to rapid death of the animal. After the first (preparing) injection a few fibrin thrombi may be found in the capillaries of the liver, lungs and spleen, but the second ("provoking") injection causes widespread blockage of capillaries, arterioles and venules by thrombi. Ischaemic damage to the adrenals, liver, lungs, bowel, pancreas, brain and pituitary occurs but the kidneys are often most severely affected and bilateral renal cortical necrosis is commonly produced. If the "provoking" injection is given into the skin a similar haemorrhagic necrotic lesion develops which is confined to the area of injection (localized Shwartzman reaction).(Shwartzman, 1931). Various permutations of the classical generalized reaction have been studied which are of interest to the understanding of human diseases in which intravascular coagulation is of pathogenetic

significance. For example, no "preparing" dose is required during pregnancy (Apitz, 1934; McKay, et al, 1960). Furthermore, the "provoking" injection may be replaced by thrombin (Lee, 1962) and antigen-antibody reactions (Lee, 1963). The consumption of coagulation factors observed in human diseases (Rodriguez-Erdmann, 1965) has also been noted, albeit sometimes transitorily, in the generalised Shwartzman reaction. Corrigan et al (1967) found a quantitative reduction in fibrinogen, Factors II, V and VIII, increased prothrombin time and decreased platelet count within the first 24 hours of the "provoking" injection after which there was a progressive return to normal values. In parallel with the coagulation changes micro-angiopathic haemolytic anaemia develops (Brain et al, 1962). This is characterised by excessive haemolysis of abnormal red cells which are distorted and fragmented by fibrin deposits in the micro-vasculature.

The generalised Shwartzman reaction therefore, has provided a convenient experimental tool whereby disseminated intravascular coagulation has been studied. Much of the same sequence of events occurs after infusion of thrombin, tissue extracts or particulate material, intravascular haemolysis, haemorrhage, anoxia, viral and bacterial infections in animals where hypotension, a bleeding diathesis, and infarct necrosis are prominent. After the intra-aortic injection of thrombin in dogs, Hardaway, Watson and Weiss (1960) noted pressure changes which may contribute to functional and pathological changes in various organs. Despite systemic hypotension, there was portal, pulmonary and renal hypertension which was attributed to obstruction

of the microcirculation by thrombi. Other authors claim, however, that vasomotor factors play a significant part, pointing out the unlikely role of coagulation in causing the peripheral vasodilata-tion accompanying the systemic hypotension. The intriguing possibil-ity exists that activation of the coagulation mechanism may indeed potentiate these vasomotor changes. Intra-arterial injection of thrombin causes peripheral vasodilatation and selective renal and pulmonary vaso-constriction (Olsson et al, 1970). Moreover, alteration in vascular tone in this situation is attributed to the adenine nucleotides released from platelets (Olsson et al, 1969). Thus, the nature of the anatomy and physiological responses of the renal micro-circulation render the kidney peculiarly susceptible to changes in coagulation — fibrinolytic balance whether local or systemic.

1.2 INTRAVASCULAR COAGULATION AND RENAL DISEASE

During the last 15 years increasing evidence has accumulated which implicates intravascular coagulation in the pathogenesis of certain renal disorders. This evidence is based mainly on immuno-fluorescent and electron microscopic studies and on changes in circu-lating coagulation factors accompanying some specific diseases in man, but mostly in experimental animals. It was widely recognised before the advent of the electron microscope and immunological staining procedures that a substance commonly referred to as fibrinoid was present within glomeruli in several types of acute glomerular diseases.

By special staining techniques fibrinoid could be seen in acute glomerulonephritis, toxaemia of pregnancy, glomerulonephritis associated with systemic lupus erythematosus, polyarteritis nodosa, thrombotic thrombocytopenic purpura and experimentally induced hypersensitivity states in animals. As it was also frequently present in a large variety of pyogenic and other inflammatory and degenerative lesions, much controversy existed as to its nature and origin within the vascular tree of the kidney. In part, this confusion resulted from the differing tinctorial properties of this material when subjected to specialised staining procedures which were unable to discriminate between degenerating collagen, altered ground substance, and deposited fibrin. The development by Coons and Kaplan (1950) of a method for the detection of antigen by means of fluorescent antibodies provided the basis for more accurate assessment of the nature of fibrinoid. Fluorescein labelled human anti-fibrinogen serum prepared in rabbits was used by Gitlin, Craig and Janeway (1957) to study fibrinoid in biopsy and autopsy specimens from patients with rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, glomerulonephritis, polyarteritis nodosa and thrombotic thrombocytopaenic purpura (Craig and Gitlin, 1957). Their findings indicated that in these lesions the fibrinoid and hyaline material was composed, at least in part, of fibrin. Moreover, using this immunological technique the extent of fibrin deposition was found to be much greater than revealed by conventional staining methods which proved very variable

when applied to experimentally produced fibrin clots (Gitlin and Craig, 1957). Similar views were expressed by Lendrum et al (1962) after a meticulous study of vascular lesions by standard and personally developed staining procedures. The reasons for the fibrin depositions were not, however, pursued by these workers.

Armed with this knowledge, Vassalli, Simon and Rouiller (1963) performed the logical experiment of inducing intravascular coagulation in animals and studied by electron microscopy the lesions which resulted from the formation and deposition of fibrin within the glomeruli. Three substances thought to activate the coagulation process by different means were infused intra-aortically into separate groups of rabbits: liquid (sodium polyanetholsulphonate) whose action was presumed to be mediated via platelets, thrombin which promotes the conversion of fibrinogen to fibrin, and thromboplastin an activator of prothrombin via the extrinsic coagulation system were used. The histological and ultrastructural appearances were similar in each group. Not only did glomerular thrombosis occur, but a variety of abnormalities commonly present in human renal diseases were observed. These consisted of varying degrees of swelling and proliferation of endothelial, mesangial, and visceral and parietal epithelial cells (sometimes sufficient to form crescents), polymorphonuclear leukocyte infiltration, clumping of platelets and deposits of fibrin and fibrinoid. Moreover, in a significant number of animals sacrificed later, progression to glomerular sclerosis had occurred. A most striking finding was the

variation in extent and severity of the lesions produced in different animals by similar stimuli. The least severely affected kidneys showed only minor focal glomerular hypercellularity whereas in others complete renal cortical necrosis occurred. More severe abnormalities were consistently seen in animals simultaneously infused with the fibrinolytic inhibitor, epsilon amino caproic acid (E.A.C.A.). Under the electron microscope, distinction was also possible between material with the fibrillar appearance of fibrin, and the amorphous, granular, densely osmiophilic fibrinoid. Their close physical proximity between and beneath endothelial cells and within the capillary lumina led to the assumption that in this experimental situation the fibrinoid was derived like the fibrin from fibrinogen and probably represented incompletely polymerized fibrin or products of the degradation of fibrinogen and fibrin.

These observations, which are the basis of the work presented in this thesis, were extended to other experimental models which more closely approximated human immunological renal diseases. Using a form of Masugi glomerulonephritis (Masugi, 1934) induced by the injection of rabbit anti-kidney serum produced in sheep, Vassalli and McCluskey (1964) demonstrated by immunofluorescent microscopy, glomerular lesions very similar to those seen after intravascular coagulation. During the latent phase of five days only minor glomerular endothelial cell swelling was observed associated with the deposition of sheep γ-globulin along the basement membrane. For the next 10-15 days an inflammatory reaction of increasing severity was seen in the glomeruli. Severe proliferative changes uniformly distributed

through all glomeruli often with crescent formation and polymorph infiltration gave way after 15 days to progressive glomerular sclerosis. Rabbit γ-globulin and fibrin were present along the basement membrane in the acute phase. Fibrin was also seen in the capillary lumina and Bowman's space. In a further group of animals treated with the oral anticoagulant warfarin many of these histological and immuno-fluorescent abnormalities did not develop. In most, the deposition of fibrin was prevented, the swelling and proliferation of glomerular cells was mild or absent and progressive sclerosis did not ensue. It was concluded that:

1. The immune reaction within the glomerulus initiated the coagulation process and resulted in the formation of fibrin and fibrinoid,
2. The fibrinoid contained γ-globulin (and presumably complement) as well as fibrin,
3. The phagocytosis of this material by endothelial, mesangial and epithelial cells was responsible for their swelling and proliferation,
4. The coagulation process was an essential factor in the development of progressive sclerosis.

Much of this work has subsequently been confirmed by other workers. Humair, Potter and Kwaan (1969a, 1969b) produced similar abnormalities in rats by intravascular coagulation and with a form of Masugi nephritis. Using a fibrin slide technique (Todd, 1959) they found that the

plasminogen activator content of the abnormal glomerular endothelial cells was markedly reduced. On this basis they used urokinase, a plasminogen activator occurring naturally in the urine, for treatment and found it to be superior to anticoagulation in preventing the inflammatory glomerular lesions.

The micro-circulatory changes after the infusion and topical administration of liquid and thromboplastin were studied in the rabbit ear-chamber and hamster cheek-pouch by Stalker et al (1969). Their findings which presumably represented similar alterations in the microvasculature elsewhere in the body, illustrated the early dynamic changes accompnaying intravascular coagulation. Blood flow initially increased but soon became sluggish and leukocytes became adherent to the vessel walls. White-emboli which sometimes contained leukocytes, red cells and platelets, but which more often were acellular, soon became detached from the wall and circulated freely or attached to similar circulating structures. The emboli were composed predominantly of fibrin and were derived from areas of vascular endothelial cells which were swollen and proliferating in response to the abnormal coagulation. Amorphous material with staining properties of fibrin began to accumulate in the capillaries of the lung and kidneys within five minutes. Leukocytes became adherent and round densely haematoxylinophilic nuclear material derived from the endothelial cells was also seen attached, or lying free within the capillary lumen (Brown, Stalker and Hall, 1969). Similar emboli were seen in the

vessels draining the placental bed of pregnant golden hamsters (Brown and Stalker, 1969). The glomeruli and intra-alveolar capillaries of the lung in both mother and foetus showed changes comparable to those seen after intravascular coagulation.

Thus, in these experimental models, lesions were produced in the kidneys which closely resemble acute renal failure, glomerulonephritis, and pre-eclampsia as seen in humans. Such experiments in man are not possible, so that deductions as to the similarity of pathogenetic mechanisms are to this time unproven.

Since immunofluorescence techniques have been more routinely applied to human renal biopsy material, the presence of fibrin in diseased glomeruli and larger renal vessels has become increasingly recognized. Its significance in specific disease states is not, however, widely appreciated. Following the initial adaptation of the method of Coons and Kaplan (1950) to renal tissue (Mellors and Ortega, 1956) perhaps the most provocative observations have concerned the role of immunoglobulins and complement components in the pathogenesis of glomerulonephritis. In particular two generally accepted theories as to its causation have been proposed by Dixon (1968) on the basis of animal experimentation. The fluorescence pattern of γ-globulin and complement takes one of two forms. In the first there is a uniform linear distribution along the glomerular capillary basement membrane which is attributed to the binding, at this site of antibodies against glomerular basement membrane (anti-G.B.M. antibodies) with the

specific antigen. In the second type lodging of freely circulating antigen-antibody complexes within the basement membrane gives rise to a characteristic granular or lumpy appearance which can also be distinguished by electron microscopy. While discrete examples of each type of glomerulonephritis have been described in humans, e.g. Goodpasture's syndrome (anti-G.B.M.) post-streptococcal, systemic lupus erythematosus, malarial and mixed cryoglobulinaemic glomerulonephritis (immune complex disease), in the large majority of cases no such distinct pathogenetic mechanism is discernable. The therapeutic implications of these studies are apparent provided the antigen(s) involved can be identified, but to date nosuch advance has been made, nor have the factors involved in rendering normal tissue antigenic been clearly defined. Immunosuppressive therapy has been widely used against many presumed immunological renal diseases with, apart from the notable exception of minimal lesion and S.L.E. glomerulonephritis, disappointing results.

Fibrin has been found consistently by immuno-fluorescence in both types of human glomerulonephritis (Sturgill and Westervelt, 1965; Michael et al, 1966) in rejecting human renal homotransplants (Busch et al, 1967; McKenzie and Whittingham, 1968), in other presumed immunological disorders such as Henoch-Schonlein purpura (Urizar and Herdman, 1970) and in conditions where no immunoglobulins are present: acute renal failure (Koffler and Paronetto, 1966), the haemolytic uraemic syndrome (Rosenmann et al, 1969), pre-eclampsia (Vassalli et al,

1963) and diabetes (Davison et al, 1971). Moreover, the varied distribution of fibrin within the glomerular capillaries, mesangial cells, Bowman's space and afferent arterioles, suggests that it is a non-specific component of many types of glomerular damage.

In electron microscopic studies, fibrillar, granular and amorphous material with the appearance of fibrin or its degradation products has often been described within the glomerulus in association with many specific renal diseases. There has been general reluctance on the part of pathologists, however, to assign the term fibrin to this material even though it occupies a corresponding intraglomerular position to fluorescing fibrin. Perhaps the lack of the characteristic periodicity (≏ 230 Å) has been a deterrent, although in the light of available evidence it is difficult to offer an alternative explanation, especially since the ultrastructural appearances of plasmin digests of fibrin formed <u>in vitro</u> are now known (Horn, Hawiger and Collins, 1969; Stewart, 1971).

Thus fibrin or some closely related substance is present within glomerular capillaries in many of the commonly occurring human renal diseases. Although a role in the pathogenesis of these conditions must remain a matter of conjecture, some clinical nephrologists have been impressed with this possibility and used anti-coagulation to combat its local formation. This form of therapy which has abundant precedent in laboratory animals (Silverskjold, 1940; Kleinerman, 1954; Vassalli and McCluskey, 1964) has usually taken the form of heparin

infusion, oral dicoumarol or warfarin administration. Encouraging results have been obtained, and in man Kincaid-Smith, Saker and Fairley (1968) have used anticoagulation in glomerular diseases which often give rise to rapid and permanent loss of renal function. The same group (Kincaid-Smith, Lavor and Fairley, 1970) are also enthusiastic about the benefit derived from such therapy in other forms of proliferative glomerulonephritis, and in human renal homotransplant rejection (Kincaid-Smith, 1970). Prevention of fibrin-thrombus formation is claimed to diminish the proliferative vascular lesions, and gradual but sometimes rapid improvement of renal function occurs. Unfortunately, much of this work has been done in an uncontrolled fashion and where more rigidly controlled studies have been reported, the results are not as impressive although in occasional cases dramatic response seems to occur (Herdman et al, 1970). While larger controlled trials are necessary to confirm the improvement in renal histology and function following anticoagulation, a more fundamental approach to the problem of coagulation and fibrinolysis in human renal diseases is equally appropriate.

A major impetus to the study of coagulation and fibrinolysis resulted from three important observations made between 1958-1962. The first of these derived from the independent work of Triantaphyllopoulos (1958) and Niewiarowski and Kowalski (1958) who observed that *in vitro* proteolysis of fibrinogen by plasmin gives rise to products which possess powerful anticoagulant properties. Secondly, the proteolysis

of fibrinogen if allowed to proceed unimpeded ultimately leads to the formation of two large polypeptide fragments (now known as fragments D and E) which each contains a single antigenic determinant in contrast to the parent fibrinogen molecule which contains multiple antigenic determinants (Nussenzweig et al, 1961; Nussenzweig et al, 1962). These studies, besides initiating work on the systematic characterisation of fibrinogen and fibrin degradation products (F.D.P.) also demonstrated that immunological techniques could be applied to the study of clinical disorders of fibrinolysis. The third important observation concerned the recording of high concentrations of F.D.P. in the plasma of patients with fibrinolytic disorders (Fletcher et al, 1962) and that the presence of these fragments, similar to those produced by plasmin _in vitro_ played a significant role in the pathogenesis of the clinically encountered haemorrhagic tendency.

The way was thus opened for the first time to study in close sequential detail the role of fibrinolysis and intravascular coagulation in the natural history of many human renal disorders.

1.3 HAEMOSTASIS

Injury to blood vessels evokes a complex response which, although it is protective in nature by facilitating repair, may also be damaging by impeding blood supply to areas distal to the injury. It is perhaps most readily studied by observing the process of haemostasis.

Haemostasis is an important protective mechanism in the body for it controls bleeding at the site of injury. Events leading to effective haemostasis may be divided into several distinct phases which occur simultaneously or in sequence.

1. Immediately after injury there is intense vaso-constriction, which initially permits only a small amount of blood to emerge from the wound. Injury stimulates an axon reflex so that contraction and retraction of the vessel occurs lasting for 15-30 seconds. Interruption of the nerve supply to the injured area renders this stage of haemostasis ineffective (Cruz and Oliveira, 1958).

2. After this time blood flows freely out of a severed vessel and as it does so, platelets become attached to the damaged vessel wall and to the surrounding peri-vascular connective tissue. This process is called <u>platelet adhesion</u>.

3. The initially adherent platelets seem to attract other platelets which aggregate (<u>platelet aggregation</u>) so that within a short time of injury (<u>bleeding time</u>) the rent in the vessel wall is filled by a platelet plug and blood flow is stopped.

4. A second vasoconstrictive episode ensues which complements the plug of aggregated platelets in securing haemostasis.

5. In small vessels of the skin the platelet plug and vaso-constriction are aften sufficient to prevent further bleeding as additional support from surrounding connective tissue is enough to withstand the blood pressure. Where this support is not available,

or if the vessel is of larger calibre, an additional haemostatic mechanism, the formation of a fibrin clot is of importance. Its formation involves the thrombin-induced conversion of fibrinogen to fibrin and results in an interlacing structure of firm tensile strength in which circulating blood elements become enmeshed. This process is called coagulation and results in the formation of a thrombus or blood clot.

6. The thrombus then slowly contracts in size (clot retraction) and over the course of days to weeks may by a process of lysis, spontaneously resolve, by an ingrowth of endothelium become recanalized, or become organized by solid connective tissue to present a permanent obstruction to the flow of blood through the injured vessel.

Thus haemostasis and thrombosis are achieved by an integration of platelet adhesion and aggregation, coagulation, and accumulation of white cells and red cells. It is perhaps important to differentiate these processes. Haemostasis is a process of great importance in the maintenance of vascular integrity, whose physiological usefulness extends far beyond the arrest of blood flow. Thrombosis is an accidental expression of haemostasis.

1.4 PLATELET FUNCTION IN HAEMOSTASIS

Study of platelet function during haemostasis has led to a wider understanding of platelet physiology, the role of the platelet in the nurture of vessel walls, as a defence mechanism against invading

micro-organisms, in phagocytosis, and in the inflammatory process. Viscous metamorphosis, a term originally used to describe changes in single platelets (Eberth and Schimmelbusch, 1886) was later applied to the fusion of platelets during clotting (Wright and Minot, 1917). Later it was suggested (Kjaerheim and Hovig, 1962) that the term should be revised because membranes of clumped platelets were seen under the electron microscope to remain intact during aggregation. More recently it has been interpreted as the whole sequence of morphological and biochemical changes in platelets during coagulation, even though it is still sometimes used in reference to only one or other aspect of these changes.

1.4.1 PLATELET ADHESION

Under normal circumstances, the blood vessels are open tubes with no obstructions, and circulating platelets are not attracted to each other, to other blood cells or the endothelium lining the vascular tree. The phenomenon of platelet adhesion occurs only if this environment is changed, if the endothelial cells are injured, or if the platelets come into contact with foreign structures such as basement membrane, elastin and collagen within the vessel wall or surrounding connective tissue. While it is possible in certain cases that hypercoagulable states brought about by increased concentrations of circulating coagulation factors or a decrease in the fibrinolytic potential may initiate thrombus formation, this seems unlikely in

the majority as changes in the endothelium are essential for platelet adhesion. Hypercoagulability may only render these changes more likely to occur.

The nature of the changes in the endothelial lining which makes platelets adhere are unknown, but almost certainly involves a change in electrical charge, and alteration of the non-wettable character of the surface. Calcium ions and fibrinogen are necessary for platelets to adhere to each other and are probably required for adherence to other surfaces. A granular, non-cellular layer, possibly fibrinogen, is necessary for adhesion to artificial membranes (Dutton et al, 1969). Adsorption of such a layer to endothelial cells *in vivo* may render the surface wettable. In support of this theory is the finding by Marr et al.(1965) of fibrin fibres in close proximity to red cells and extravascular collagen as soon as 30 seconds after vessel transection. Also, the platelet is surrounded by a "plasmatic atmosphere" (Roskam, 1922) in which there are high concentrations of proteins of coagulation factors, adsorbed with varying grades of affinity. Hence, abnormality of the vessel wall may activate the contact factor (Factor XII) of the coagulation system early in the course of haemostasis. The resultant thrombin activation and fibrin formation potentiates the adhesion of platelets to the damaged surface and to each other. Adenosine diphosphate (A.D.P.) is released from red blood cells early in the process of haemostasis possibly due to microhaemolysis (Hellem et al, 1961). Thus the early and simultaneous appearance of A.D.P.

and thrombin is of unknown but potentially important significance to platelet adhesion. Their role in the ensuing platelet aggregation is more clearly defined.

1.4.2. PLATELET AGGREGATION

Platelet aggregation has been extensively studied since Born (1962) developed simple means of measuring the response to aggregating agents *in vitro*. By using such techniques aggregation induced by A.D.P. and thrombin is seen to be a biphasic process with distinct primary and secondary components. The prime moversof platelet aggregation *in vivo* are thought to be A.D.P., thrombin and collagen. A.D.P. is released from injured endothelium and vascular smooth muscle, whence it diffuses towards the luminal surface (Honour and Mitchell, 1964), and also from damaged erythrocytes. Once aggregation under this stimulus has commenced, further aggregation occurs at a rapid rate due to the release of A.D.P. from the initially aggregated platelets. A similar response is thought to occur after thrombin activation, but whereas *in vitro* studies of A.D.P. induced aggregation suggest that it is a reversible process (Johnson, 1970), that caused by thrombin may be irreversible. Thus disaggregation possibly does not play an important part in *in vivo* haemostasis as aggregation is simultaneously induced by A.D.P. and thrombin. Critical to this concept is the concentration of thrombin at the site of aggregation. If it is low the process may be reversible. However, when only minor endo-

thelial cell damage occurs, platelet aggregation may be induced almost solely by A.D.P. in which case disaggregation may take place. Larger degrees of vascular damage not only incite thrombin activation, but also expose circulating platelets to the other powerful aggregating agents present in connective tissue and vessel walls. Of these collagen is the most powerful. The ability of collagen to cause aggregation resides in the positively charged epsilon-amino groups of lysine (Wilner et al, 1968) and can be differentiated from the Hageman factor (Factor XII) activating property which resides in the negatively charged carboxyl groups of glutamic and aspartic acids. Release of endogenous A.D.P. completes the aggregation process started by exposure to collagen.

Other physiologically active substances also cause aggregation, during the second phase of which A.D.P. plays a vital role. These include adrenaline, nor-adrenaline, and serotonine (5-hydroxytryptamine). Adrenaline produces a primary and secondary response and the platelets develop pseudopodia (Mannucci and Sharp, 1967). Nor-adrenaline is approximately one tenth as potent. Phentolamine will inhibit the primary response (MacMillan, 1966) whereas β -adrenergic blockers inhibit the secondary response to adrenaline (Thomas, 1967). It is generally accepted that the larger, younger, more metabolically active platelets are more responsive to adrenaline than the smaller older cells. Serotonine is preferentially taken up by platelets and is almost exclusively contained within circulating platelets. It

initiates platelet aggregation but there is no secondary phase. Unlike aggregation caused by A.D.P. and thrombin, that produced by serotonin is not dose dependent and indeed, platelets become refractory to it as further aggregation does not occur on subsequent addition. The precise roles of adrenaline, nor-adrenaline and serotonine in aggregation *in vivo* are unknown, but they may be more important than previously recognized.

For some time it has been known that antigen-antibody complexes cause platelet aggregation (Robbins and Stetson, 1959; Siquiera and Nelson, 1961; Mustard 1964; Movat et al, 1965) accompanied by degranulation and release of A.D.P. and serotonine. Platelets have also been observed to phagocytose these complexes (Mustard and Packham, 1968).

Fatty acids cause calcium dependent platelet aggregation. As free fatty acids (F.F.A.) are readily taken up by platelet membranes in an unesterified form, the aggregation may be due to changes in the membrane caused by large amounts of F.F.A. The platelet aggregating effect of fibrin/fibrinogen degradation products is thought to reside in the smaller molecular weight, late stage proteolysis fragments, whereas the higher molecular weight early products and complexes have an inhibitory effect on aggregation (Kowalski, 1968).

Electron microscopy of platelets aggregating spontaneously in tubes has revealed structural changes of importance to the understanding of haemostasis (Rodman et al, 1962; Castaldi et al, 1962; White et al, 1962). In the normal situation platelets may be simply described as round or ovoid bodies contained by a distinct membrane system

inside which there are randomly distributed mitochondria, granules, vacuoles and glycogen. Just prior to aggregation, granules move towards the centre of the platelet and projections (pseudopodia) jut from the surface. In the early phases of agglutination these features become more pronounced, fibrin strands appear at the periphery of the mass of cells, although not between individual platelets, and the membrane remains intact. As soon as aggregates form, granules and mitochondria disappear, the membranes of platelets in the centre of the mass disintegrate (at the periphery they remain intact), and fibrin becomes more prominent. Later, no intact platelets are seen, but there is an abundance of granular debris, membrane remnants and fibrin. Similar alterations in platelet structure have been observed in platelet plugs observed in vivo with the exception that membranes are preserved for long periods (Kjaerhaim and Hovig, 1962; Marr et al, 1965).

1.4.3. THE PLATELET RELEASE REACTION

During the phase of aggregation, agents with powerful chemical properties are released into the surrounding medium which potentiate the haemostatic process (Grette, 1962). The mechanisms of this release are the subject of much research in the fields of biochemical, microvascular and ultrastructural endeavour. Already it is apparent that irreversible platelet aggregation with structural disintegration is not necessary for release to take place, and it is now assumed that the platelet has a secretary mechanism analagous to that responsible

for the release of adrenaline from its storage granules in the adrenal medullary cells. Secretion is essential for the secondary phase of aggregation as studied by nephelometry and is a physiological process not necessarily resulting from injury to the cells. It results from stimulation of the contractile mechanisms and leads to the release via the open canalicular system, of endogenous chemical products which are normally confined to the storage organelles inside the cells (White, 1970).

Products of the platelet release reaction include the adenine nucleotides ATP and ADP, serotonin, catecholamines, potassium, calcium, platelet factors 3 and 4, hydrolytic enzymes, mucopolysaccharides, amino acids and fibrinogen (Holmsen et al, 1969). Not all appear simultaneously as adenine nucleotides, serotonin and catecholamines are released more rapidly than other products. Platelet factor 3, a strongly procoagulant lipoprotein (platelet thromboplastin) is similar to the lipoproteins of the intrinsic and extrinsic (tissue thromboplastin) coagulation systems so necessary for prothrombin activation. Platelet factor 4, or platelet antiheparin substance, is a platelet specific protein and presumably a granule constituent. It neutralises the anticoagulant effects of heparin and fibrinogen degradation products, causes paracoagulation of soluble fibrin monomer complexes and may function in the adherence of platelets to fibrin threads to consolidate the platelet plug. A permeability factor of cationic protein nature has been isolated from platelet granules (Nachman et al, 1970) and may also be secreted during the release reaction.

The release reaction is an energy-dependent process and possibly consists in the activation of a contractile tubular system of which thrombosthenin is an important element. Thrombosthenin is a contractile protein which also may act as an ATP-splitting enzyme giving rise to ADP formation. Its activation may be dependent on the local concentration of prostaglandins which are known to facilitate the influx and release of platelet bound calcium (Emmons et al, 1967). Little is known about intraplatelet calcium and its state during platelet aggregation and how it affects platelet contraction. Aggregation is a high-energy requiring process for which high concentrations of calcium are necessary and it is possible that any substance regulating calcium flux may be important in its activation. Thrombosthenin plays a direct role in clot retraction which is important to the stability of the final product of the haemostatic process — the blood clot. Intricately linked with the formation of the blood clot is the coagulation system.

1.5 THE COAGULATION SYSTEM

Events leading to the formation of fibrin from fibrinogen constitute the coagulation system. It is a complex, dynamic process dependent on the activation of a succession of enzyme systems, whose sequence has been reasonably well documented *in vitro*. The relevance of this series of events occurring in glass tubes, to *in vivo* coagulation must however remain conjectural. A description of the coagulation system is perhaps facilitated by a brief synopsis of the historical development of the modern concept of coagulation.

Hewson (1771) was the first to demonstrate that the coagulating portion of blood resided in the plasma rather than in the red cells. In 1856, Virchow postulated the existence of fibrinogen which was isolated 3 years later by Denis. It was soon recognized, however, that a further factor was necessary: this active coagulant was called thrombin by Schmidt (1872) and shown by Gamgee (1879) to be a protein of the globulin variety. In a series of classical observations Morawitz (1905) noticed that tissue extracts accelerated clotting and that serum contained a coagulant of fibrinogen not present in plasma. He was therefore able to propose a theory why the blood remained fluid within vessels, but coagulation occurred on its release. On exposure to tissue extract a precursor substance, prothrombin, was converted to an enzyme, thrombin, which altered fibrinogen in some way to make it clot. As blood did not contain tissue extracts, it remained fluid until exposed to damaged tissues. The validity of this simple theory has never been seriously questioned although further developments revealed it to be too facile an explanation of the clotting process. The one-stage prothrombin time test devised by Quick (1935) was the first such development to stimulate further thought. As it had been previously demonstrated that the conversion of prothrombin to thrombin was a quantitative reaction, and the clotting time of fibrinogen was directly related to the concentration of thrombin, Quick argued that the clotting time of a system including plasma, optimum concentrations of calcium and tissue extract and excess fibrinogen, should represent the prothrombin concentration of the plasma. Patients were soon found whose plasma had prolonged clotting

times by this assay, but who were believed for other reasons not to be deficient in prothrombin. As a result a great deal of discussion and confusion existed in the literature for some time before the existence of three further clotting factors (V, VII, and X) were recognized. Thus the extrinsic coagulation system was defined.

In his original paper, Quick (1935) recorded that the one-stage prothrombin time was normal in patients with haemophilia, by far the commonest of the congenital coagulation defects. Haemophilic blood, however, was known to have along whole blood clotting time and it was assumed that haemophilic patients must either be deficient in thromboplastin or an alternative pathway for the activation of prothrombin must exist. The finding between 1937 and 1954 of four further clotting factors which did not require tissue extract to make a clot established the existence of the alternative pathway or "intrinsic" clotting system. These included Factor VIII (anti-haemophilic globulin), Factor IX (Christmas Factor) and Factors XII and XI the contact factors. Much effort has gone into working out the sequence of events in which these factors react. The general deduction from this work is that Factor X rather than thromboplastin is the central factor which eventually activates prothrombin to thrombin. It has also become apparent that the process of coagulation consists of a series of stages in a chain reaction in which coagulation factors act as pro-enzymes for other factors. Such a scheme as outlined in Fig. 1 was proposed by MacFarlane in 1964 and is now referred to as the "cascade theory". Thrombin has an auto-catalytic effect on earlier reactions, and this, together with the sequential enzyme nature of the total reaction

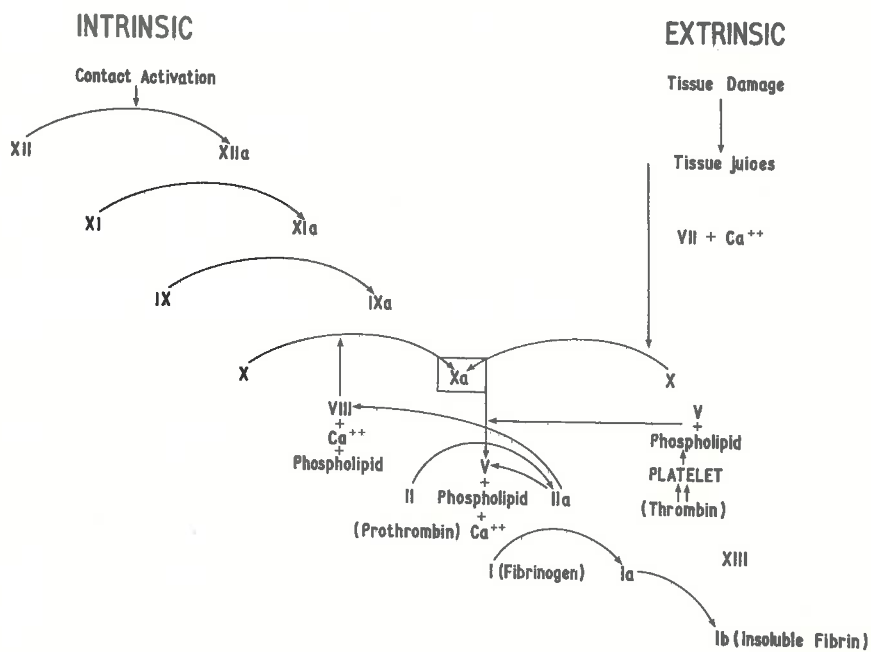


Fig. 1. Current concept of the coagulation system. The interaction between thrombin and platelets, and the activation of other coagulation factors by thrombin is illustrated.

gives rise to progressive acceleration of the reaction speed as it proceeds (MacFarlane, 1964). This amplification of a long chain reaction leads to an initial delay and sudden explosive finale of such power that it is surprising how blood remains fluid. Fluidity is favoured by several factors, however: firstly, initiation requires an active surface which is usually lacking; secondly, all of the active coagulants are normally countered by naturally occurring anticoagulants; and thirdly, activation of the fibrinolytic enzyme system probably occurs simultaneously with that of the coagulation system.

A further factor, Factor XIII, or fibrin stabilising factor is essential for the polymerization of fibrin to form insoluble fibrin so necessary for integrity of clot structure.

1.5.1. CONTACT ACTIVATION

Factor XII (Hageman Factor) is present in the plasma in trace amounts and has a molecular weight between 100-200,000. Biochemical analyses indicate that it is possibly a glycoprotein. Patients with Factor XII deficiency have no haemorrhagic diathesis but present because of a greatly prolonged clotting time, due to a deficit confined to the initial stages of activation of the intrinsic pathway. While contact activation can be achieved by many substances *in vitro*, the stimulus for activation *in vivo* is not fully understood, although collagen fibrils are known to be one possible source (Niewiarowski et al, 1964). Factor XII is thought to be adsorbed on to the activating

surface where it complexes with Factor XI and converts it to its activated form (Ratnoff et al, 1961). Factor XI is present in plasma and serum and its absence causes a mild haemorrhagic disorder. In its activated form it activates Factor IX (Christmas Factor) (Ratnoff and Davie, 1962).

It has been recognized for some time that the enzymes involved in contact activation may have several substrates of varying specificity. For example Niewiarowski and Prou-Wartelle, (1959) and Iatridis and Ferguson (1961) have demonstrated increasing fibrinolytic activity of euglobulin fractions from normal plasma incubated in glass, or in the presence of kaolin, whereas plasma from a Hageman factor deficient patient did not show such activity. Moreover, polybrene (Hexadimethrine bromide), which inhibits Hageman factor also inhibits the fibrinolytic activity in these circumstances (Eisen, 1964). Conflicting reports about the fibrinolytic activity of patients with Factor XII deficiency indicate that fibrinolysis is not mediated solely through the contact factors, but it does seem that fibrinolytic activity is dependent in some manner on activated Hageman factor.

It is implied from several observations that contact factors also play a role in inflammatory reactions. Following exposure to glass, plasma develops the ability to produce pain and increase vascular permeability in experimental animals (Margolis, 1958) and man (Ratnoff and Miels, 1964). Polybrene inhibits this phenomenon. Activated Hageman factor also induces in plasma the formation of agents capable of contracting smooth muscle, dilating blood vessels and causing leukocytes to adhere to and migrate through vascular walls (Graham

et al, 1965). Responsibility for these actions seems to rest with the kinins and it is now assumed that Hageman factor activates kallikreinogen to form kallikrein which in turn activates prokinin to the active kinins.

An alternative pathway for the generation of kinins and permeability factor by Factor XII, via the complement system has recently been proposed (Pondman, 1969). Hageman factor is capable of transforming the first component of complement (C1) into its activated form (C_1 esterase). Once activated the other components respond in a chain reaction giving rise to permeability factors (from C_3 and C_5) and kinins due to the activation of kallikrein by C_1 esterase.

Activation of Factor XII may give rise in turn, therefore, to activation of the coagulation, fibrinolytic, kinin and complement systems.

In the intrinsic coagulation system activated Factor IX is necessary together with Factor VIII, calcium ions and phospholipid, for the conversion of Factor X to its active form. Factor IX is a β-globulin synthesized in the liver and its deficiency leads to a severe bleeding disease (Christmas disease). Factor VIII (antihaemophilic globulin) is present in trace amounts in normal blood, and is very susceptible to the action of both thrombin and plasmin (Triantaphyllopoulos and Triantaphyllopoulos, 1967), as there is an apparent relation between fibrinogen degradation products and Factor VIII concentrations. Recent work suggests that Factor VIII is produced in the spleen and other reticulo-endothelial cells (Webster et al, 1967).

1.5.2 TISSUE ACTIVATION

The extrinsic coagulation system provides an alternative pathway for the activation of Factor X and may serve as a rapid means of generating small amounts of thrombin necessary for activation of the slower intrinsic system. The reaction initiating this pathway is the activation of Factor VII by tissue thromboplastin (Factor III). Tissue thromboplastin is a protein-phospholipid complex connected with the endoplasmic reticulum which can be split into its two components (Deutsch et al, 1964). It has a membrane-like structure which is believed important in its procoagulant activity. Current thinking is that tissue thromboplastin binds calcium ions and Factor VII to form a complex which has enzymatic activity towards Factor X. Factor VII is a glycoprotein which has a remarkable affinity for binding divalent cations, and has been purified from serum (Pryds, 1965). Besides being of importance in the extrinsic system, it may, with tissue thromboplastin, activate Factor IX in the same way as the contact factors do in the intrinsic system. In this way, it is possible to explain why patients with Hageman Factor deficiency do not bleed.

1.5.3 THE PROTHROMBINASE CONCEPT

The physiological conversion of prothrombin to thrombin requires two coagulation factors, activated Factor X and Factor V as well as bivalent cations and phospholipid. Activated Factor X is derived from the interaction of components of both extrinsic and intrinsic systems and as such is the cornerstone of the coagulation cascade. Activated

Factor X is an enzyme with a molecular weight of approximately 24,000 and possesses esterolytic as well as procoagulant properties. It will slowly convert prothrombin to thrombin in the absence of calcium ions, but this reaction is greatly accelerated by calcium ions, Factor V and phospholipid. Factor V is probably a high molecular weight compound whose stability is dependent on calcium ions. Neither Factor V nor phospholipids can activate prothrombin however (Barton et al, 1967), and it is now proposed (Hanahan et al, 1969) that prothrombin is adsorbed on to phospholipid and Factor V by which process its configuration is changed rendering it more susceptible to the enzymic action of adsorbed activated Factor X. The adsorption is calcium dependent.

1.5.4. FIBRINOGEN TO FIBRIN CONVERSION

Initiation of the process leading ultimately to the formation of fibrin is through the proteolytic enzyme thrombin which causes a limited degradation of the fibrinogen molecule. Fibrinogen is composed of three chains of peptides α, β and γ, which are linked at the N-terminal end by a strong disulphide knot. The molecule is a dimeric unit as shown in Fig. 2. By using a reagent, cyanogen bromide, which cleaves peptide bonds involving methionine, the N-terminal disulfide knots have been obtained in a relatively pure form. Thrombin acts by splitting fibrino-peptides (A and B) from the α and β chains at the N-terminal ends. It has a narrow specificity of action and hydrolyses arginylglycine bonds linking the fibrinopeptides to the rest of the fibrinogen molecule. Blomback and Blomback (1970) have

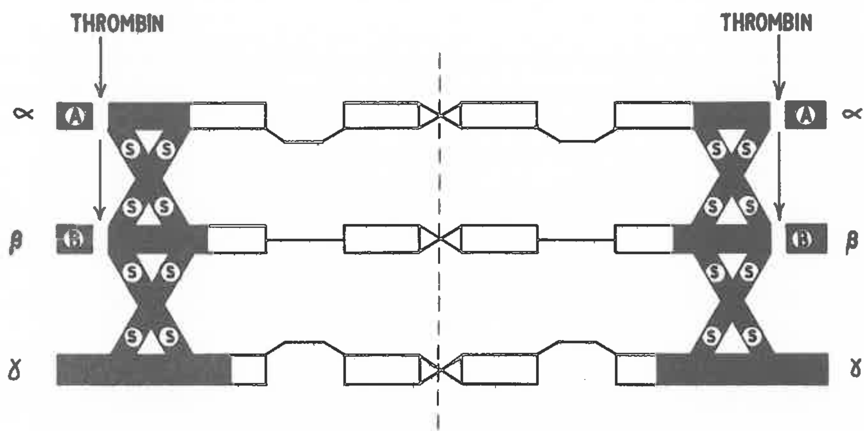


Fig. 2. A schematic model of the fibrinogen molecule. The sites of action of thrombin are indicated by arrows.

proposed that the configuration of the fibrino-peptides is important for the substrate specificity of thrombin, and that this abnormality may be responsible for the bleeding tendencies of persons possessing such a molecule.

It is possible that the fibrinopeptides have specific physiological roles, for they may act as homeostatic inhibitors in the clotting mechanism (Silver and Murray, 1966). Moreover, their release may be of some importance in the haemodynamic control of the microcirculation as they potentiate the actions of bradykinin and histamine (Osbahr et al, 1967) and effect changes in blood flow of heart, lung and uterus when injected in minute amounts (Bayley et al, 1967).

Once fibrinopeptides A and B are split from fibrinogen the fibrin monomers aggregate and form a three dimensional network of fibrin polymers. This fibrin is unstable, is soluble in urea and monochloracetic acid, but may be stabilized by the action of Factor XIII or fibrin stabilising factor which converts it to an insoluble polymer with normal haemostatic function. Thrombin is thought to play a dual role in this process for besides preparing fibrinogen for the reaction with Factor XIII, it activates Factor XIII itself, by limited proteolysis and uncovering new sulphydryl groups (Buluk et al, 1961). For its activation and function Factor XIII needs calcium ions. Recent findings are in agreement concerning the role of Factor XIII. Following the work of Lorand et al, (1962), who found that certain amines inhibited fibrin formation Loewy (1963) ascribed the action of Factor XIII to a transamidation process. Further work (Lorand and Jacobsen, 1964) suggested that it was a glutaminase enzyme which catalysed

a reaction resulting in the formation γ-glutamyl glycine peptide bonds between the γ-carbonyl of the glutamine residues of the fibrinogen molecule. This covalent bonding converts unstable soluble fibrin polymer into physiological fibrin. The sequence of reactions resulting in the formation of fibrin from fibrinogen are represented schematically in Fig. 3.

1.5.5. FIBRIN MONOMER COMPLEXES

The normal polymerization of fibrinogen may be interrupted by the complexing of fibrin monomers with fibrinogen, and other fibrinogen derivatives. Existence of such complexes was first suspected after Morrison (1946) demonstrated the presence of a type of fibrinogen in plasma of patients with high erythrocyte sedimentation rates which precipitated in the cold (Cryofibrinogen). Heparin (Smith and von Korff, 1957), protamine (Lipinski et al, 1967) and alcohol (Godal and Abildgaard, 1966) also precipitate the complexes from plasma. Ultracentrifugation studies demonstrated two peaks of sedimentation in purified cryofibrinogen (Korst and Kratochvil, 1955) and on this basis the protein was thought to be a form of partially polymerised fibrinogen produced by the action of thrombin *in vivo*, in combination with fibrinogen and other proteins. According to Shainoff and Page (1960, 1962) amounts of thrombin, insufficient to cause fibrin formation, split fibrinopeptide A from fibrinogen and the altered fibrinogen then complexes with unaltered fibrinogen. These conjugates were thus of a molecular weight higher than fibrinogen. However, cryofibrinogen is not simply a complex of altered fibrinogen with fibrinogen as

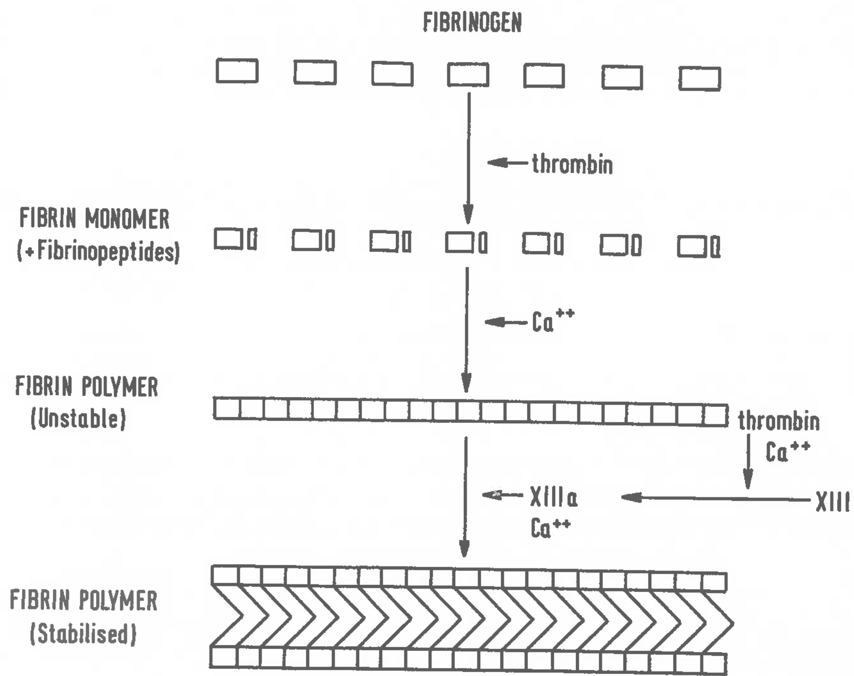


Fig. 3. A scheme of the conversion of fibrinogen to fibrin.

Lipinski et al (1964) demonstrated that fibrinogen degradation products, produced by the action of plasmin on the fibrinogen molecule, were also capable of complexing with fibrin monomers. The same group of workers (Latallo et al, 1970) demonstrated that fibrinogen altered by removal of either fibrinopeptide A, or A and B, was capable of complexing with fibrinogen and fragment X of fibrinogen proteolysis and still retaining its clottability. At present it can be assumed that soluble complexes may be formed as a result of interaction between fibrinogen and its other derivatives. These complexes are in the main not clottable with thrombin and result in the formation of defective fibrin polymers. Once formed, soluble fibrin monomer complexes (S.F.M.C.) are very labile, and changes in ionic strength, temperature or pH are capable of precipitating them. It is possible that in certain clinical situations these physical factors may be of some importance, e.g. acidosis and hypothermia. However, mutual interaction between platelets and S.F.M.C. may be of greater importance (Wegrzynowicz et al, 1970). Certain types of fibrin monomer complexes are potent platelet aggregating agents. The release reaction following aggregation seems in turn to affect the solubility of fibrin, due to release of platelet factor 4 (PF_4)(Niewiarowski et al, 1968). The precipitation of fibrin complexes has considerable practical interest, for only by the understanding of this process will the causes of fibrin deposition within the body be explicable.

1.6 PARACOAGULATION

The process whereby fibrinogen, altered by the action of thrombin, is precipitable (or rendered insoluble) is called paracoagulation. There seem to be three important pre-requisites for paracoagulation to occur:

1. Alteration of fibrinogen molecules to form derivatives capable of spontaneous association.

2. Prevention of these derivatives from undergoing spontaneous polymerization.

3. Induction of spontaneous polymerization by paracoagulating agents.

There seems no doubt that the agent most likely to alter the fibrinogen molecule *in vivo* is thrombin. By splitting off fibrinopeptides A and B, thrombin alters the electrical charge of fibrinogen allowing not only spontaneous polymerization but also complexing with other proteins which if it occurs inhibits spontaneous polymerization. Paracoagulating agents also possess strong electrical charge and precipitate fibrin by dissociating complexes and allowing normal spontaneous polymerization to occur. It has been suggested by Stewart (1970) after electron microscopic study of the polymerization of fibrinogen and its derivatives, that the paracoagulation phenomenon consists primarily of spontaneous polymerization of the X fragment derived from lysis of fibrin (as against fibrinogen) and also fibrin monomer, upon their release from complexes with fragments Y,

D and possibly fibrinogen. Fragment X derived from the plasmin digestion of fibrinogen retains its fibrinopeptides A and B and is prevented from undergoing spontaneous polymerization by the excess negative charge imparted to it by their retention.

1.6.1 ELECTRON MICROSCOPY OF FIBRINOGEN TO FIBRIN CONVERSION

Thrombin induced fibrinogen conversion results in the formation of interlacing fibrin fibres consisting of relatively short broad strands in which a characteristic cross-striation of periodicity ≏ 230 Å is seen. Electron microscopy of the polymerization of fibrinogen and its derivatives has been recently studied (Niewiarowski et al, 1970) and offers some insight into the extent to which the fibrinogen molecule may be altered before derivatives lose the ability to polymerize. Only fragment X is capable of forming highly organised polymers, but retention of fibrinopeptides inhibits this. Of considerable clinical significance was the observation that fibrin/fibrinogen fragments Y, D and E, if precipitated with paracoagulating agents, formed only amorphous precipitates, globules or rudimentary fibrils with no periodicity (Stewart, 1970).

1.7 FIBRINOLYSIS

Fibrinolysis, the physiological antithesis of coagulation, is the process whereby a blood or fibrin clot undergoes liquefaction. For many reasons it has been customary to think of fibrinolysis as a system isolated from its counterpart, but it is perhaps most instructive to look on coagulation and fibrinolysis as two aspects of a

a single system concerned with the deposition and removal of fibrin. Interest in the fibrinolytic mechanism is comparatively recent, despite fundamental observations made as long ago as 1761 by Morgagni who noticed that the blood of persons dying suddenly, remained fluid, and 1838 by Denis who described lysis of whole blood clots. The discovery of streptokinase, the product of streptococci which produces lysis of fibrin, by Tillet and Garner (1933) stimulated the definition of components of the fibrinolytic enzyme system by Milstone (1941) and Christensen and MacLeod (1945), and provided a basis for modern research into fibrinolysis. It has become apparent that fibrinolysis is a basic physiological process concerned not only with maintaining vascular and excretory duct patency but also with inflammation and wound healing. But the study seems only to be in its infancy. If the hypothesis that coagulation and fibrinolysis are continuous and integrated processes in dynamic equilibrium is correct, it must be assumed that the present concepts of the fibrinolytic system represent only the skeleton to which the body will be added in the future. A simplified scheme of the fibrinolytic system is shown in Fig. 4.

1.7.1 COMPONENTS OF THE FIBRINOLYTIC SYSTEM

PLASMIN

Plasmin is a relatively broad-spectrum protease which hydrolyses many proteins including coagulation factors II, V, VIII and IX, complement components and other blood proteins, although in biological situations it has a fairly specific fibrinolytic action. In pure systems fibrinogen and fibrin are equally susceptible to plasmin but

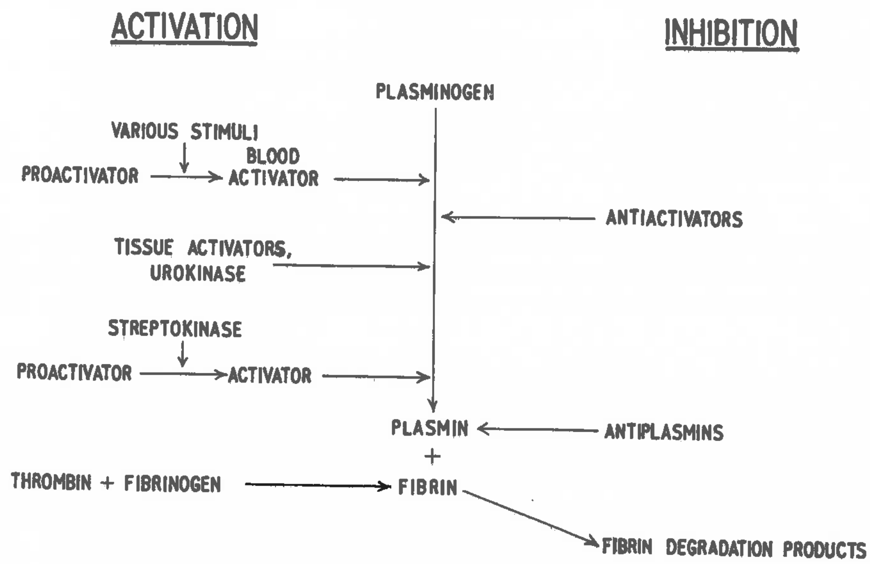


Fig. 4. A simplified scheme of fibrinolysis.

in vivo fibrin is split more readily presumably because the presence of inhibitors in plasma inhibits the reaction with fibrinogen. The adsorption of fibrinolytic components on to fibrin during clotting probably accounts for its relative specificity towards fibrin. Plasmin is known to attack bonds containing arginine or lysine carbonyl (Troll and Sherry, 1955; Ronwin, 1956; Beck and Jackson, 1966). It has a molecular weight of approximately 107,000 (Shulman et al, 1958) but its precise structure is not known. The proteolytic digestion of fibrin and fibrinogen by plasmin will be considered in detail later.

PLASMINOGEN

The inactive precursor of plasmin is a β-globulin contained in the euglobulin fraction of plasma. It has a strong affinity for fibrinogen, so that most purified fibrinogen preparations are contaminated with it. However, separation can be achieved by gel filtration, and purification procedures following this reveal a protein with molecular weight of approximately 90,000. Besides being present in the blood it is widely distributed throughout the body tissues, excretions and secretions. The liver is probably responsible for its production (Sherry, 1965) although immunofluorescent studies suggest that bone marrow cells and particularly eosinophils may contribute (Barnhart and Riddle, 1963).

The conversion of plasminogen to plasmin requires the presence of activators, for plasminogen in plasma exhibits stability over a fairly wide range of pH and temperature, while after isolation it shows no loss of activity after acidification to pH below 4, after

heating to 100°C, and after storage at -30° for as long as 8 weeks.

PLASMINOGEN ACTIVATORS

The plasminogen to plasmin conversion involves removal of part of the plasminogen molecule by the splitting of arginine and lysine bonds (Alkjaersig et al, 1958a). In this way "active sites" are exposed. Activators exist in three forms. Spontaneous or complete activators are substances which act directly on the plasminogen molecule and are present in blood, tissues and body fluids, secretions and excretions. Pro-activators are substances which first must be transformed to their effective form by lysokinases present in tissues, vascular endothelium and bacteria such as streptococci and staphylococci. After removal of inhibitors plasmin itself may also activate plasminogen by a process of autocatalysis (Alkjaersig et al, 1958b).

PLASMA PLASMINOGEN ACTIVATOR

When sufficiently sensitive methods are used, plasminogen activator can be detected in plasma under normal conditions. Increased levels are found under circumstances of stress, anxiety, after exertion or following adrenaline infusion. Flute (1960) was able to distinguish two types of plasma activator by electrophoresis of plasma. One is temperature stable and resembles tissue activator, the other is temperature labile. The nature of these activators is unclear. The labile fraction is rapidly destroyed by heating and acid pH. Most of it is precipitated in the euglobulin fraction of plasma and advantage is taken of this in the commonly performed assay for plasma activator, the euglobulin lysis time. Negligible amounts are found

in serum euglobulin (MacFarlane and Pilling, 1946) as it is adsorbed on to fibrin during clotting (Lassen, 1958).

There now seems little doubt that the blood vessel walls are the major source of plasma activator as originally suggested by Holf (1904). Vascular connective tissue was noticed to have high activator content by Astrup (1961) and Kwaan and Astrup (1964), but use of the technique of fibrinolysis autography developed by Todd (1958) has demonstrated that the intimal surfaces of veins, venules and capillaries are largely responsible for its production (Todd, 1959, 1964; Warren, 1964). It is possible that the especially large contribution of activator to the circulation by such organs as the kidney (Buluk and Furman, 1962; Niewiarowski et al, 1964) and uterus (Maki et al, 1965) is attributable to their vascularity.

The physiological control of plasma activator release has recently attracted much attention. However, as yet, little is known about the factors controlling the release and maintenance of circulating levels. There is diurnal variation in plasma activator concentration with the peak during the day and trough at night (Fearnley et al, 1957). It is probably degraded by the liver and may be excreted in the urine. Many physiological and pharmacological stimuli promote an increase in circulating activator content but Kwaan and McFadzean (1956, 1957) were the first to suggest that its release may be under neurological control. Following this postulate numerous attempts were made to study this neurological mechanism. However, controversial and contradictory observations were made as it was found there was marked

variability from individual to individual both of resting levels and response to stimulation. Moreover, reproducible "stressing events" were not used. A new approach to this subject was introduced by Cash (1966) and Cash and Woodfield, (1968) who by using standardised exercise procedures demonstrated that the fibrinolytic reactivity in any one individual was reproducible, but was variable between individuals. In particular, a group of "poor responders" was isolated. Moreover, standardised adrenaline infusions in the same subjects revealed similar reproducible results (Cash and Allan, 1967). These authors concluded that "when rapid dynamic changes of fibrinolysis are required the ability of the individual to augment the concentration of plasminogen activator could be the single most important factor in maintaining homeostasis" (Cash and Woodfield, 1967). Further study of the adrenergic mechanisms in the systemic plasminogen activator response revealed that it is probably derived from two separate components (Cash, Woodfield and Allan, 1970) which may share the same adrenergic receptor site. A relatively minor one may be secondary to vasoactive changes whereas the second major component is quite independent of this phenomenon. Using selective α and β-adrenergic agonists it has been found that activator release follows β_2 receptor stimulation (Gader et al, 1971). The roles of prostaglandins and adenyl cyclase in activator release are as yet uncertain.

Several theories have been advanced to explain the mechanism of thrombolysis by plasma activator. Each has taken into account the fundamental observation that the dissolution of fibrin can occur

without the appearance of free plasmin in the circulating blood, i.e. fibrin is attacked but fibrinogen is protected. Deposition of fibrin provides a focus for fibrinolysis, and Fearnley (1953) and Sherry et al (1959) have proposed that activator diffuses into the interstices of the clot, is actively adsorbed on to fibrin, and converts plasminogen, entrapped with its closely associated fibrinogen, into plasmin. Local lysis thus ensues and any free plasmin managing to escape is neutralized by circulating anti-plasmins. A different view was put forward by Ambrus and Markus (1960) who suggested that fibrin is able to dissociate plasmin from a plasmin-antiplasmin complex. Wolf (1968) postulated that plasminogen activator and anti-activator exist as a loosely formed complex which dissociates during the diffusion of plasma through a thrombus. As anti-plasmins and anti-activators diffuse less rapidly due to their large size and asymmetrical shape, activator is freed and plasminogen activation occurs unimpeded. While each theory is attractive they are somewhat irreconcilable but not necessarily mutually exclusive. However, further work is necessary for firm conclusions to be drawn. Strikingly, each theory proposes a key role for plasma activator in the physiological control of fibrin lysis.

TISSUE ACTIVATORS

Most tissues of the body contain a spontaneous plasminogen activator which is localized in the microsomes of cell cytoplasm. It possesses marked stability to heat, drying, acidification and to chloroform and is bound firmly to structural proteins. Extraction

and partial purification has been achieved due to its solubility in potassium thiocyanate (Astrup and Stage, 1952) allowing quantitative determination of its concentration in various tissues. It is released from cells after tissue injury and contact with blood or fluids originating from the blood results in plasminogen transformation. Little is known of the chemical properties of tissue activator, but if behaves in a different way to streptokinase.

Its physiological role is probably concerned with tissue maintenance and repair as it seems to be the fibrinolytic counterpart of tissue thromboplastin in the extrinsic coagulation system. Fibrin participates in tissue repair by serving as a matrix for the formation of reparative connective tissue. Tissue activator is of biological significance in preventing overgrowth of reparative tissues as resolution and removal of the fibrin is required for re-establishment of normal structure.

UROKINASE

Specific fibrinolytic activity of the urine was recognized by MacFarlane and Pilling (1947) and an activator of plasminogen — urokinase — was demonstrated in 1951 (Williams, 1951). Urokinase has now been purified and seems to activate plasminogen to plasmin by first order kinetics. Its role in the regulation of endogenous fibrinolysis homeostasis has been difficult to evaluate due to the considerable difference of opinion as to its origin. As the content in renal pelvic urine is the same as excreted urine (Bjerrehus, 1952), it seems unlikely that the lower urinary tract contributes

significantly to its production. Interesting relations with variations in blood activator levels are compatible with the thesis that urokinase is derived at least in part from excreted blood activator (Guest and Celander, 1961). For example, urokinase excretion is increased after physical exercise and cardiac surgery, and is low in renal insufficiency, cardiac failure, and carcinomatosis. Of interest is the observation that persons on a high-salt diet excrete less than those on a restricted salt regime (Celander and Guest, 1960). On the other hand, increased levels of plasma activator induced for example by nicotinic acid injection are not accompanied by changes in urokinase excretion (Holemans et al, 1966). Thus there may be several variants of urokinase. It seems that one of these is produced in the kidney as Kucinski et al (1968) using immunological methods could detect no urokinase activity in renal and peripheral venous blood. Nor did urinary urokinase exhibit immunological identity with plasma, milk or tissue activators. The site of production within the kidney is not clear. Using fibrinolysis autography, activator activity can be detected in glomerular and peritubular capillaries of the renal cortex, but its greatest concentration is in the medulla where presumably it is concentrated. Recent work with tissue cultures has suggested that a soluble activator probably originates in the juxta-medullary apparatus (Painter et al, 1962; Prokopowicz et al, 1964). While these observations excite conjecture as to some relation between salt homeostasis, renin production and fibrinolysis, until more refined technology is available it is perhaps wise to ascribe no physiological function to urokinase apart from maintenance

of the patency of renal tubules and lower urinary tract.

PLASMINOGEN ACTIVATOR IN OTHER BODY FLUIDS

Activators of plasminogen are present in tears, saliva, milk, seminal fluid, cerebrospinal fluid, but their physiological importance is unknown.

NON-PHYSIOLOGICAL ACTIVATORS

Streptokinase identified in 1933 has been purified for use in thrombolytic therapy. Plasminogen is not activated by streptokinase unless proteins from the globulin series are also present (Müllertz and Lassen, 1953). From this was inferred the presence of a pro-activator in human blood.

Other non-specific substances which may cause activation *in vitro* include peptones, urea, heparin and protamine while liquid cultures of Aspergillus aryzae and cell free filtrates of staphylococci contain plasminogen activators.

INHIBITORS OF FIBRINOLYSIS

The fibrinolytic system is kept in check by a mechanism comprised of inhibitors against the formation and action of activators and plasmin. In addition a great number of inhibitors of exogenous origin are known.

PHYSIOLOGICAL ANTIACTIVATORS

Inhibitors of activator seem essential for fibrinolysis regulation, but their separate identity from anti-plasmins is questioned, as although some evidence exists for their presence it is not conclusive. The formation of an anti-activator during incubation of blood in

glass was described by Flute (1960) and found to be enhanced by calcium ions. However, it has no influence on pre-formed activator. Selective impairment of urokinase induced lysis is found in pregnant women during the second and third trimesters, but there is no evidence of inhibition of streptokinase induced activity (Brakman et al, 1963).

PHYSIOLOGICAL ANTIPLASMINS

There are at least four antiplasmins in blood. The α-1 and α-2 antiplasmins in the globulin fraction are the best characterised. Alpha-2 antiplasmin is an immediately effective competitive inhibitor of plasmin action, while α-$_1$, antiplasmin reacts slowly by forming a stable complex with plasmin. The α-1 antitrypsin which accounts for 90% of the trypsin inhibiting capacity of plasma has only negligible activity against plasmin. Another anti-plasmin may exist in the γ-fraction of plasma proteins (Morian et al, 1964). Platelets also possess antiplasmin activity and this may be of importance to *in vivo* resistance to clot lysis, especially as a retracted clot is much more resistant to lysis than one not yet retracted.

MISCELLANEOUS EXOGENOUS ANTI-FIBRINOLYSINS

Several unrelated substances are known to inhibit plasmin action directly or indirectly by virtue of anti-activator activity. These include soya-bean trypsin inhibitor, basic amino acids, heparin, heavy metals, trasylol, epsilon amino carpoic acid (EACA), amino-methyl cyclohexane carboxylic acid (A.M.C.H.A.) and para-amino methyl benzoic acid (P.A.M.B.A.)

1.8 THE PROTEOLYSIS OF FIBRINOGEN AND FIBRIN BY PLASMIN

During the degradation of fibrinogen and fibrin by the fibrinolytic enzyme plasmin, the substrate molecule is split up into several soluble polypeptide fragments known as fibrin/fibrinogen degradation products (F.D.P.). The nature of the products formed *in vitro*, and presumably *in vivo*, by this reaction depends on the time it is allowed to proceed. Early lysis products are of high molecular weight, remain clottable with thrombin, possess powerful anticoagulant properties and are still susceptible to further lysis by plasmin. Continued lysis results in smaller molecular weight non-clottable fragments, with negligible anticoagulant action and resistance to further lysis.

1.8.1 CHARACTERIZATION OF F.D.P.

Five distinct fractions labelled A, B, C, D and E were obtained by Nussenzweig et al (1961) after subjecting fibrinogen digests to D.E.A.E. cellulose. Two of these, D and E precipitated with anti-fibrinogen serum but the remainder appeared to have lost antigenic similarity during digestion. Fletcher et al (1962, 1966) found that only D and E fragments could be reliably produced with prolonged digestion and these workers were able to estimate the molecular weight of these plasmin resistant fragments at 88,000 and 33,000 respectively. On the other hand Jamieson and Pert (1963) using starch gel electrophoresis found 10 different bands.

From observations based on the biological and physical properties of F.D.P., Marder Shulman and Carroll (1969) claim that the lysis of fibrinogen and fibrin is a sequential process in which three phases are

distinguishable. By using immune-electrophoresis, ultracentrifugation and gel filtration techniques it has been able to characterize the nature of the products formed at each stage of digestion. Moreover, using pevikin-block electrophoresis these have been separated in quantities large enough for purification, concentration and subsequent immunological studies (Marder, James and Sherry, 1969). In the first stage, a number of small molecular weight peptides are rapidly split from the fibrinogen molecule (M.W. 300,000) leaving a single, large clottable fragment of M.W. 240-270,000, which has been termed X. The second stage is slower and the sequence of events less clear than in the first phase. The clottability of the products is lost and the anticoagulant properties increased as measured by prolongation of the thrombin time. It is Marder's view that during the second stage, fragment X is cleaved into 2 further parts of molecular weights 155,000 and 90,000 which represent fragments Y and D respectively. The third stage is the slowest and results in the formation of the plasmin resistant fragments D and E (M.W. 30-50,000).

There still remains some disagreement concerning this proteolytic reaction, especially the second and third stages. For example Fletcher et al (1966) proposed that further lysis of fragment X resulted in a large number of products of progressively smaller molecular weights. However, subsequent work with G-200 Sephadex column chromatography provided substantial evidence for the existence of two distinct intermediate degradation products (Marder, Shulman and Carroll, 1967, 1969) and does not lend support to the view that numerous intermediate products other than fragment X appear during plasmin

digestion. It thus seems that plasmin causes an asymmetrical fragmentation of the fibrinogen molecule as represented in Fig. 5. Fibrinogen is first converted into fragment X with the release of the minor fragments A, B and C. Fragment X then splits asymmetrically to fragment Y and fragment D, and fragment Y in turn is split unevenly to a second fragment D and the fragment E. This scheme is comparable with the known information regarding the antigenic determinants (Nussenzweig et al, 1961), the molecular weights of the fragments, the concentrations of fragments D and E in "end-stage" digests where the concentration of D has been found by several workers to be double that of E, and the changes in the electrophoretic patterns of digests during progressive enzyme action.

Recently it has been shown that there is strong immunological identity between the "N-terminal di-sulphide knot" and fragment E (Marder, 1970). As the M.W. of E is double that ofthe knot, there is some conjecture now that both knot moieties may be contained in the centre of the fibrinogen molecule rather than at either end. This hypothesis would be in agreement with the data of Hall et al (1950) who depicted a 3-sphere structure for fibrinogen, as fragment E would then represent the central sphere. It also concurs with the biochemical data of Wallen (1970) who proposed a molecular scheme with the knot moieties joined in the centre of the fibrinogen molecule by fibrin bonds.

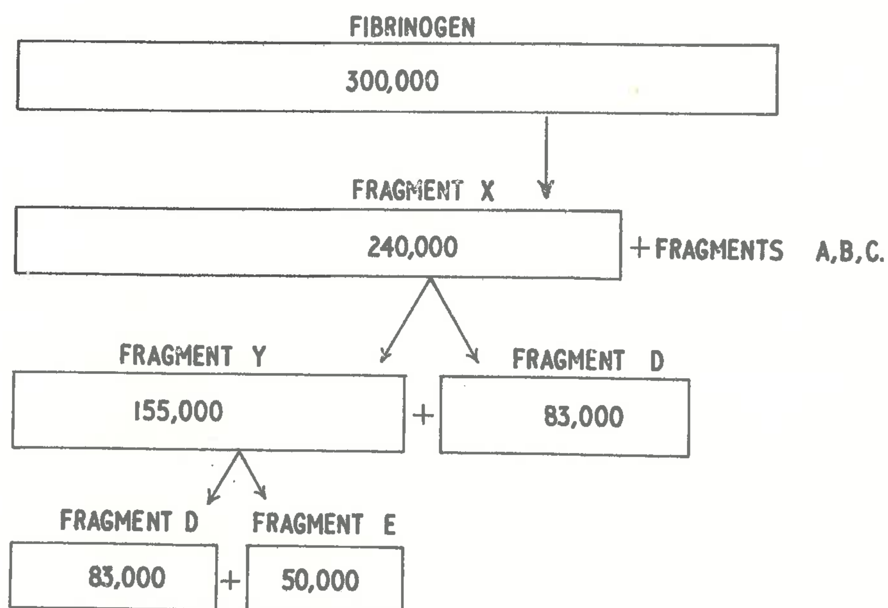


Fig. 5. Schematic representation of the asymmetrical fragmentation of fibrinogen by plasmin (after Marder, V.J., 1970).

1.8.2 BIOLOGICAL PROPERTIES OF F.D.P.

Products formed in the early stages of fibrinogen degradation are strongly anticoagulant, a property originally attributed to inhibition of the action of thrombin (Triantaphyllopoulos, 1958; Niewiarowski and Kowalski, 1958). However, with increased understanding of the fibrinogen to fibrin conversion, it is now possible to attribute other anticoagulant properties to first and second stage F.D.P. Polymerization of fibrin is inhibited (Alkjaersig et al, 1962; Latallo et al, 1962) as F.D.P. complex with normally formed fibrin polymers to form abnormal polymers of diminished tensile strength (Bang et al, 1962; Hirsh et al, 1965) which may themselves be incoagulable (Lipinski et al, 1967). They also complex with fibrinogen, fibrin monomer and activated coagulation factors (Kowalski, 1960; Miller and Sanchez-Avalos, 1968), and may inhibit platelet aggregation (Kowalski et al, 1964, Larrieu et al, 1966) as previously mentioned. This anticoagulant action is of obvious importance in clinical situations of excessive fibrinolysis secondary to intravascular coagulation, where a life-threatening bleeding tendency sometimes develops.

F.D.P. also possess actions of pharmacological importance in that they potentiate the hypotensive effect of bradykinin, and hypertensive effect of angiotensin in the rat, enhance the permeability of capillaries, and the action of adrenaline on the rat myometrium (Malofiejew, 1970). It is not as yet clear whether these properties are due to F.D.P. alone, as pharmacologically active F.D.P. were probably small molecular weight polypeptides similar in size to those potentiating

or inducing platelet aggregation.

1.8.3 QUANTITATION OF F.D.P.

F.D.P. can be detected by measurement of their anticoagulant effect (Worowski et al, 1964) but this method is non specific. The introduction of immunological methods to measure F.D.P. has created great interest, as for the first time quantitation of actual *in vivo* fibrinolysis has been made possible. Anti-fibrinogen serum has been employed in all immuno-assays for F.D.P. as the F.D.P. retain common antigenicity to fibrinogen. Such methods as precipitation by flocculation (Ferreira and Murat, 1963), immuno-electrophoresis (Nilehn and Nilsson, 1964) and radio-immuno-assay (Catt et al, 1968) have been used, but the tanned red cell haemagglutination inhibition immuno-assay (T.R.C.H.I.I.) (Merskey et al, 1966) has gained widest popularity. With the T.R.C.H.I.I. accurate assessment of F.D.P. concentrations previously unattainable have become available. More recently a non-immunological method has been evolved based on the ability of fibrinogen and F.D.P. to clump certain strains of staphylococci (Allington, 1967; Hawiger et al, 1970). This method also provides a sensitive quantitative assay for F.D.P. and it has been compared with the T.R.C.H.I.I. by several groups (Thomas et al, 1970; Thuot and Larrieu, 1971). Both tests are equally sensitive to fibrinogen and higher molecular weight early derivatives. However, the T.R.C.H.I.I. is far more sensitive to the lower molecular weight products D and E. One of the drawbacks of the T.R.C.H.I.I. therefore is its inability to distinguish between and therefore provide a method for

quantitation of the individual fragments in biological fluids as each fragment possesses some antigen(s) in common with fibrinogen and fibrin. For these purposes more laborious methods such as chromatography with various media have to be employed.

1.8.4 CLEARANCE OF PRODUCTS OF FIBRINOLYSIS

The mechanisms available for clearing the products of fibrinolysis include the reticulo-endothelial system, neutrophil leukocytes and the kidney. On the basis of immunofluorescent studies in rabbits after infusion of endotoxin or thrombin (Lee and McCluskey, 1962), the reticulo-endothelial cells of the liver and spleen were implicated in the removal of fibrin. In states of low grade intravascular coagulation it was suggested that the R-E system removed the bulk of fibrin. Under certain circumstances however, neutrophils contribute to this removal (Barnhart and Cress, 1967). It also seems that when renal damage is present, urinary excretion also takes place.

1.9 AIMS OF PROJECT

Personal interest in the role of intravascular coagulation in the pathogenesis and natural history of certain human renal diseases was initiated by a close clinical study of cases of the Haemolytic Uraemic Syndrome. In this uncommon condition features of intravascular coagulation predominate and in our experience almost invariably led to renal failure of acute onset and poor prognosis. A most striking histological abnormality consisted of occlusive thrombus formation within the interlobular arteries, afferent

arterioles and glomeruli of the kidneys; and a histological picture which resembled certain features of malignant hypertension, acute tubular necrosis, renal cortical necrosis, pre-eclampsia and certain types of glomerulonephritis. If, as was postulated (Clarkson, Sage and Lawrence, 1969; Clarkson, Meadows and Lawrence, 1969; Clarkson et al, 1970) intravascular coagulation caused these gross abnormalities in the haemolytic uraemic syndrome, it might play a significant role in the production of similar but less pronounced histological lesions in other renal diseases.

Much of the evidence for the occurrence of intravascular coagulation in human renal disease has been based on histological, ultra-structural and immunofluorescent identification of fibrin in renal biopsy or autopsy material, and the sequence of patho-physiological changes occurring in experimental animals. This has been sufficient to convince several groups of workers of the possible benefit to patients of anti-coagulant therapy despite its potential hazards and the lack of firm evidence for a dynamic coagulation abnormality in these cases. Moreover, the claims of success with this form of treatment by some authors, perhaps provides indirect evidence of the role of coagulation in the evolution of these diseases.

Until recently, the sensitivity of coagulation methods in routine use has been such that intravascular coagulation can only be detected when coagulation is severe and disseminated. A larger number of cases may be associated with either lower grades of disseminated

intravascular coagulation or coagulation localized to a particular organ such as the kidney.

The primary aims of this study were:

1. To investigate the relationship between the results of sensitive coagulation and fibrinolytic assays with clinical, haematological and biochemical features of patients suffering from a wide variety of acute and chronic renal diseases, in order to establish in which of these conditions abnormal coagulation plays a significant role.

2. To correlate these findings with electron microscopic, immunofluorescent and histopathological appearances of renal biopsies and post-mortem tissues.

3. To use appropriate techniques to study the value of appropriate therapeutic regimes.

Section 2.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BUFFERS

1. Aronsson and Gronwall (1957) buffer for fractionation studies was prepared by adding equal parts of 0.5M (60.5G/litre) "tris" (tris (hydroxymethyl) aminomethane)(free base), 0.021M E.D.T.A. (free acid) (6.0G/litre) and 0.075M boric acid (4.6G/litre) and adjusting the pH to 8.9. For elution one part of this buffer was mixed with 19 parts physiological saline and sodium azide (200mg/litre).

2. Veronal Acetate buffer pH 7.4. 9.714G of sodium acetate trihydrate and 14.714G of sodium diethylbarbiturate are dissolved in 500ml distilled water. This stock solution is stored at +4°C. A working solution is made by mixing 25 ml of stock and 25ml of 0.1 N HCl and the volume made up to 500 ml with 0.9% saline. The pH is adjusted to 7.4 with either 0.1N HCl or 0.1 N NaoH.

3. Tris buffer pH 7.8. 73G of tris (hydroxymethyl) amino methane, are dissolved in 2 litres of distilled water and the pH adjusted to 7.8 with $\frac{N}{1}$ HCl. The solution is then made up to 4 litres with distilled water and the pH checked.

4. Barbitone-Saline buffer pH 7.22. Sodium barbitone (5.71G) and sodium chloride (2.93G) are dissolved in distilled water (≏ 960ml), the pH is adjusted to 7.22 with $\frac{N}{1}$ HCL (≏ 25mls) and the volume made up to 1 litre with distilled water.

5. Barbitone-Acetate buffer pH 8.6. Sodium barbitone (50G) and sodium acetate (50G) are dissolved in approximately 4 litres of distilled water and the pH adjusted to 8.6 using $\frac{N}{1}$ HCl. Volume is then made up to 5 litres with distilled water.

6. Phosphate buffered saline pH 8.0. This buffer is made by mixing 9 volumes of 0.15M NaCl (8.767G/litre), one volume of 0.15M anhydrous disodium hydrogen phosphate (21.295G/litre) and 5 volumes of distilled water. The pH is adjusted to 8.0 with either $\frac{N}{1}$ NaOH or $\frac{N}{1}$ HCl.

7. Citrate buffer pH 6.4. A 2 litre volume of this buffer is prepared by mixing 350ml of 0.15M hydrated disodium hydrogen phosphate (20.4135G/ 26.70G/litre, 650ml of 0.15M potassium dihydrogen phosphate (20.4135G/ litre) and 1 litre of 0.1M tri-sodium citrate (29.410G/litre) and adjusting the pH to 6.4 with either citric acid or NaOH.

8. Diluting fluid for F.D.P. assay. To each litre of citrate buffer pH 6.4 was added 1.0G sodium azide as a preservative and bovine serum albumin to a final concentration of 2% as a stabiliser.

2.1.2 REAGENTS

Fibrinogen: A preparation of human fibrinogen of 97% clottability (Kabi Pharmaceutical Company, Stockholm, Sweden) was used on all occasions.

Thrombin: Topical thrombin (Parke Davis and Co., Detroit, Michigan, U.S.A.) was made into varying strengths for different assays: 50 units/ml in tris buffer for fibrin plates; 5 units

per ml in veronal acetate buffer for englobulin lysis time; and 100 units per ml in 0.9% saline for fibrinogen estimation and preparation of the serum for F.D.P. assay. Aliquots were stored at -40°C.

Heparin: (Evans Medical, Ltd., Liverpool, U.K.) in a concentration of 5,000 I.U. per ml.

Urokinase: (Leo Pharmaceuticals, Denmark) 5,000 units per vial were used. Solutions of 3 units per ml in tris buffer and stored at -40°C in plastic containers were used as standards in assays for plasma activator and urinary urokinase.

Trasylol: (Bayer, Germany) containing 25,000 Kallikrein Inhibitor units in 5 mls was used to inhibit fibrinolysis.

Protamine Sulphate: (Roche Products Ltd., Herts, U.K.) was used in concentration of 1, 2, 3 and 4% in saline (weight for volume) in the assay for soluble fibrin monomer complexes.

Anticoagulants:

a. Sodium Citrate B.P. 3.8% (Boots Pure Drug Co., Ltd.)

b. Ammonium Oxalate 0.1M.

Folin and Ciocalteu's Reagent: (British Drug Houses, Ltd., England).

Fibrinogen Anti-Serum: The preparation used for the F.D.P. assay was that of Behringwerke (Germany) prepared in rabbits against human fibrinogen.

Glutaraldehyde: was obtained from Koch-Light Laboratories, Ltd., Buckinghamshire, U.K., in 25% solution in water.

Polyethylene Glycol ("Carbowax"): was obtained from Union

Carbide, U.K. Ltd., Southhampton.

Microtiter equipment: including microtiter plates (V-bottom wells) microtiter pipettes (delivery volume 0.025ml), microdiluter gun and heads (delivery volume 0.025ml) and mirror were obtained from Cooke Engineering Co., 735, N. St. Asaph Street, Alexandria, Virginia, U.S.A.

Agarose 8% ("Bio-Gel A-1.5M") for column chromatography was obtained from Bio-Rad Laboratories, 32nd and Griffin, Richmond, California, U.S.A.

G-200 Sephadex was obtained from Pharmacia, London, U.K. Dextran Blue and the Chromatographic Column were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Agar (Difco) for immunoelectrophoresis. 1.5G of agar and 0.1G sodium azide are mixed into a solution with 50ml distilled water and 50ml of sodium acetate buffer. The mixture is heated in a boiling water bath until the agar dissolves and gives a clear solution. The agar is used immediately or stored in suitable aliquots at room temperature.

Ponceau-S-Solution for staining immuno-electrophoresis slides. 0.2% Ponceau-S stain is mixed in equal volumes with 6% sulpho-salicylic acid.

2.2 METHODS

2.2.1 PLASMA FIBRINOGEN

Two methods were used.

a. Fibrinogen assay as described by Ratnoff and Menzies (1951) as modified by Alkjaersig (1960).

This method was used as the standard reference assay with which other fibrinogen assay results could be compared. The test specimen is diluted in E.A.C.A. solution to prevent proteolysis, and clotted with thrombin. The resultant fibrin is precipitated, separated and hydrolysed with sodium hydroxide and boiling. The amount of tyrosine released by hydrolysis is estimated with Folin Ciocalteu's reagent and the fibrinogen concentration calculated from a standard tyrosine curve.

Into each of four 5' x ⅝" non-siliconized test tubes is added 6.0ml E.A.C.A. saline and 0.2 ml of 2.5% calcium chloride solution. 0.1 ml of thrombin (100 units/ml in 0.9% saline) is then added to 3 tubes (the fourth remaining as the blank) after which 0.2 ml of the test solution is added to each tube and thoroughly mixed. To ensure complete coagulation, the tubes are incubated at 4°C for 12 hours before the addition of constant amounts of well-washed glassbeads (diameter 0.15mm) which aids the subsequent precipitation of the fibrin by centrifugation at 3,400 r.p.m. at 4°C for 5 minutes. After removal of the supernatant, the glass beads and entrapped fibrin are washed 3 times with 0.9% saline and then hydrolysed by the addition

of 0.4ml of 10% sodium hydroxide. Hydrolysis is completed by boiling the tubes in a water bath for 20 minutes. The tubes are cooled, 2ml of 0.5N sodium hydroxide and then 0.6ml Folin and Ciocalteu's reagent (diluted 1:2 in distilled water) are added in sequence and mixed, and the tubes centrifuged at 4,000 r.p.m. at room temperature for 2 minutes. The tyrosine released by hydrolysis is assessed by measuring optical density in a Unicam S.P.600 at 650mμ using the red filter, the non-clotted tube acting as the blank. The tyrosine released is calculated from a previously prepared standard tyrosine curve, and converted to fibrinogen concentration by multiplying by 11.7. This technique is time consuming and tedious and not easily adapted for routine work.

b. Fibrinogen Assay as described by Ellis and Stransky (1961).

This provided a rapid reproducible assay easy to perform and was used for routine purposes. The increase in optical density resulting from the formation of fibrin is measured, in citrated plasma, recalcified and clotted with thrombin. By comparison with a previously prepared standard fibrinogen curve, the fibrinogen content of the test plasma can be calculated.

0.5ml of test plasma is added to 5.5ml barbitone saline buffer (pH 7.22) in a glass test tube, and mixed by inversion. 3.0ml aliquots of this solution is added to Unicam cells one of which is used for the estimation, the other for the blank. 0.5ml of calcium-thrombin solution is then added to the test cell and mixed by inversion. After incubation at room temperature for 20 minutes, the optimal

density of the test cell is compared with the blank cell in a Unicam S.P. 600, set at 470mμ with the blue filter.

2.2.2 SOLUBLE FIBRIN MONOMER COMPLEXES

The technique of Lipinski and Worowski (1968) has been used for detecting soluble fibrin monomer complexes in plasma. Blood samples are placed in oxalated tubes (1 part Ammonium Oxalate to 9 parts blood) and the plasma separated after centrifugation at 3,000 r.p.m. at room temperature for 15 minutes. Assay may be performed immediately or after storage at -40°C. 0.25ml of test plasma is placed in each of 2 cuvettes and diluted with 0.75ml and 0.85ml of saline respectively. To the test cuvette 0.1ml of protamine sulphate is added and mixed. After 5 minutes, the optical density of the test is read in a Unicam S.P. 600 at 619mμ, with the cuvette to which no protamine sulphate has been added serving as the blank. Results are expressed in optical density (O.D.) units. During the preliminary investigations of this technique, the optimal concentrations and different types of protamine sulphate commercially available were assessed on a series of individual plasmas. As can be seen from Fig. 6 comparable results were obtained using 8 separate sources of protamine sulphate. The relationship held if the precipitation was performed with 1%, 2%, 3% or 4% protamine sulphate, but the studies revealed that there was an increasing precipitation with increasing protamine sulphate concentration (Fig. 7). It was concluded therefore that it would be more appropriate to work on the

Protamine SO4 Preparation (1%)	Fish	Plasma Sample No.									
		1	2	3	4	5	6	7	8	9	10
Roche	S	0.61	0.72	0.53	0.53	0.51	0.75	0.65	0.65	0.43	0.55
B-D	S	0.59	0.70	0.52	0.52	0.55	0.82	0.72	0.68	0.45	0.57
Koch-Light	H	0.72	0.74	0.59	0.53	0.52	0.86	0.75	0.72	0.45	0.59
Sigma I	S	0.63	0.74	0.62	0.57	0.58	0.77	0.67	0.68	0.41	0.57
Sigma III	H	-	-	-	-	-	0.71	0.60	0.61	0.42	0.51
B.D.H.	S	-	-	-	-	-	-	0.67	0.68	0.44	0.57
B.D.H.	H	-	-	-	-	-	0.77	0.65	0.62	0.44	0.52
K.K. Lab.	H	-	-	-	-	-	0.77	0.65	0.55	0.42	0.54

Fig. 6. Optical densities obtained after the addition of 1% protamine sulphate from different sources to a series of individual plasmas.

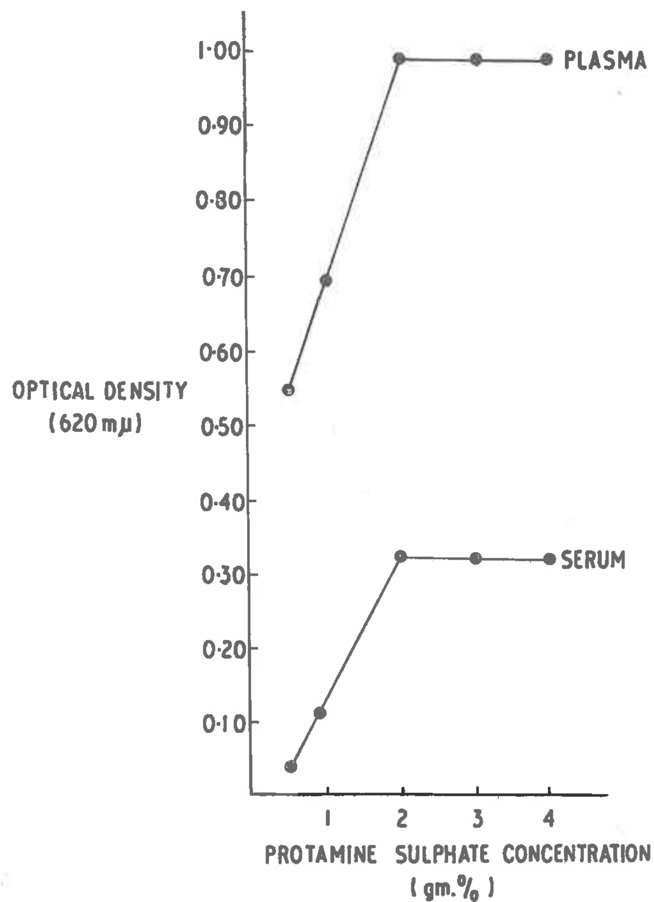


Fig. 7. Changes in optical density in a citrated plasma and serum sample with increasing concentrations of protamine sulphate (Roche).

more stable part of the curve and use a 5% protamine sulphate solution in the test rather than the 1% recommended by Lipinski and Worowski (1968). Subsequent work on this technique has demonstrated that only fibrin and fragment X are precipitated after protamine sulphate addition to test plasma, so that it seems that the prior action of thrombin on fibrinogen is necessary to cause significant increase in optical density (Palester-Chlebowczyk et al, 1970).

2.2.3 PLASMA PLASMINOGEN ACTIVATOR

In the early stages of this investigation 2 separate assays were run in parallel for themeasurement of plasma activator content. These were the euglobulin lysis time (E.L.T.) and the fibrin plate assay, which gave comparable results. Later, for routine work, the E.L.T. was used because of its greater sensitivity and ease of handling in the face of multiple specimens.

1. Euglobulin Lysis Time. The lytic activity of the euglobulin fraction of plasma, in which fibrinolytic inhibitors are virtually absent, is regarded as being a measure of plasma plasminogen activator concentration provided free plasmin is not present. During the preparation of euglobulin by dilution and acidification of plasma, fibrinolytic inhibitors are discarded in the supernatant, leaving plasminogen activator, plasminogen, fibrinogen and other plasma proteins. After precipitation of euglobulin, it is resuspended, clotted with thrombin and the time taken for lysis to occur observed and recorded. The method is as described by Cash (1967). Venous blood is collected, if possible without stasis and placed in citrated

tubes (1 part 3.8% sodium citrate to 9 parts blood) kept on ice to prevent the deterioration of activator which occurs at higher temperatures. As short a time as possible (less than 10 minutes) should be taken in transportation to a refrigerated (+4°C) centrifuge where it is spun for 20 minutes at 3,400 r.p.m. One ml of the supernatant plasma is placed in a siliconised centrifuge tube containing 19.0ml of dilute acetic acid. The pH is adjusted to 6.0 with 0.25% acetic acid and euglobulin precipitation continued at 4°C for 10 minutes. After further centrifugation at 3,400 r.p.m. for 10 minutes and decanting of the supernatant, the euglobulin precipitate is resuspended in 1.0ml veronal acetate buffer (pH 7.4). The assay is performed in triplicate by placing 0.24ml of the euglobulin suspension in each of three 3" x ½" siliconised tubes and adding 0.24ml thrombin (5 units per ml). Lysis time at 37°C is recorded by eye or automatically in a clot lysis recorder (Cash and Leask, 1967). In all studies, lysis times were converted to activator units by using the formula of Januszko and Dubinska (1964), $\frac{1500}{T}$ where T is the euglobulin lysis time in minutes. This adjustment is probably valid in physiological situations, but some doubts must arise concerning this concept in circumstances where the presence of free plasmin cannot be excluded.

2. Fibrin Plate Assay. This assay provides confirmation of euglobulin lysis time assay but should be regarded as semi-quantitative at best and relatively insensitive.

Fibrin plates are prepared on a level table by the addition of 0.2ml thrombin (50 units per ml in tris buffer pH 7.8) to 10ml of 0.150% human fibrinogen (Kabi) in a plastic disposable petri dish.

When clotting is complete ($\simeq$20 minutes), 0.025ml of resuspended englobulin precipitate is dropped in triplicate on to the fibrin films. After incubation at 37°C for 20 hours, the perpendicular diameters of the lysed areas are measured and compared with the area of lysis produced by urokinase (3.0 units per ml in tris buffer pH 7.8) on a separate fibrin plate.

2.2.4 UROKINASE

Urokinase was measured on fibrin plates prepared by methods similar to those described for plasma activator. An aliquot of the urine specimen to be tested is dialysed against tap water for 6 hours and diluted 1 in 5 in tris buffer pH 7.8 before assay. Assay is performed in triplicate by dropping 0.025 ml of the diluted sample on to the plate which is then incubated at 37°C for 20 hours. The area of lysis is compared with that produced by 0.025ml of urokinase (3 Ploug units per ml in tris buffer pH 7.8), and the results expressed in terms of concentration or as an excretion rate depending on the nature of the study.

2.2.5 FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS (F.D.P.).

The method used for estimation of F.D.P. in this study was the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) as described originally by Merskey et al (1966) and which has been fully investigated in these laboratories (Das, 1968). In the early part of the present study sensitised sheep red cells were used

which necessitated a time-consuming absorption procedure of all human samples prior to their assay. However, when it became possible to prepare stable and sensitive human red cells (Hoq and Das, 1970) this absorption stage was eliminated for serum samples, and human red cells were used in the routine assay. These cells provide the end-point or indicator for the assay, for when conjugated with fibrinogen they can be agglutinated by fibrinogen antiserum. Haemagglutination can be inhibited by the presence of substances which carry antigens capable of combining with the antiserum, and this degree of inhibition can therefore be titrated for quantitation of such substances as fibrinogen, fibrin or their degradation products. The assay which is specific has been increased in sensitivity by the use of human cells.

TECHNIQUE OF TANNED RED CELL HAEMAGGLUTINATION INHIBITION IMMUNOASSAY

1. Preparation of sensitised cells:

Fixing: The preparation of sensitised sheep cells has been fully documented by Das (1970) and will not be discussed here. Human group O Rhesus negative cells are washed three times in 20 times the volume of 0.15M sodium chloride to render the cells plasma free. A 2% suspension of cells in phosphate buffered saline pH 8 (P.B.S. 8) is then mixed with an equal volume of 1% glutaraldehyde solution in P.B.S. 8. The fixing cells are kept in iced water for 30 to 60 minutes and mixed frequently. An extensive washing procedure in 0.15M saline (3 times), distilled water (3 times) and P.B.S. 8 (3 times) is then undertaken to remove residual

glutaraldehyde and provide the optimum conditions for tanning.

Tanning: Equal parts of an 8% cell suspension in P.B.S. 8 are mixed with a 1:10,000 (weight:volume) solution of tannic acid in P.B.S. 8 at 56°C for 60 minutes. The cells are then washed three times in P.B.S. 8 to remove free tannic acid from the suspension prior to coating.

Coating: After a further wash with citrate buffer pH 6.4 which provides the optimum pH for coating, an 8% suspension of cells in citrate buffer is incubated at 37°C for 30 minutes with an equal volume of human fibrinogen solution (30µg/ml). The cells are washed three times in citrate buffer pH 6.4 and resuspended in diluting fluid in a 10% concentration. The cells are stored in plastic tubes at 4°C and diluted to 2.5% suspension prior to use in the assay. Just before use further washing with citrate buffer and diluting fluid is necessary to ensure that any fibrinogen eluted from the cells on storage is not present in the assay system.

2. Collection and preparation of specimens:

Serum: 2.5ml of blood is collected into plastic tubes containing 0.1ml Trasylol (5,000 K.I. units/ml) as a fibrinolytic inhibitor, and thoroughly mixed. These tubes are incubated for at least 4 hours at 37°C to allow clot retraction and the serum separated by centrifugation and stored at -40°C in small plastic tubes.

Prior to assay of serum samples, thrombin (100 units per ml in saline), in a volume one-eleventh that of sample volume

is added to each and incubated for 1 hour at 37°C. This procedure is crucial as incomplete removal of fibrinogen from the test sample invalidates the results of the assay.

Urine: Aliquots of 24 hour urine or early morning urine specimens are collected in universal containers with no fibrinolytic inhibitor added. It was felt that any inhibitor inserted into the collection bottle would be superfluous as maximum lysis probably occurs in the urinary tract. After the volume of the specimen is measured it is dialysed against running tap-water in Visking dialysis tubing for between 6-8 hours to establish a relatively constant pH and ionic strength. It is then concentrated in polyethylene glycol (Carbowax). This material was chosen because of its ability to concentrate many specimens simultaneously at a cost much lower than other available concentration procedures. It is, however, comparatively inefficient in terms of time, and usually specimens are left for between 12-16 hours before adequate (1 in 20 to 1 in 40 times) concentration of protein is achieved. The concentration factor is calculated after recording the volume of the concentrated specimen, which is stored at -40°C in small plastic tubes. The concentration factor was determined on 20 specimens by both buiret determination of urinary protein and by volume measurements. As there was a highly significant degree of agreement between the 2 methods (Fig. 8), the more simple and less time consuming procedure of volume measurement was adopted for routine use.

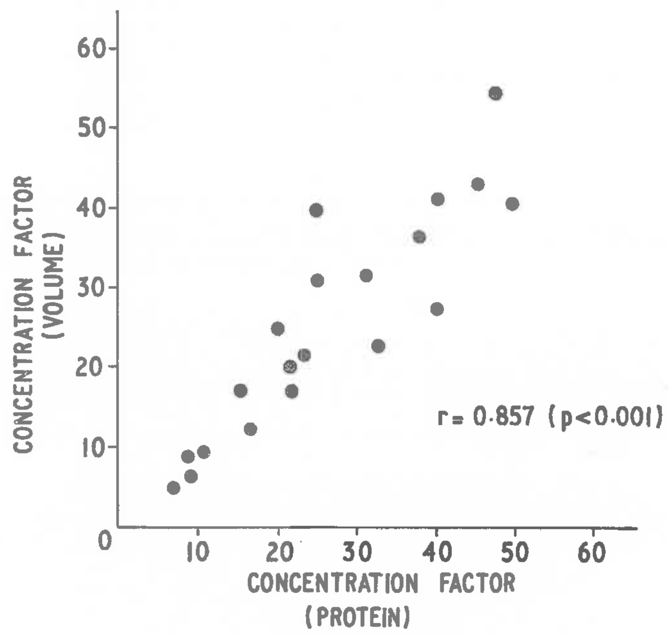


Fig. 8. Correlation of the two methods evaluated for determining the concentration factor.

Polyethylene glycol is known to pass through dialysis tubing and theoretically therefore, might interfere with the agglutination reaction in the F.R.C.H.I.I. In 20 samples to which minute concentrations of polyethylene glycol were added there was no difference in haemagglutination inhibition titre when compared with the uncontaminated specimen.

Despite the use of human cells, absorption of concentration urine samples was found to be necessary because in 60% of 80 samples assayed without prior absorption, the titre of non-specific agglutinine was sufficiently high to interfere with recording of the specific haemagglutination inhibition end-point. To each 2 volumes of specimen is added one volume of packed, glutaraldehyde fixed human group O, Rh negative cells and the mixture incubated overnight at 4°C. The suspension is then centrifuged, the absorbed urine separated and assayed, or stored at -40°C.

3. Assay of Test samples: Doubling (1:2:4:8, etc) or alternate doubling (2:3:4:6, etc.) dilutions of the test sample in diluting fluid are prepared in microtiter plates using the microtiter multiple diluting gun (Fig. 9). This is facilitated by the use of a microtiter pipette which is designed to deliver 0.025ml per drop, and the heads of the gun which delivers the same volume. To each cup of the microtiter plate is then added 0.025ml of fibrinogen antiserum of predetermined strength. The plates are mixed and incubated for 4 hours at room temperature. After incubation, 0.025ml of thrice-washed, 2.5% fibrinogen-coated red cell suspension is added to each cup. The plates are mixed and left at

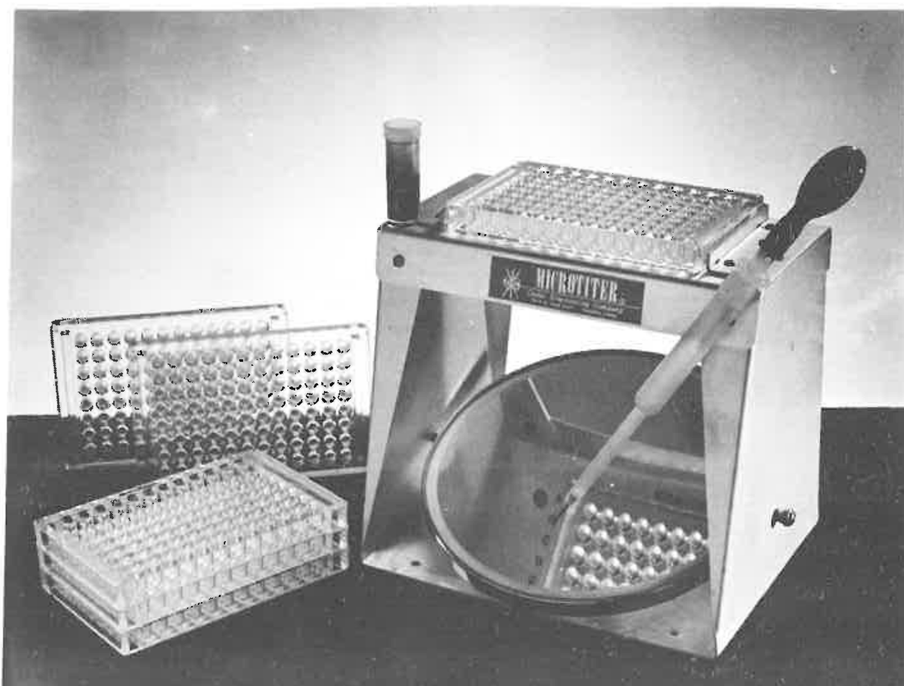


Fig. 9. Equipment used in the Tanned Red Cell Haemagglutination Inhibition Immunoassay for F.D.P.

room temperature overnight.

Fibrinogen standards (1.25, 2.5 and 5.0mg/ml) and a control serum sample are included with each assay and are treated identically to the samples being assayed. The fibrinogen content of these standards is checked regularly using the reference method of Ratnoff and Menzies (1951). Positive and negative controls are included in each assay.

CALCULATION OF RESULTS

The mean sensitivity of the system is calculated from the fibrinogen standards after recording the haemagglutination inhibition titre in each. Thus, division of the fibrinogen content (in μg/ml) by the inhibition titre, gives the quantity of fibrinogen causing haemagglutination inhibition in the test system. The mean of the 3 standards is taken as the sensitivity for that day, and F.D.P. content of each unknown is calculated by multiplying the sensitivity by the inhibition titre of the unknown. A control serum sample is included with each assay as a further check against gross errors in techniques.

For urine samples, the F.D.P. content derived from the assay is that of the concentrated specimen, and accordingly has to be divided by the concentration factor in order to ascertain the F.D.P. concentration of the original urine specimen.

$$\text{Sensitivity} = \frac{\text{Fibrinogen content of standard}}{\text{Haemagglutination inhibition titre of that standard}} \ \mu g/ml$$

$$\text{F.D.P. content of unknown} = \text{Sensitivity} \times \text{Haemagglutination inhibition titre of the unknown}$$

$$\text{F.D.P. content of urine} = \frac{\text{F.D.P. of unknown}}{\text{Concentration factor}} \ \mu g/ml$$

Although the sensitivity of the assay system varied from day to day the reproducibility was remarkably consistent. For example the mean value and standard deviation of the control serum in 168 separate assays over the course of 12 months was 7.04 ± 0.86 $\mu g/ml$. Sensitivity was influenced by several factors including the concentration of antiserum used in the assay and the time of incubation of sample with the antiserum.

ANTISERUM CONCENTRATION

The optimum concentration of antiserum to be used in the assay was calculated from an antiserum dilution test. The fibrinogen antiserum was stored in small aliquots of 1:500 dilution in diluting fluid at -40°C. In this test a $\frac{1}{500}$ dilution of antiserum is diluted progressively in a microtiter plate and fibrinogen coated cells added to each well. The concentration of antiserum most suitable for assay with those cells was approximately twice that of the minimum concentration causing haemagglutination in this test.

2.2.6 PLATELET FACTOR 4 (PF_4)

Platelet anti-heparin substance is assayed by virtue of its heparin neutralizing properties. The assay, although specific, is insensitive and unreliable and its poor reproducibility allows only crude changes in its concentration to be measured. In this regard it is perhaps significant to reflect that 15 years passed between the original description of PF_4 by Von Crovold and Paulssen (1951) and the first successful assay procedure (Farbiszowski et al, 1966). Since then several authors have described its use in measurement of PF_4 release after platelet aggregation in vitro (O'Brien, 1969; Niewiarowski et al, 1968; Youssef and Barkhan, 1969; Niewiarowski and Thomas, 1969). To this day however, no accurate and sensitive assay exists for the measurement of plasma PF_4 concentrations. If this was possible valuable information may be obtained concerning platelet function in vivo. The method used in this study is a modification (Fuster, 1971) of the method described by Farbiszewski et al (1966), in which the anti-heparin activity of the test plasma is assayed in a modified heparin-thrombin time system.

COLLECTION AND PREPARATION OF SPECIMENS

By clean venepuncture, 5mls blood is withdrawn, placed in citrated tubes (9 parts blood to 1 part 3.8% sodium citrate) and thoroughly mixed. Platelet poor plasma is separated by centrifugation at 3,400 r.p.m. for 15 minutes and then heated to 60°C in a water bath for 10 minutes to denature fibrinogen and anti-thrombin materials present. These are removed after further centrifugation

at 3,400 r.p.m. for 10 minutes and the supernatant plasma is ready for assay or is stored at -40°C in small aliquots.

ASSAY PROCEDURE

Test plasma (0.1ml), pooled unheated human platelet poor plasma (0.3ml), heparin (0.1ml of 1.0 unit/ml in veronal acetate buffer pH 7.4) and thrombin (0.1ml of 30 units/ml in veronal acetate buffer pH 7.4) are placed together in a siliconised glass tube kept at 37°C and the subsequent clotting time noted and compared with the clotting time of a control in which distilled water 0.1 ml is substituted for the test plasma. The results are expressed as an inverse percentage of the control, so that low clotting times (high PF_4 content) are expressed as percentages higher than the control. The clotting assay is performed in an automatic coagulometer thermostatically controlled to 37°C in which four simultaneous assays could be performed. The mean result of 100 healthy subjects was 106 ± 25.6%.

2.2.7 COLUMN CHROMATOGRAPHY

Column Chromatography was performed with both G-200 Sephadex and 8% Agarose in a column 100 x 2.5cm. Prior to use, the gel was thoroughly washed in azide saline, and then poured on to the column. Packing was achieved by continuous flow of Aronsson and Gronwall (1957) Buffer, after which the column was ready for use. Samples were inserted on to the top of the column with a constant flow infusion pump and elution was achieved in Aronsson and Gronwall

buffer by a similar pump inserted into the outflow system of the column. Eluates were collected into tubes placed on an automatically timed fraction collector.

Controls built into the chromatographic procedure included:

1. A marker dye (Dextran Blue) introduced into each assay specimen in such quantity as to render its peak concentration in the eluates easy to determine by eye. The tube in which this occurred was taken as the void volume (V_o), each subsequent tube containing eluates of the sample.

2. Glucose added to the specimen before elution so that the tube containing maximum glucose concentration as measured by standard reduction reaction was readily identified. This marked the termination of the sample elution (V_T).

3. Frequent checks were made to ensure that the fraction volume remained constant, the column length was static, there were no bacteria growing within the gel, and that the pump tubing in particular was not leaking.

2.2.8 IMMUNE-ELECTROPHORESIS

Ten ml of dissolved 1.5% agar is poured on to 3 microscope slides held in a slat placed on a level table. Once the agar has set the slats are stored in a moist chamber following which troughs and wells are cut. Samples to be electrophoresed are placed in the wells, and the slides placed in position in the apparatus so that electrical contact is made between the gel and buffer solution in

the electrophoresis tank. Electrophoresis is carried out at 7.5 m.amps per section or 15m.amps per slat for 2 hours after which anti-serum is placed in the troughs and the racks placed in a moist box for 2 days, then covered with azide saline for a further 2 days. After drying with filter paper and washing with tap-water the slides are placed in Ponceau-S stain for 10 minutes. After the stain is poured off, the slide is washed in 2% acetic acid until all excess stain is removed, andthen dried in air.

2.2.9 URINARY PROTEIN

The buiret method of Hiller et al (1948) was used to determine the concentration and thus the total 24 hour protein loss in the urine. These estimations were all performed in the Department of Clinical Chemistry, University of Edinburgh. Urinary protein is first precipitated with an equal volume of 10% w/v trichloracetic acid, the precipitate dissolved in 30% w.v. NaOH, and the buiret colour developed by 5% w/v $CuSO_4$. The concentration of protein is determined by a Lovibond comparator.

2.2.10 PROTEIN SELECTIVITY

The method used in this study was that of immuno-diffusion as described by Blainey et al (1960) and was performed in the labor-atories of the Medical Renal Unit, Department of Medicine, The Royal Infirmary, Edinburgh, as modified by MacLean (1966). A

solution of 1% w/v agar in 0.2M phosphate buffered saline pH 7.0 prepared by heating over a boiling water bath and subsequent filtering, is poured into flat-bottomed Petri dishes. A pattern of 6 large holes each surrounded by 6 small holes is cut in the agar with a special template. Dilutions of serum are placed in 4 of the large holes and urine (often previously concentrated) in the remaining 2 large holes. Dilutions are made up in the 0.2M phosphate buffered saline. In early work immunological studies using 5 anti-sera, albumin, transferrin, γ-globulin, α_2-macroglobulin and β-lipoprotein were performed, but latterly only transferrin and α_2-macroglobulin were used (MacLean and Robson, 1967). After 24-48 hours the antigen-antibody precipitation lines were seen as hexagons surrounding each large hole. By comparing the thickness, intensity and position of the precipitation lines in relation to the antibody dilutions it is possible to match the urine and serum hexagons, and obtain a urine:serum ratio for the protein under study. The clearance of all the proteins could then be expressed as a percentage of the albumin urine:serum ratio and the relative clearances from serum and urine samples of different protein concentrations could be compared. When only transferrin and α_2-macroglobulin were used, the clearance of the latter was compared with that of transferrin which was taken as 100%. Using a log:log scale the relative clearances of the individual proteins are plotted against the molecular weight and an approximately linear relationship is found. The slope of this line (k) is an index of the selectivity of proteinuria and is calculated by estimating the

regression line by the method of least squares.

A selective proteinuria contains a small proportion of high molecular weight protein and the slope of the line (k) is very steep. Conversely when urine contains a large proportion of high molecular weight protein the slope of the line (k) is flatter and the proteinuria is said to be unselective. Besides reflecting the permeability of the glomerulus to macromolecules, the selectivity of proteinuria has been shown to be of clinical value in the differential diagnosis of patients with nephrotic syndrome, and in predicting which of these patients are likely to undergo remission with corticosteroid therapy. The normal glomerulus is thought to be highly selective (Petrie et al, 1970).

2.2.11 HISTOLOGICAL STUDIES

With few exceptions all the patients studied had a variety of renal diseases which were diagnosed on the basis of clinical and pathological evidence. Renal histology was evaluated in most instances on biopsy specimens obtained with a modified Vim Silverman needle. If biopsy was not procured during life, autopsy specimens were obtained.

Sections for light microscopy were routinely stained with haematoxylin and eosin and periodic acid-Schiff stains. In selected cases M.S.B. and picro-Mallory stains were also employed. Material for electron microscopy was fixed in 1% buffered osmium tetroxide and embedded in Araldite. Sections were cut on a Porter Blum microtome

with glass knives, stained with lead citrate and alcoholic uranyl acetate, and viewed in an A.E.I. EM6 electron microscope.

For immunofluorescent studies frozen sections were cut at 2 thickness, fixed in 95% ethanol for 10 minutes, rehydrated with phosphate buffered saline (pH 7.4) and then exposed to fluorescein conjugated human fibrinogen anti-serum for 30 minutes at room temperature. The anti-serum was prepared in rabbits and obtained commercially from Behringwerke. Prior to use the anti-serum was absorbed with rat liver powder and its specificity confirmed by immuno-electrophoresis. Throughout the study, blocking techniques were carried out randomly to confirm uniformity. Sections were viewed with a Leitz Ortholux microscope employing incident light from a Wotan HBO 200 W/4 lamp with BG 38 and UG 12 excitation filters and a K530 barrier filter. Photographs were taken with a Leitz Orthomat camera using Kodak Ektachrome daylight film.

Section 3.

ACUTE RENAL DISEASE

An acute decrease in renal function may arise in a large number of clinical circumstances and results in a sudden breakdown of the kidneys' contribution to body metabolism. The complexities of the underlying causes often cloud the clinical features of acute renal failure, so that recognition of the event is sometimes delayed. Since the introduction of a more physiological approach to the management of acute renal failure, (Bull et al, 1950) and the more recent advent of dialysis, the essential unity of the clinical syndrome has, however, been more widely recognised. The excretion of urea and other end products of protein metabolism does not keep pace with their rate of production and they accumulate; the agencies responsible for water, sodium and potassium balance no longer operate, and in the face of unrestricted intake oedema and hyperkalaemia are inevitable; the renal contribution to acid-base balance is deficient and metabolic acidosis develops. These metabolic abnormalities which are the universal accompaniment of acute renal failure are supplemented by the clinical features of uraemia, and if not relieved may result in the death of the patient. With successful treatment, however, gradual recovery of renal function usually occurs after a variable period of oliguria or even anuria and is accompanied by massive diuresis. In autopsy specimens of patients dying during such an illness, or in renal biopsies taken during the phase of oliguria the light microscopy reveals lesions of patchy and variable distribution throughout the cortex involving the renal tubules. The tubular lining cells are necrotic and there is disruption of the tubular basement membrane so that free communication between adjacent capillaries and the tubular lumen may be seen. Often the tubular lumen appears

blocked by cellular debris and casts. Early workers coined the phrase lower nephron nephrosis, but more recently the term acute tubular necrosis has been used to describe these lesions which have been held responsible for the loss of renal function. The lack of obvious glomerular abnormality and the regeneration of renal tubular cells demonstrable during recovery seem to support this thesis.

While clinical recovery occurs in the majority it is not a universal feature of such cases. Uncommonly renal failure persists or only partial recovery of function occurs, perhaps after a protracted course of treatment. In these circumstances histological examination usually discloses obvious inflammatory lesions of the glomerulus, or more proximal renal vascular tree in association with the tubular lesions which may be so diffuse that they coalesce.

In this section, the more common form of acute renal failure will be discussed in the first part, and the second part deals with a condition which tends to cause irrecoverable renal failure, viz, the haemolytic uraemic syndrome. Glomerulonephritis will be discussed in a later chapter.

3.1 ACUTE ISCHAEMIC RENAL FAILURE

The mechanism of the prolonged depression of renal function in acute ischaemic renal failure has been the subject of speculation since the original descriptions of the syndrome by Baker and Dodds (1925) and Bywaters and Beall,(1941), and a number of theories have been proposed to account for it. The weight of clinical evidence rather than the direct measurement of renal blood flow has led to the hypothesis

that renal ischaemia is the common precipitating factor in the human disease (van Slyke, 1948; Simeone et al, 1950; Brun and Munck, 1957). On the basis of morphological observations in man, Peters (1945) Oliver et al (1951) and Merrill (1960) have suggested that the resultant oliguria is due to compression of vessels and tubules by interstitial oedema, which when it resolves allows normal function to resume. However, several lines of evidence do not support this view. Renal decapsulation does not encourage diuresis (Peters, 1945) and intra-renal pressure measurements determined by the insertion of needles (Reubi, 1956) or by catheters wedged into the renal vein (Brun, et al, 1956; Munck, 1958) do not differ from normal. Obstruction of tubules by cellular debris and casts has also been put forward by several workers (Baker and Dodds, 1925; Bywaters and Beall, 1941; Meroney and Rubini, 1959) but the inconstancy of this histological finding has rendered this theory inconclusive. The other classical view based on histological and microdissection observations is that oliguria results from passive reabsorption of normally formed glomerular filtrate through the damaged tubules (Dunn et al, 1941; Govaerts, 1948; Oliver et al, 1951).

Theories advanced to explain the loss of renal function on changes in blood flow to the kidney have been based, until recently, on somewhat controversial evidence. For example, the marked reduction of renal blood flow during the oliguric phase determined by clearance techniques (Bull et al, 1950) must now be regarded as a considerable underestimate since these methods are not valid at low arterio-venous

extraction ratios. However, measurements based on the diffusion of krypton and other inert gases indicate that the renal blood flow is reduced to between one-third and one-half of normal values during oliguria (Munck, 1958; Reubi et al, 1962; Walker et al, 1963; Gomori, 1964). Using Xenon washout techniques (Hollenburg et al, (1968 and 1970) have confirmed the supposition of Finckh et al (1962) that there is preferential renal cortical ischaemia, and by selective renal arteriography have demonstrated failure of filling in cortical arterial vessels. Thus glomerular filtration rate is negligible.

Similar conclusions have been drawn by Oken and his colleagues (Flanigan and Oken, 1965; Oken et al, 1966; Wilson et al, 1969) after studying rats in which renal failure was induced by mercury poisoning or glycerol-induced haemoglobinuria. By micropuncture techniques these workers confirmed that glomerular filtration rate was reduced during the oliguric phase and suggested, on the basis of presumedly normal glomerular structure, that alterations in the tone of glomerular afferent-efferent arterioles may be responsible. Their experiments also indicated that passive reabsorption of glomerular filtrate did not play a role in experimentally induced oliguria. The similarity of the haemodynamic and functional pattern in patients with acute renal failure of widely different aetiologies suggests a pathogenetic final common pathway involving mediators that induce severe, sustained preglomerular vasoconstriction. From a study of the redistribution of intra-renal blood flow following haemorrhagic hypotension in dogs, it has been concluded that sympatho-adrenergic factors are mainly responsible (Grandchamp, Ayer and Truniger, 1971). Furthermore, this cortical

hypo-perfusion does not depend on intact innervation of the kidney, but rather is mainly mediated via circulating catecholamines (Truniger et al, 1971). It is also possible that the elevated circulating levels of renin in acute renal failure contribute to the intense vaso-constriction by stimulating the conversion of angiotensin I to angiotensin II within the kidney (Brown et al, 1970). However, the question of mediators is unresolved and it is possible that other factors may play an important role in the genesis of this prolonged loss of renal function.

Acute renal failure may be produced in animals by inducing intra-vascular coagulation (Hardaway and McKay, 1958) and it has been suggested that such a mechanism might contribute to the renal failure in man, but there has been little direct evidence to support this view. Because of this, studies of coagulation and fibrinolysis together with electron microscopy of renal biopsies obtained during the illness were carried out with specific reference to the occurrence of intra-vascular coagulation in the syndrome occurring in man.

3.1.1 PATIENTS AND METHODS

Brief clinical details of the 47 patients studied are shown in Table 1. The causes of the renal failure were similar to other reported series and although infection, drug overdose, and post-operative hypotension predominate, many of the less frequently seen causes were encountered. Twelve patients died, usually as the result ofthe pre-disposing cause of the renal failure. All were treated by intermittent haemodialysis until recovery of renal function or death.

TABLE 1.

Clinical and Investigative Data in the 47 Patients Studied

Patient No.	Sex	Age	Outcome	Cause of Renal Failure	Coagulation Study	Phase when Biopsy Performed	Time Between Onset and Biopsy (days)
1	M	47	S	Hepato-renal syndrome	—	Recovery	17
2	M	18	S	Alcoholic poisoning	—	Recovery	9
3	M	35	S	Barbiturate overdose	—	Failure	19
4	F	33	S	Ruptured ectopic: hypotension	—	Failure	42
5	F	26	S	Caesarian Section: Hypotension	—	Failure	30
6	M	38	S	Alcoholic poisoning	—	Failure	16
7	F	29	S	Incomplete abortion	—	Recovery	28
8	M	47	S	Carbon monoxide poisoning	—	Failure	4
9	F	51	S	Morphine poisoning	—	Failure	10
10	M	57	S	Septicaemia. Esch. Coli	—	Recovery	17
11	M	62	S	Barbiturate overdose	—	Failure	16
12	M	47	S	Barbiturate overdose	+	Failure	13
13	M	47	S	Barbiturate overdose	+	Failure	8
14	F	48	S	Barbiturate, paracetamol Overdose	+	Recovery	7
15	M	47	S	Barbiturate overdose	+	Failure	12
16	M	62	S	Post-op. ruptured iliac aneurysm	+	—	—
17	M	63	D	Septicaemia. Esch. Coli	+	—	—
18	F	62	S	Post-op. mesenteric thrombosis	+	Failure	8
19	M	66	D	Septicaemia. Esch. Coli	+	—	—
20	F	60	S	Post-op. Ca. Colon	+	—	—
21	F	57	S	Rh. Arthritis: septicaemia	+	—	—
22	F	48	S	Post-op: Ca. Cervix	+	—	—
23	M	59	D	Post-op: hypotension	+	—	—
24	F	61	S	Post-op: hypotension: Ca. Stomach	+	—	—

TABLE 1. (Continued)

Patient No.	Sex	Age	Outcome	Cause of Renal Failure	Coagulation Study	Phase when Biopsy Performed	Time Between Onset and Biopsy (days)
25	F	45	D	Paraquat ingestion	+	—	—
26	M	16	D	Multiple trauma	+	—	—
27	M	75	D	Pneumonia, pneumococcal septicaemia	+	—	—
28	M	72	S	Septicaemia: Esch. coli	+	—	—
29	M	62	S	Septicaemia: Esch. coli	+	—	—
30	M	35	S	Septicaemia: Esch. coli	+	—	—
31	M	42	S	Multiple trauma	+	Failure	18 & 32
32	M	37	S	Paraquat ingestion	+	Failure	12
33	F	70	S	Pneumonia: septicaemia. H. influenzae	+	—	—
34	M	20	S	Septicaemia: Esch. coli	+	—	—
35	M	22	S	Multiple trauma	+	—	—
36	F	50	D	Septicaemia: Esch. coli	+	—	—
37	M	52	S	Multiple trauma	+	—	—
38	M	29	D	Septicaemia: Esch. coli	+	—	—
39	M	35	S	Cardiomyopathy	+	—	—
40	M	63	D	Multiple myeloma	+	Failure	8
41	M	53	S	Lysol poisoning	+	Failure	11
42	M	53	D	Acute pancreatitis	+	—	—
43	M	68	S	Pneumonia: dehydration	+	—	—
44	F	30	S	Septic abortion	+	Failure	12
45	F	40	S	Post partum haemorrhage	+	Failure	14
46	F	57	D	S.B.E. staphylococcus aureus	+	Failure	8
47	F	28	D	Paracetamol overdose	+	—	—

S = Survived
D = Died

Coagulation and fibrinolytic studies were undertaken daily in most instances in 36 patients. Predialysis samples of blood were assayed to avoid contamination with anti-coagulant. Renal biopsies were carried out in 13 of this group of 36 and in a further 11 patients in whom coagulation studies were not performed. Biopsies were taken at intervals after the onset of renal failure and in both the oliguric and diuretic phases of the illness.

CONTROL PATIENTS

For the purposes of control and comparison, daily studies of coagulation and fibrinolysis were performed in four groups of patients.

1. Serum F.D.P. concentrations were measured in 20 patients during uneventful convalescence from abdominal surgery.

2. Serum and urinary F.D.P. and plasma soluble fibrin monomer complexes (SFMC) were measured in 11 women during the post-partum period. Ten of these suffered from pre-eclamptic toxaemia of sufficient severity to necessitate early induction of labour.

3. In 19 patients with coma following drug overdosage, serum and urinary F.D.P., plasma fibrinogen, S.F.M.C., Platelet Factor 4 (PF_4) and plasminogen activator concentrations were serially measured. In this latter group the study was extended to include the serial measurement of certain serum enzymes: creatine phosphokinase, lactic dehydrogenase, hydroxybutyric dehydrogenase, glutamic pyruvic and oxalic transaminases and amylase. Grossly elevated levels of serum creatine phosphokinase and hydroxy-butyric dehydrogenase have been reported in acute renal failure (Kemp et al, 1964) and this part of the study was

included to determine the co-relation of enzyme changes with those of coagulation and fibrinolysis.

These groups were chosen for study because of the relative frequency with which patients in this category develop acute renal failure. Renal biopsy was not performed.

4. Serum and urinary F.D.P. concentrations were measured in five patients with urinary tract obstruction — one with calculus anuria, three with unilateral calculus ureteric obstruction and one with urinary retention due to prostatic hypertrophy. Care was taken to exclude from this group patients with evidence of urinary or systemic infection or haematuria.

3.1.2 RESULTS

The course of the illness was divided arbitrarily into 2 phases: a phase of oliguric renal failure in which haemodialysis was necessary to support life, and a phase of recovering renal function in which no further haemodialysis was needed and during which a progressive fall in the concentration of blood urea occurred. All patients were studied during the phase of renal failure and those surviving were followed into recovery. In 2 patients blood and urine was available during what might be regarded as the onset phase.

3.1.3 COAGULATION: FIBRINOLYTIC STUDIES

(a) ACUTE ISCHAEMIC RENAL FAILURE

The concentrations of plasma fibrinogen, S.F.M.C., PF_4 and serum F.D.P. were elevated in each patient during the early oliguric phase. Euglobulin lysis times were uniformly prolonged indicating a diminished plasma concentration of plasminogen activator. Although individual patients showed variable degrees of abnormality in each index studied, a remarkably consistent pattern of change emerged which was independent of cause of the renal failure.

Serum F.D.P. attained peak concentrations between 2 and 8 days after onset of renal failure and thereafter although gradually falling, remained above the normal range during the oliguric phase and returned to control levels at or about the onset of diuresis (Fig. 10). Comparison between the concentration of serum F.D.P. in the 36 patients studied during the two phases and the values from 106 healthy control subjects is shown in Fig. 11. There was a highly significant difference between the mean concentration in the phase of renal failure and the mean concentration in the recovery phase ($p < 0.001$) and the mean concentration of the healthy controls ($p < 0.001$). No significant difference was found between the mean value during the recovery phase and the mean of the controls.

Plasma fibrinogen concentrations were often in excess of 1000mg/100ml early in the oliguric phase, but fell to within the normal range at a somewhat early stage than the serum F.D.P. (Fig. 10 and 12).

Values for PF_4 and S.F.M.C. remained elevated throughout the oliguric phase (Fig. 13).

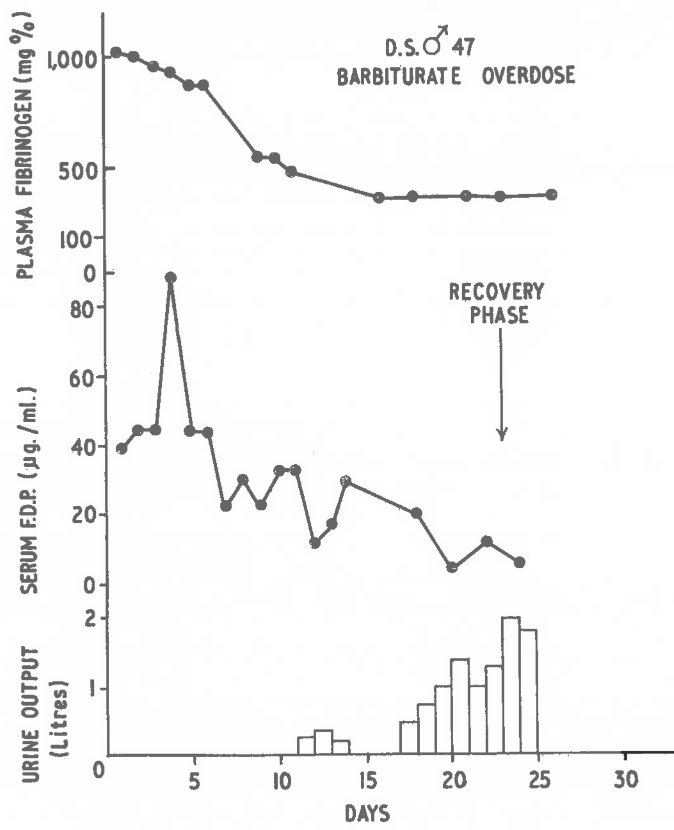


Fig. 10. Case 12. Serial plasma fibrinogen and serum F.D.P. concentrations are shown in relation to urinary output. Onset of the recovery phase is indicated by the arrow. Plasma fibrinogen and serum F.D.P. concentrations are elevated during renal failure but return to normal with the onset of diuresis.

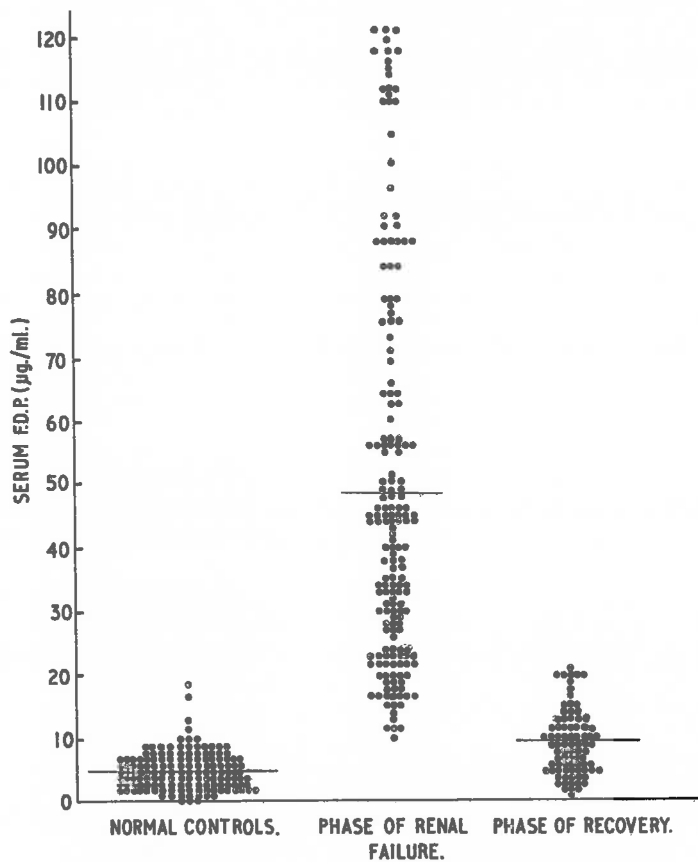


Fig. 11. Serum F.D.P. concentrations in 36 patients in the phases of renal failure and recovery are compared with those from 106 healthy controls. There is a significant difference between the mean concentration in the phase of renal failure and the mean concentration in the recovery phase ($p > 0.001$) and the mean concentration of the controls ($p > 0.001$).

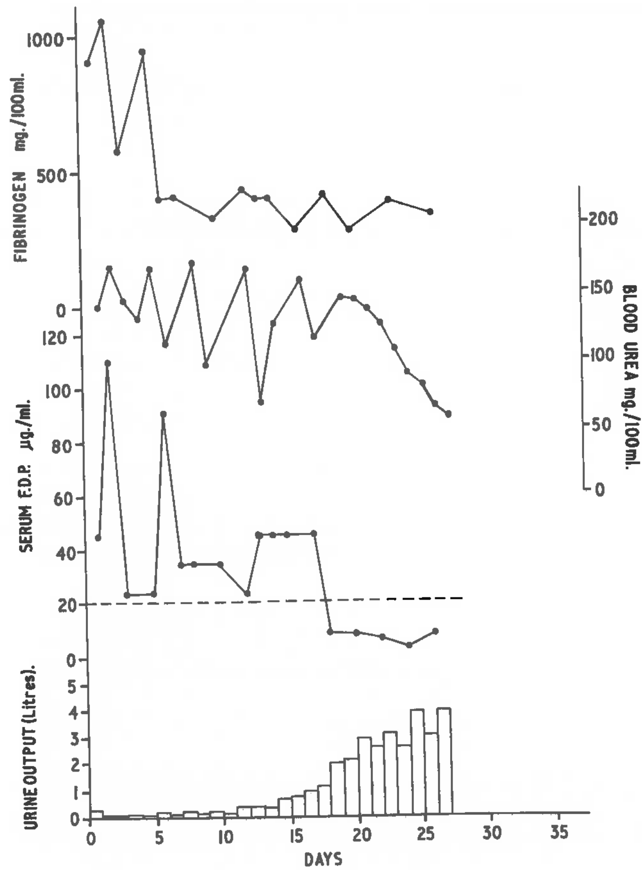


Fig. 12. Case 13. Serum F.D.P. and plasma fibrinogen concentrations in relation to urinary output and daily blood urea concentrations. Plasma fibrinogen concentration fell to within the normal range at an earlier stage than serum F.D.P.

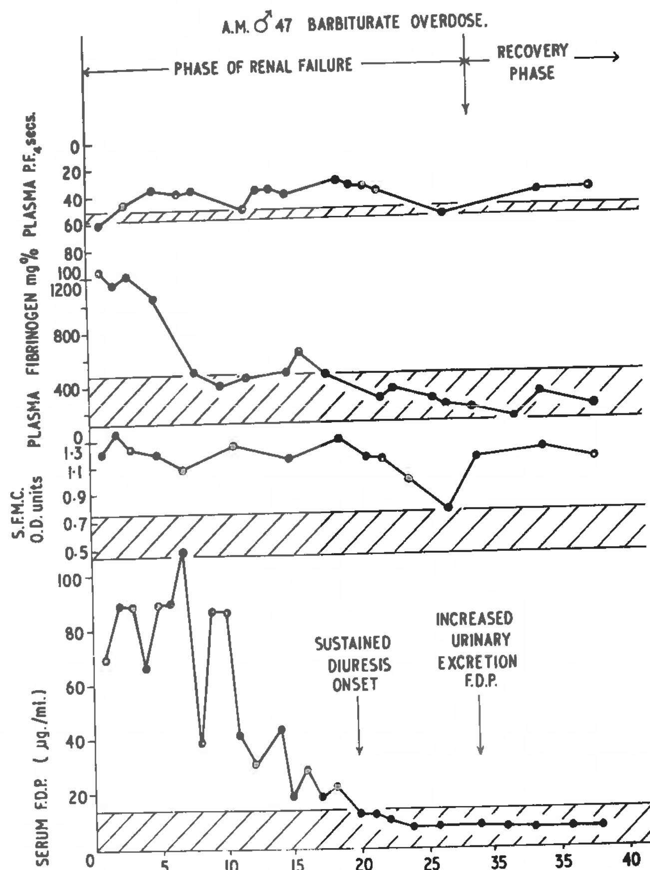


Fig. 13. <u>Case 15</u>. Serial values for serum F.D.P., plasma soluble fibrin monomer complex, fibrinogen and platelet factor 4, are shown in relation to both phases of the disease. The observations were similar to those made in other patients. Normal ranges are cross-hatched.

F.D.P. were found in the urine (when available) throughout the illness, but there was a pronounced increase in excretion during the diuretic phase at the time of recovery of renal function when the concentration of blood urea began to fall. Fig. 14 illustrates the progress in patient 15, a 47 year-old labourer who, following an overdose of Nitrazepam and barbiturate, became hypotensive and developed acute renal failure. A striking relation is shown between the fall of serum F.D.P. to control values and the onset of sustained diuresis (see also Fig. 10.). The appearances of large amounts of urinary F.D.P. coincided with the onset of spontaneous recovery. (See also Fig. 15). Significant excretion of F.D.P. continued well into the recovery phase.

Plasma plasminogen activator content remained depressed during oliguria but rose during recovery (Fig. 16).

Similar sequential changes in serum and urine F.D.P. were noted in patients with "pre-renal" renal failure, "polyuric" acute renal failure and "acute on chronic" renal failure. Fig. 17 demonstrates these changes in a patient whose renal failure responded to conservative management.

In only one patient with the fully developed syndrome of acute renal failure was there a deviation from this pattern. This was in Case 31, a 42 year-old railway foreman who became anuric after being knocked down by a train. Serum F.D.P. did not return to base-line after 7 weeks of constant dialysis, during which diuresis did not occur. Plasma activator remained depressed and soluble fibrin monomer complexes

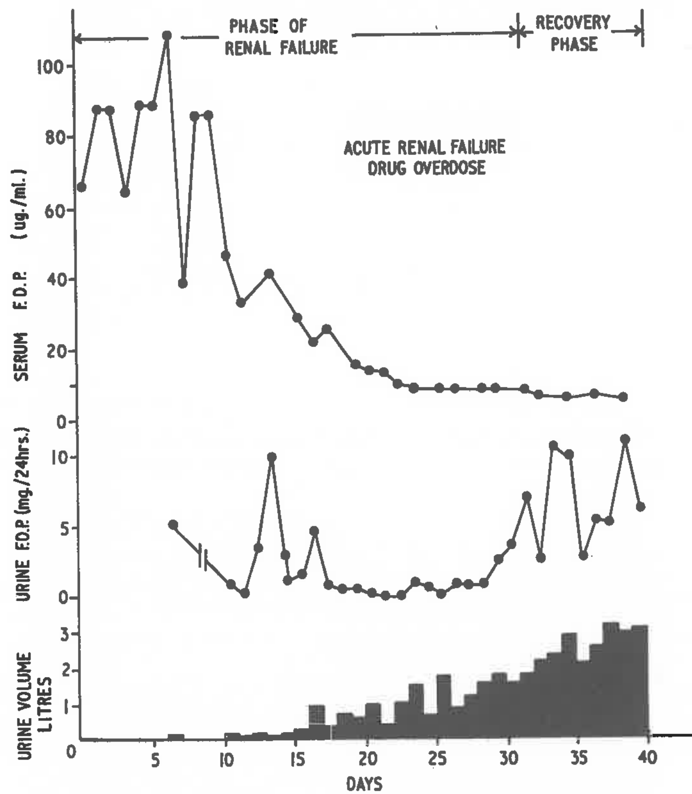


Fig. 14. Case 15. The relation between abnormal elevation of serum F.D.P. concentration and the oliguric phase of acute renal failure. Serum F.D.P. concentration fell to the normal range with the onset of sustained diuresis which is accompanied by a release of large amounts of F.D.P. into the urine.

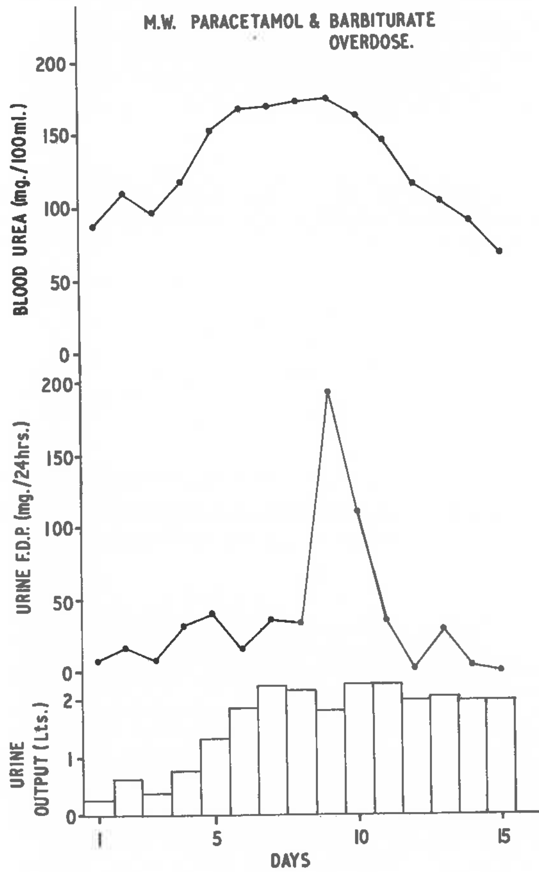


Fig. 15. Case 14. A further illustration of the sharp increase in urinary F.D.P. excretion coinciding with the onset of renal recovery. This may be some time after diuresis has commenced.

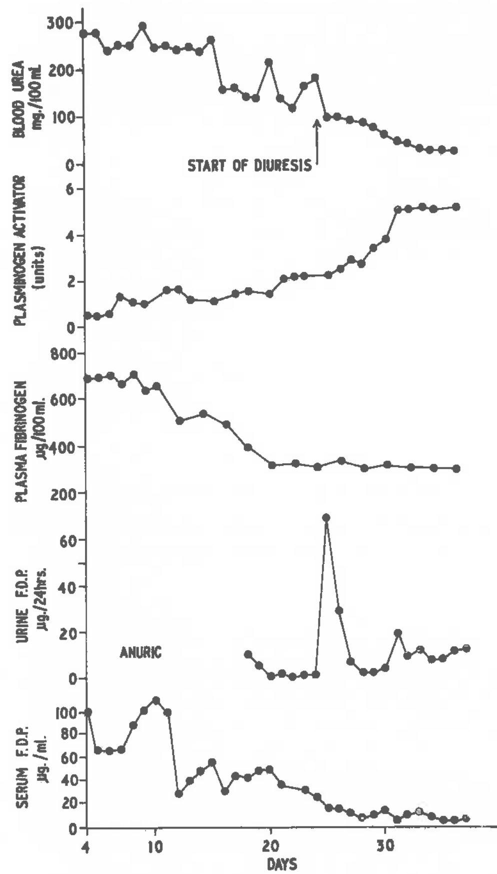


Fig. 16. Case 37. Serial studies in a 52 year-old man who developed acute renal failure following a traffic accident. Plasma plasminogen activator concentration remained depressed during oliguria but rose during recovery.

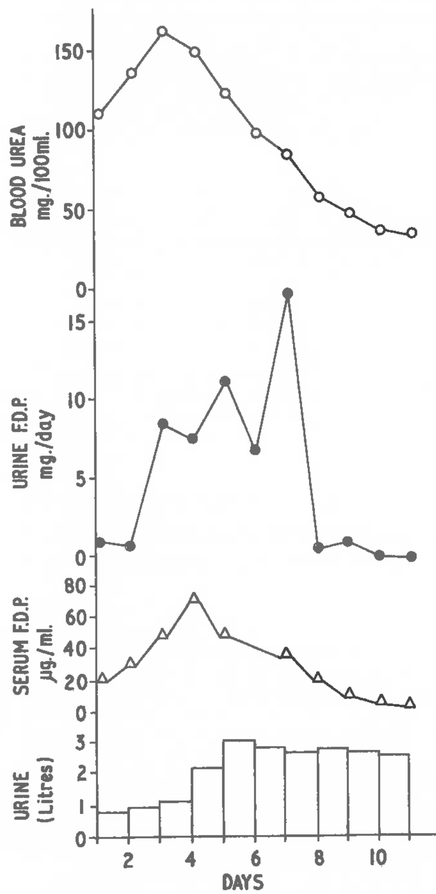


Fig. 17. Case 33. Changes in blood urea and serum F.D.P. concentrations and urinary F.D.P. excretion in a patient who presented in a dehydrated state because of Haemophilus influenzae pneumonia. She responded to conservative management.

remained elevated. Renal biopsy on the 18th and 32nd day following injury confirmed the clinical suspicion of bilateral renal cortical necrosis, and he underwent renal homotransplantation on the 50th day. The data are shown in Fig. 18.

In 2 patients both suffering from self-poisoning with drugs, blood and urine collected in the early stages of development of renal failure was available for retrospective study due to the courtesy of a group of workers studying drug metabolism in these patients. Although blood specimens, timed to coincide with the onset of oliguria were not available, there appeared to be a significant rise in serum F.D.P. concentration at about the time oliguria developed. However, consecutive urine samples were available for assay and it was observed that oliguria was preceded by or coincided with a large increase in excreted F.D.P. (Figs. 19 and 20).

(b) CONTROL PATIENTS

Distinctive patterns were seen in three of the four control groups of patients, and probably reflect a non-specific response to the stress of surgery, child-birth and poisoning respectively. Similar changes have been reported after severe trauma and burns (Innes and Sevitt, 1964) and myocardial infarction (Chakrabarti et al, 1969).

POST-OPERATIVE GROUP

In the post-operative patients there was prompt elevation of serum F.D.P. to a mean value of 21.77 ± 8.19ug/ml on the third post-operative day, and the elevation lasted between 5 and 14 days. In no instance, however, including four patients who underwent colonic and rectal resection for carcinoma did the peak value reach the mean of

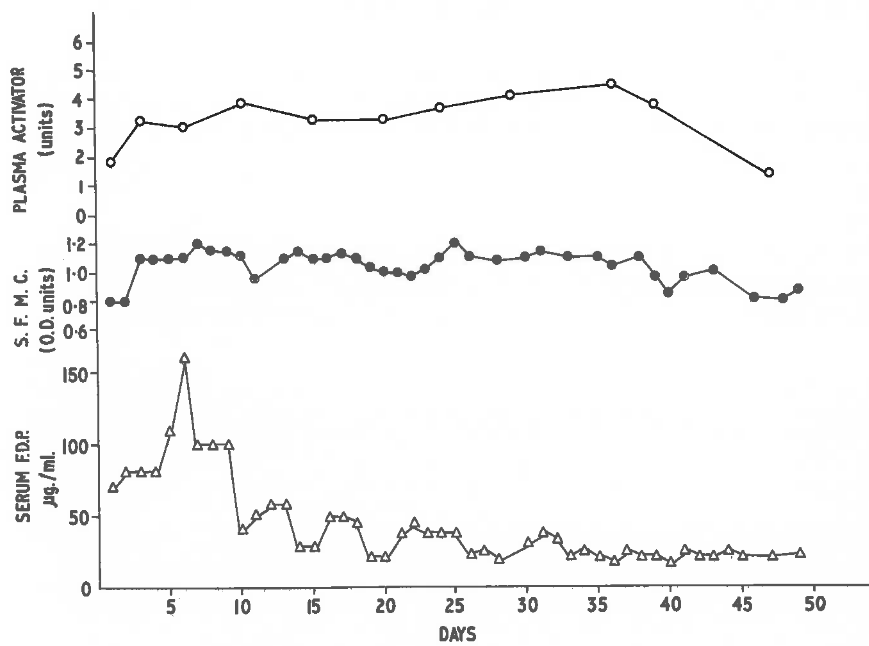


Fig. 18. <u>Case 31</u>. Renal cortical necrosis. Serum F.D.P. concentrations at no time fell to the levels seen in the recovery phase of acute renal failure. Plasma S.F.M.C. and plasma activator activities also remained at abnormal levels during the period of study.

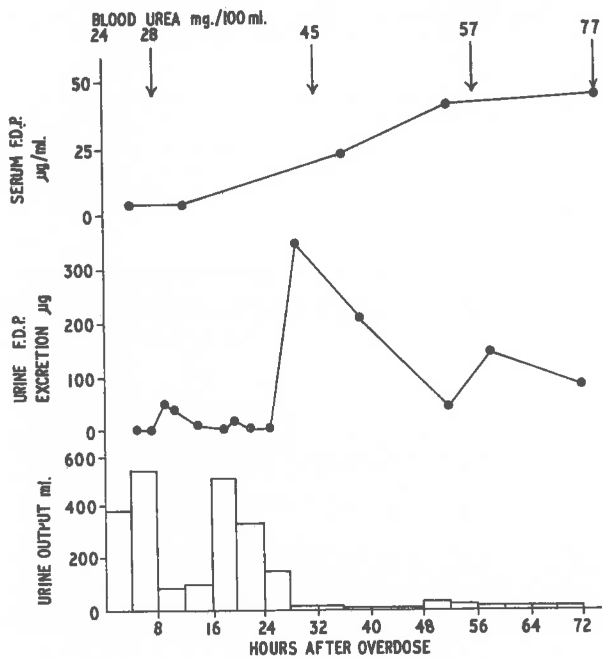


Fig. 19. Case 47. Serum and urinary F.D.P. values recorded during the onset phase of acute renal failure in a 28 year-old woman who took an overdose of paracetamol.

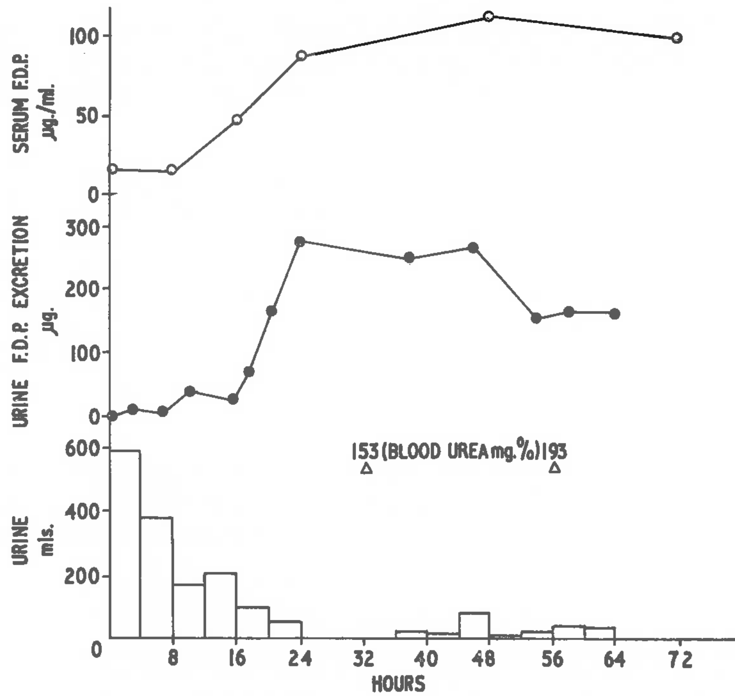


Fig. 20. <u>Case 41</u>. Serum and urinary F.D.P. values in the onset phase of acute renal failure in a 53 year-old man who ingested lysol and barbiturate.

the renal failure group and the difference between the mean of the peak values in each groups was highly significant ($p < 0.001$). The changes in serum F.D.P. during the initial 8 days of study for all patients in both groups is shown in Fig. 21.

POST PARTUM GROUP

Results of serum F.D.P. in the first 8 days post partum are shown in Table 2 and those for urine F.D.P. in Table 3. Values for plasma S.F.M.C. commonly rose post-partum reaching a peak on the third and fourth days, and often remaining above normal values for the period of observation. Despite the fact that pre-eclampsia is associated with deposition of fibrin within glomeruli, and elevated serum concentrations of F.D.P. (Henderson et al, 1970) the mean peak value for serum F.D.P. (22.9 µg/ml on day 4 post-partum) was significantly different ($p < 0.001$) from the mean peak value in acute renal failure (63.8 µg/ml on day 3 after admission to haemodialysis unit). Moreover, in most cases the rise in serum F.D.P. was short lived in contrast to the persistent elevation seen in acute renal failure. Although it is perhaps unwise to compare the excessive urine F.D.P. excretion seen in some of these women with that observed in acute renal failure, the finding is of particular interest in that it may represent clearance of the intraglomerular fibrin which accumulated during the latter part of pregnancy.

DRUG OVERDOSAGE GROUP

Nineteen patients who developed grade IV coma (Matthew and Lawson, 1970) after overdosage with a variety of hypnotic and tricyclic

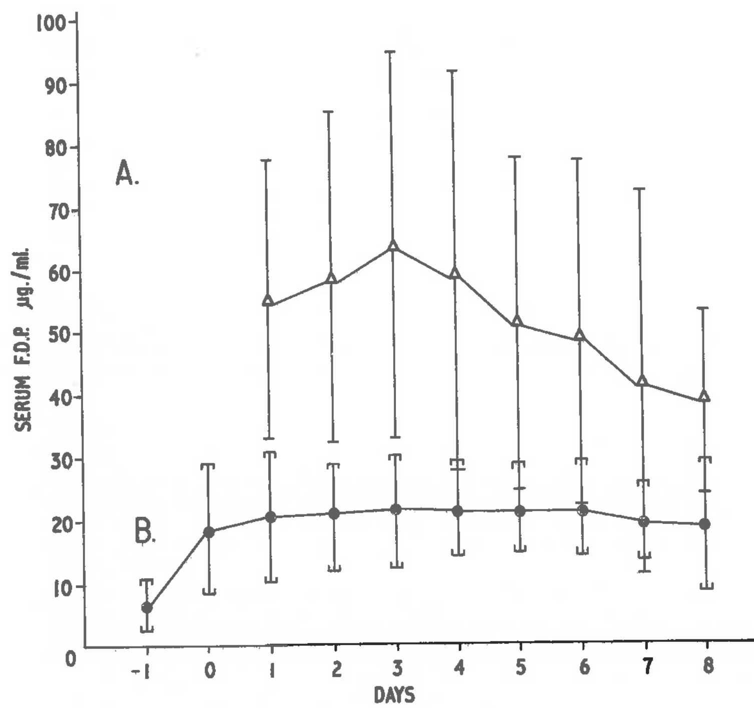


Fig. 21. The means and standard deviations of serum F.D.P. concentrations during the first 8 days of study for all patients with acute renal failure (A) is compared with that of the post-operative patients (B) studied before and after surgery.

TABLE 2.

Serial changes in Serum F.D.P. (μg/ml) in the post partum period in 9 women with pre-eclampsia, one with eclampsia and one after normal delivery

	Days Post Partum	1	2	3	4	5	6	7	8
Patient									
1	Severe Pre-eclampsia	30.7	23.0	23.0	23.0	15.4	15.4	11.5	14.1
2	Severe Pre-eclampsia	13.4	16.1	46.1	46.1	61.4	30.7	30.7	15.4
3	Severe Pre-eclampsia	22.4	—	33.6	33.6	44.8	44.8	33.6	20.6
4	Moderate Pre-eclampsia	29.8	22.3	29.8	29.8	—	19.8	—	29.8
5	Moderate Pre-eclampsia	44.6	29.8	44.6	—	22.3	29.8	29.8	18.8
6	Moderate Pre-eclampsia	16.3	21.8	10.9	22.9	10.9	12.0	10.9	—
7	Moderate Pre-eclampsia	7.4	14.7	29.4	14.7	—	22.1	22.1	7.5
8	Moderate Pre-eclampsia	15.4	13.2	8.9	8.8	10.9	15.4	—	—
9	Moderate Pre-eclampsia	3.7	5.6	8.9	16.6	16.6	14.9	15.5	3.7
10	Eclamptic	21.8	10.9	21.8	10.9	10.9	7.6	7.6	9.4
11	Normal Delivery	7.4	7.4	7.4	22.8	7.4	—	7.4	7.0
MEAN		17.7	15.0	22.0	22.9	22.3	20.9	18.8	14.1

TABLE 3.

Serial changes in urinary F.D.P. concentration (μg/ml of urine) in the post partum period.

Patient	Days Post Partum	1	2	3	4	5	6	7	8
1	Severe Pre-eclampsia	1.13	1.21	0.25	1.69	0.02	Nil	Nil	0.14
2	Severe Pre-eclampsia	0.06	0.23	24.68	0.11	0.22	0.09	0.10	Nil
3	Severe Pre-eclampsia	0.20	1.57	1.13	6.77	9.17	14.81	11.55	14.44
4	Moderate Pre-eclampsia	1.12	0.45	1.01	1.82	0.82	Nil	—	0.15
5	Moderate Pre-eclampsia	0.57	2.48	3.62	2.38	0.48	1.07	0.42	0.24
6	Moderate Pre-eclampsia	0.25	0.17	0.23	0.52	0.14	0.15	0.10	—
7	Moderate Pre-eclampsia	1.47	0.15	0.24	9.04	8.64	3.53	2.21	3.68
8	Moderate Pre-eclampsia	2.09	0.55	3.62	2.88	—	0.43	—	—
9	Moderate Pre-eclampsia	2.23	1.70	6.80	0.66	Nil	0.15	Nil	—
10	Eclamptic	0.08	0.14	0.03	0.20	0.49	0.58	0.54	0.44
11	Normal Delivery	3.38	0.39	Nil	1.76	0.11	Nil	0.17	0.10

anti-depressant drugs were studied and the length of coma determined as the period between the ingestion and the time when the patient responded to loud vocal commands. In none was there evidence of renal insufficiency. In 5 patients in whom the length of coma was less than 24 hours, no significant changes in coagulation and fibrinolytic indices were observed. In the remaining 14, all of whom were unconscious for longer than 24 hours, significant abnormalities were found which were similar in degree and sequence to those seen in the post-operative and post-partum groups. The sequence of the mean coagulation and fibrinolytic values for these 14 patients for 5 days following overdosage is shown in Table 4. There was a significant difference between the peak mean of the serum F.D.P. in this group and that of the renal failure group. The changes in other indices were also short-lived and dissimilar to the prolonged periods of abnormality seen during the oliguric phase of acute renal failure.

Of interest and perhaps of some bearing on the pathogenesis and clinical manifestations of acute renal failure, are the results of a study of changes in serum enzyme activities performed in conjunction with those of coagulation and fibrinolysis in these patients. The sequence of changes in serum enzymes was strikingly similar to those of coagulation and fibrinolysis. In particular, those patients who showed nocoagulation abnormality also showed no change in enzyme activities, whilst in the 14 patients with coagulation-fibrinolysis changes as described above, similarly timed changes in serum creatine kinase, and alanine and aspartate transaminases occurred. Most significant was the relation between serum creatine-kinase and serum F.D.P.

Table 4.

Sequence of Mean Coagulation and Fibrinolytic Changes

in 14 Patients with Prolonged Coma Following Drug Overdosage

Day	Serum F.D.P. (μg/ml)			Plasma Fibrinogen (mg/100ml)			Plasminogen Activator Content (Units)			Plasma S.F.M.C. (O.D. units)		Platelet Factor 4 %
	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	Range
1	4.5-25	11	(12)	270-640	430	(150)	12-18	15	(15)	0.55-0.91	0.76	64-94
2	4.8-50	25	(28)	220-1030	560	(600)	1.5-9.0	5.3	(5.5)	0.54-1.15	0.87	92-125
3	14-49	26	(27)	320-1030	700	(720)	3.1-8.6	5.5	(5.4)	0.65-1.16	1.01	101-120
4	8.0-74	23	(29)	300-1170	620	(660)	2.1-13.0	6.0	(6.4)	0.69-1.13	0.93	68-120
5	4.8-33	14	(16)	350-1140	640	(700)	4.4-13.0	8.3	(8.7)	0.70-1.30	0.94	66-96

(Fig. 22). The overall pattern of enzyme and coagulation changes for a typical patient in the latter group is shown in Fig. 23.

GROUP WITH URINARY TRACT OBSTRUCTION

In the five patients with urinary tract obstruction no significant change in serum or urinary F.D.P. was observed.

3.1.4 HISTOLOGICAL AND ELECTRON MICROSCOPIC STUDIES

LIGHT MICROSCOPY

In all cases focal tubular changes characteristic of acute tubular necrosis were seen. These included tubulorrhexis, interstitial oedema with inflammatory cell infiltration and occasional tubulo-capillary communications. The glomeruli appeared histologically normal with the exception of one case in which a few glomerular capillaries were plugged with a pale granular material which, however, did not give positive results for fibrin with Picro-Mallory or M.S.B. stains, and another in which glomeruli showed unusual congestion.

ELECTRON MICROSCOPY

TUBULES: Some tubular cells showed mitochondrial lesions, these structures being swollen with ill-defined and often disrupted cristae. In other tubules, rupture of the basement membrane occurred and cell cytoplasm and organelles were observed to spill into the interstitial tissues (Figs. 24a and b). These findings were noted in the cortex and medulla, in both convoluted and collecting tubules, and possibly in ascending limbs of the loop of Henle, and are consistent with the changes often described in acute tubular necrosis.

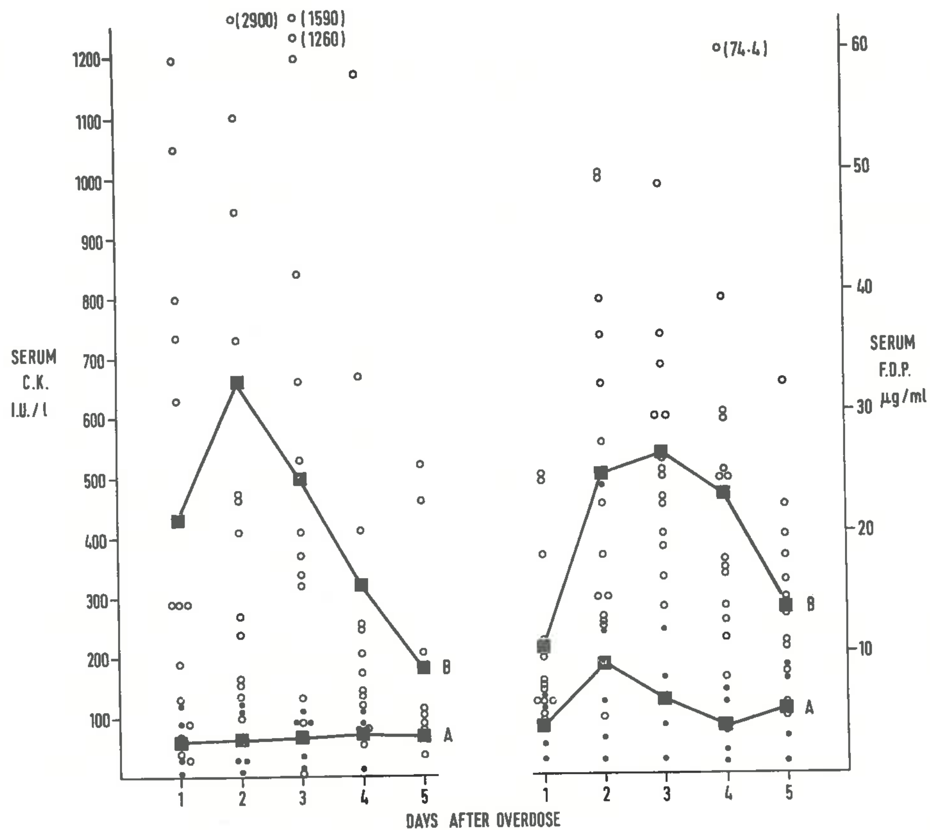


Fig. 22. Daily values for the activity of serum creatine kinase and for the concentration of serum F.D.P. in 5 patients unconscious for less than 24 hours (closed circles), and 14 patients unconscious for longer periods (open circles). The daily means for the two groups are indicated by squares (A-short duration of coma, B-long duration of coma). The normal range for serum creatine kinase activity is 10-100 I.U. per litre.

GLOMERULI: Examination of the glomeruli by electron microscopy revealed lesions unsuspected on light microscopy. Some glomeruli were normal, but on average almost half of the glomeruli examined in any one case showed pathological changes.

The most impressive abnormality consisted of occlusive intra-capillary fibrin deposition and thrombus formation. The affected capillaries were usually narrower than normal and the lining endothelium appeared swollen. Even on low-power examination it was apparent that strands of dark fibrillar material were present in these narrowed capillaries (Fig. 25). More detailed examination revealed this material characteristically situated between the endothelium and basement membrane often apparently lifting the endothelium from the latter (Fig. 26). Not uncommonly, the fibrils seemed to be in continuity with the basement membrane (Fig. 27). When the fragmented lumina of these capillaries were visible they were seen to be filled with dark material which was mostly fibrillar but sometimes granular or amorphous (Figs. 28, 29 and 30). Occasionally, irregular strands of basement membrane-like material passed between islands of what appeared to be endothelial cytoplasm, and crowded in the lumen with the fibrillar or granular material were numerous rounded cytoplasmic bodies (Fig. 31). Most of these probably represented projecting portions of endothelial cytoplasm, but it is possible that in some cases the intraluminal bodies were degranulated platelets (Fig. 32). Such changes in platelet ultrastructure have been described for example by Jorgensen et al (1967). Some of these intraluminal bodies would be better described as

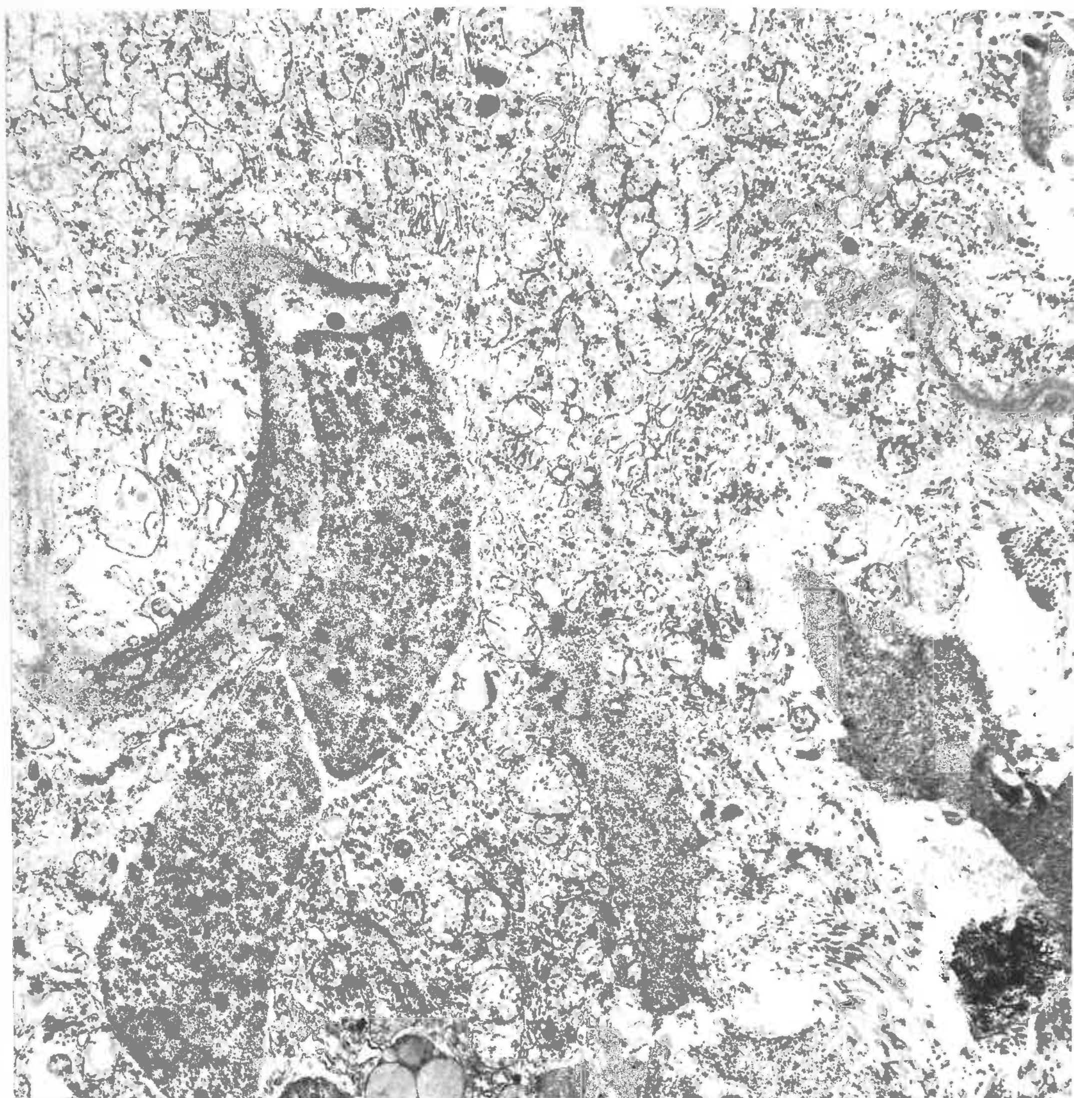


Fig. 24a. Electronmicrograph of a tubule in a case of acute tubular necrosis. The tubular lining cells are in the upper part of the field and the intertubular tissue in the lower. The basement membrane of the tubule can be seen running across the picture from both lateral margins as a dense line, but there is a complete break in its continuity in the centre. Through this break the cytoplasm of a tubular cell is streaming into the interstitial tissue. (Case 6; x 8,000)

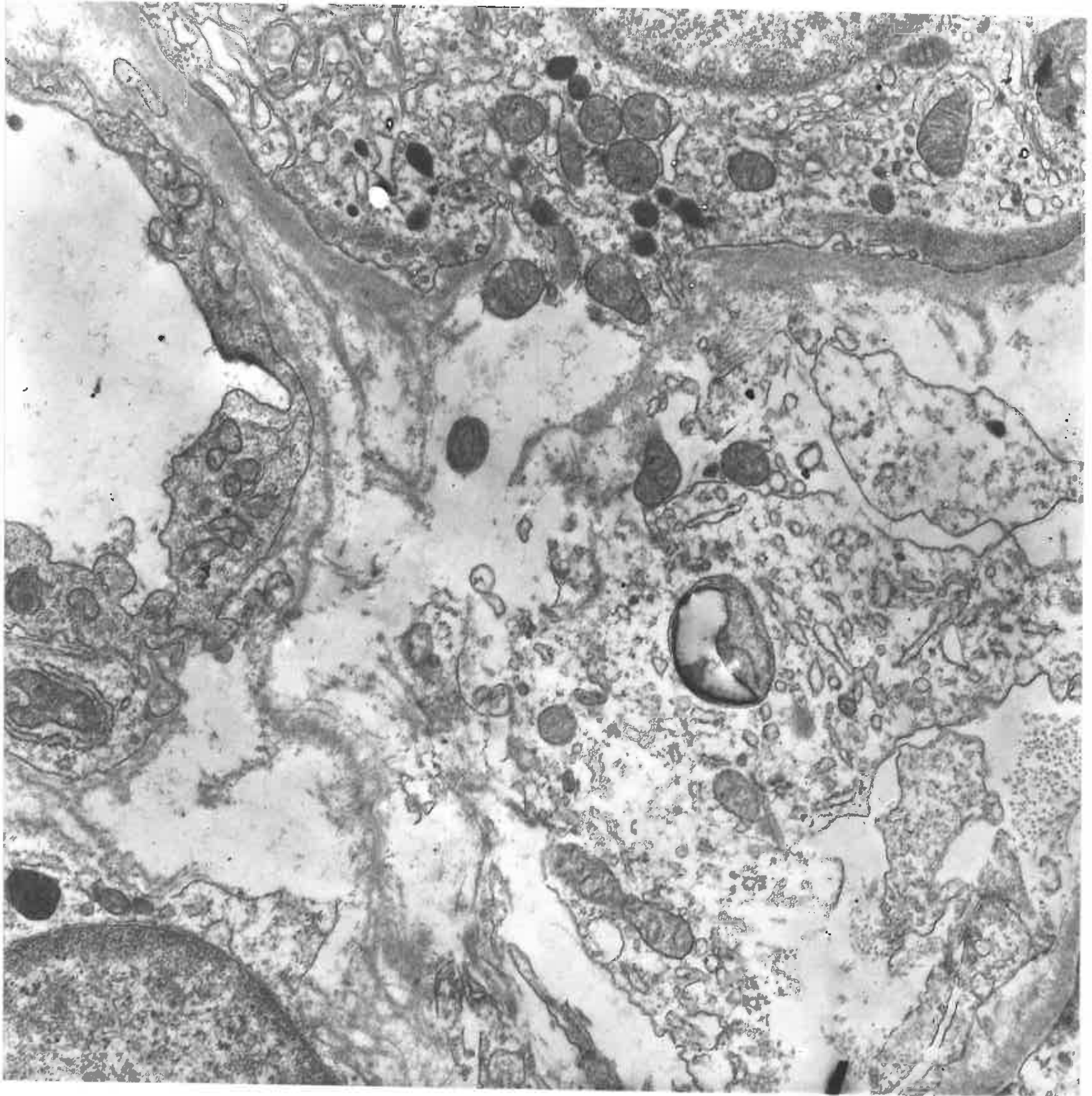


Fig. 24b. Acute tubular necrosis. The intracellular processes of a tubular cell can be seen bursting through a rent in the basement membrane. (Case 11; x 12,000)

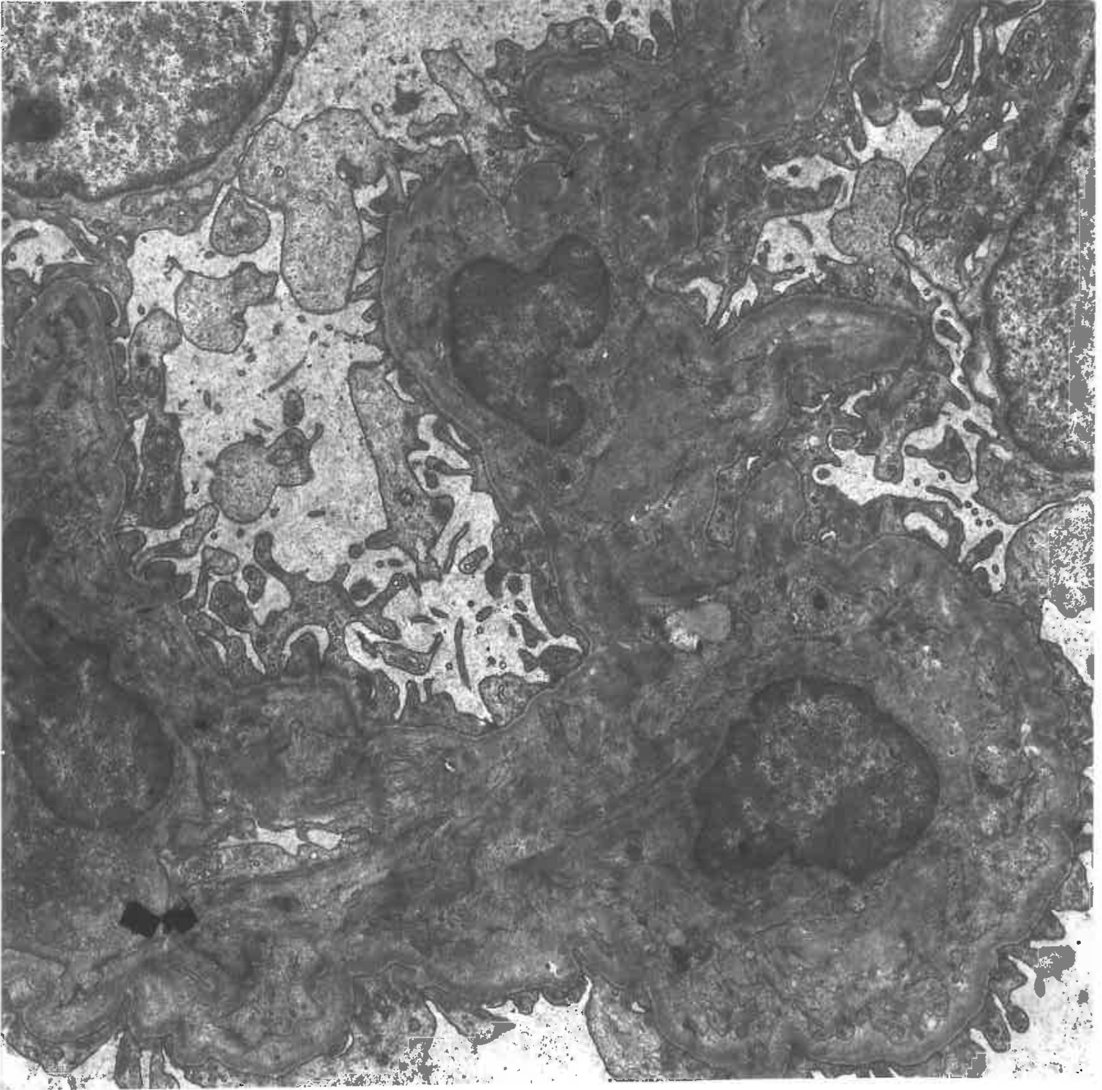


Fig. 25. The glomerular capillary in this micrograph is considerably narrowed and the endothelium is relatively thick. Groups of dark fibrils are seen in the capillary between portions of endothelial cytoplasm. (Case 14; x 5,000)

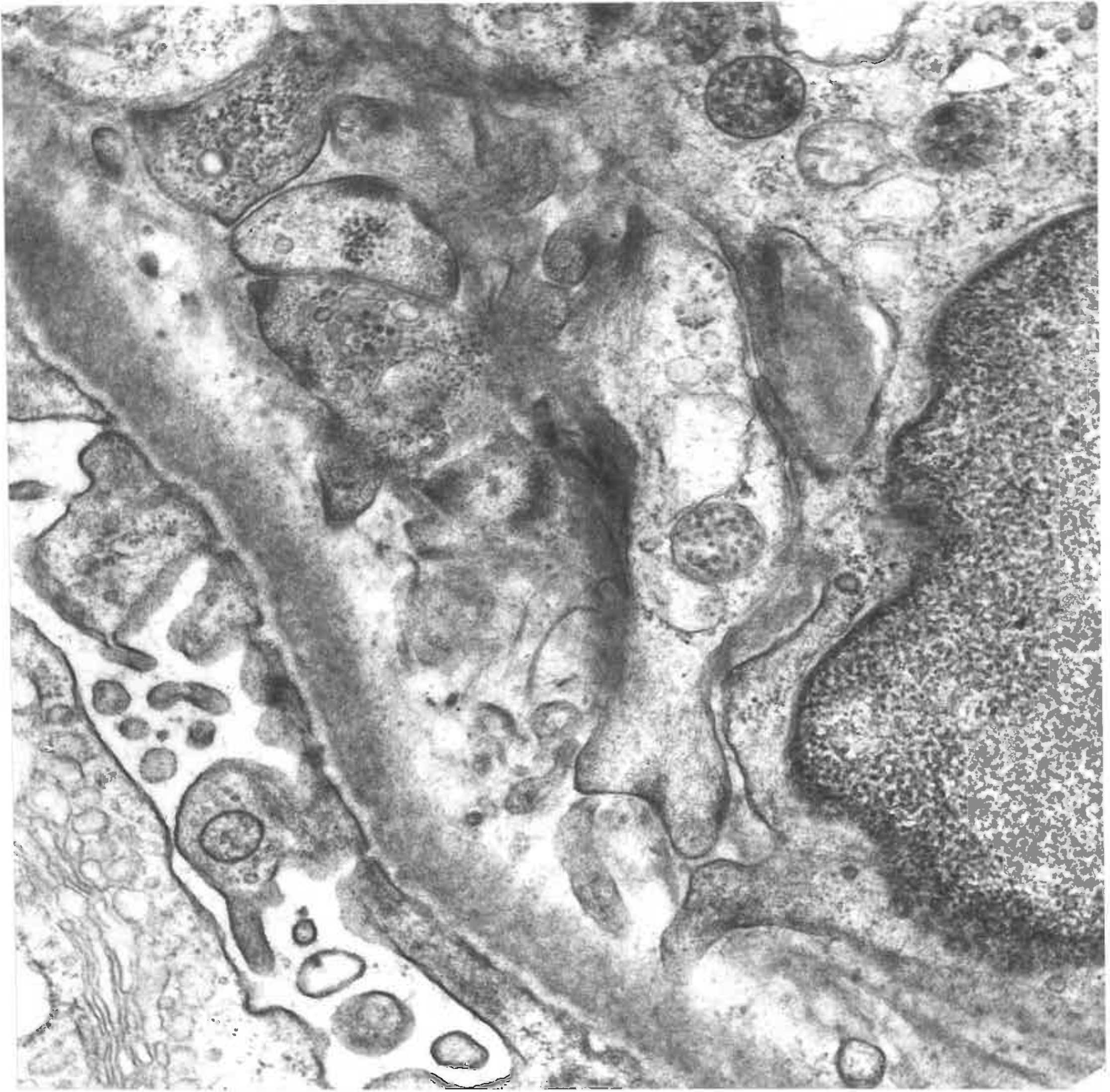


Fig. 26. At higher magnification, fibrillar material is seen in the interstices between cytoplasmic structures, and often between the basement membrane and endothelium (Case 13; x 32,000)

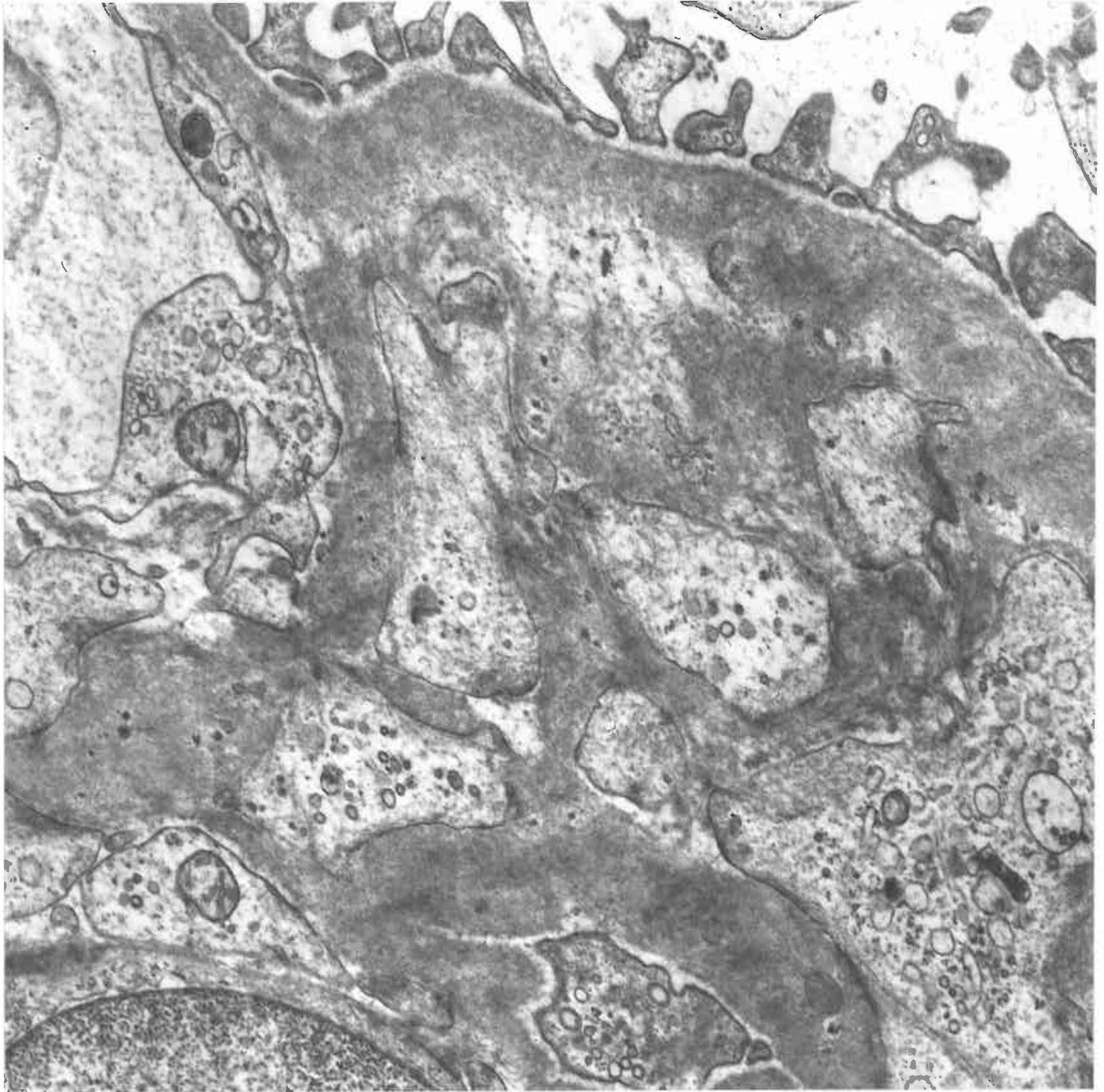


Fig. 27. The fibrin fibres are seen lying between basement membrane and endothelium, as well as between islands of endothelial cytoplasm, and in many places there seems to be continuity between fibrin and basement membrane (Case 11; x 16,000)

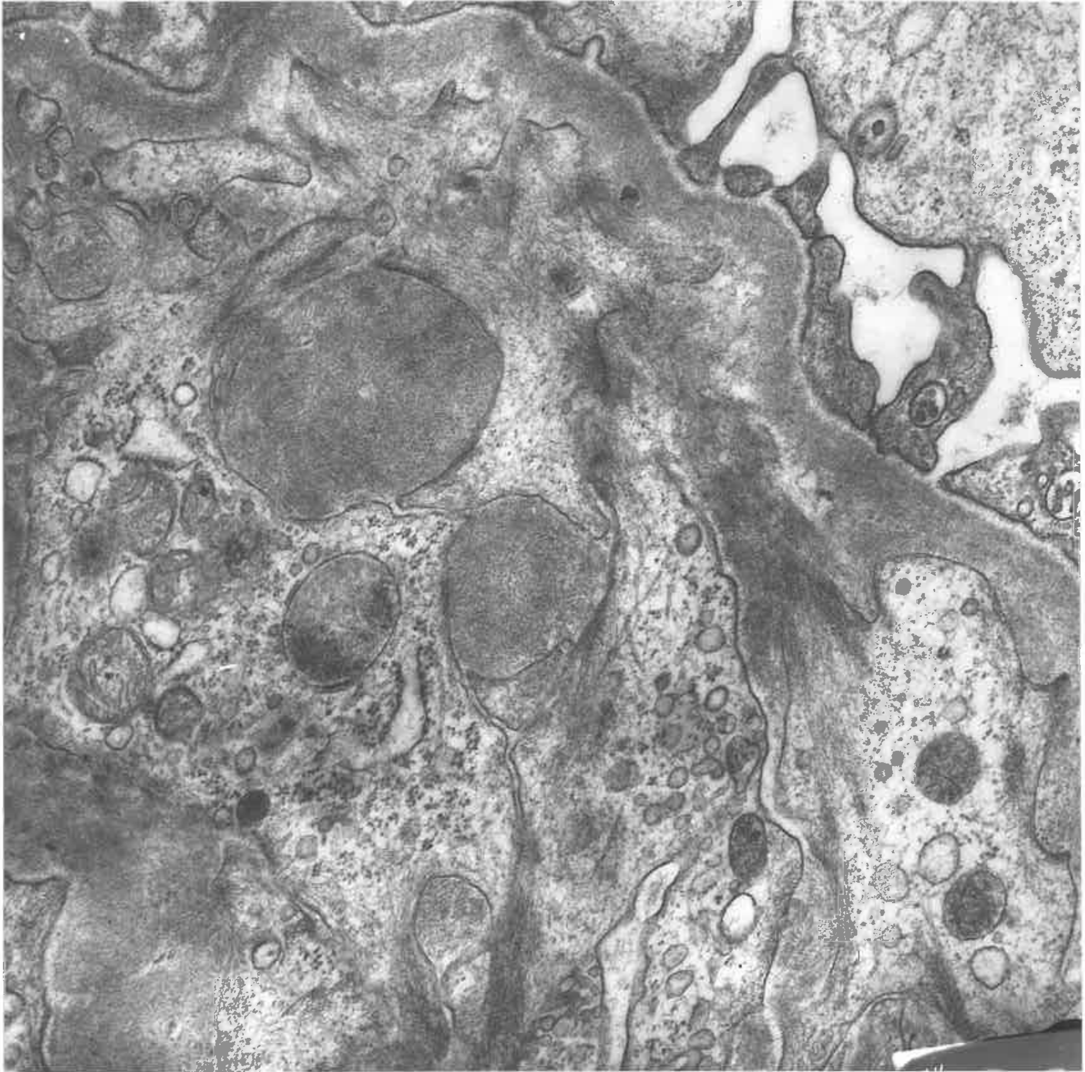


Fig. 28. Definitely fibrillar material is present just deep to the basement membrane, while rounded masses of a more granular material are also visible (Case 9; x 32,000)

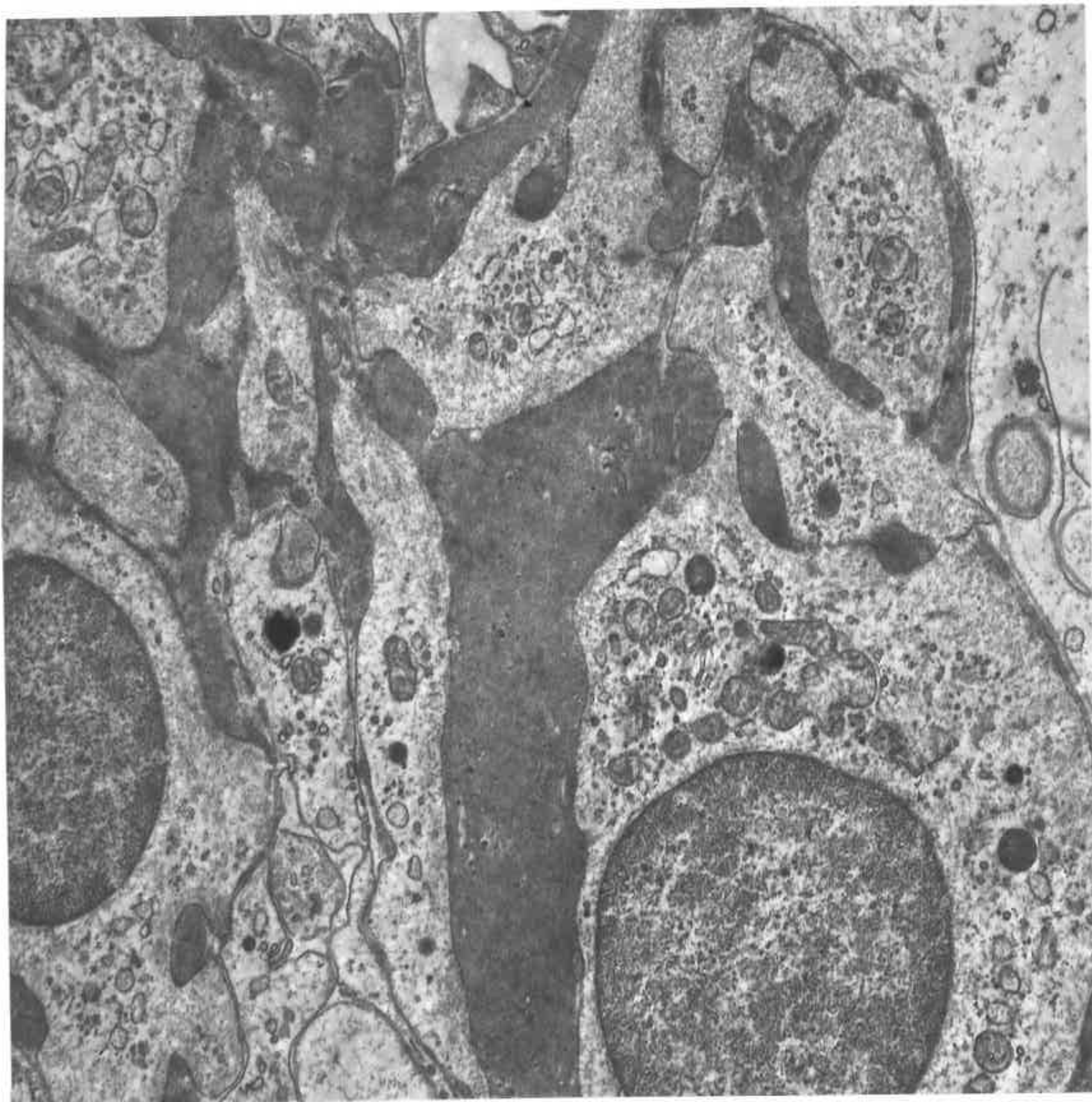


Fig. 29. In the lumen of this glomerular capillary are elongated masses of granular or structureless material, filling the spaces between cytoplasmic bodies (Case 15; x 8,750)

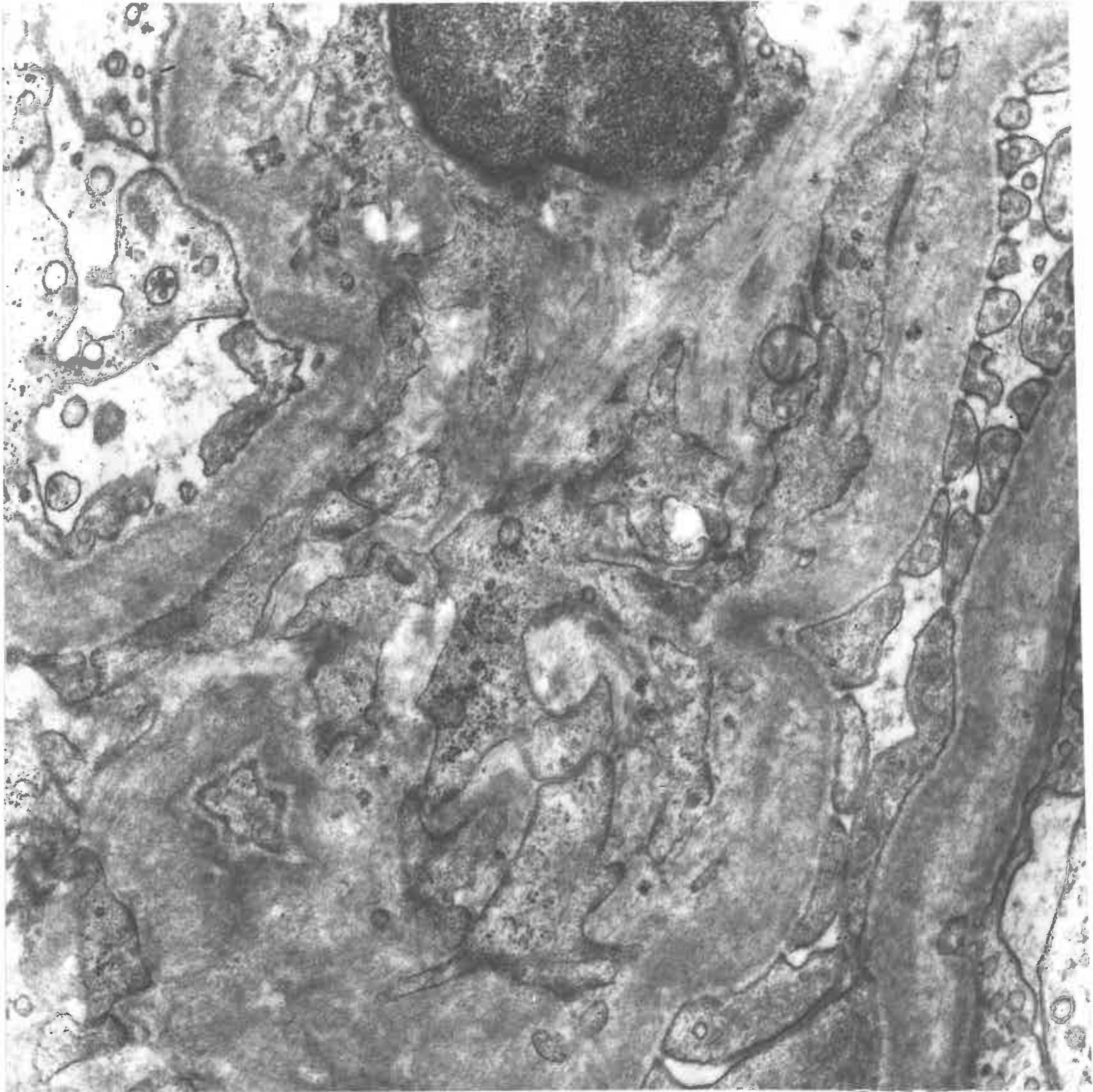


Fig. 30. In this narrowed capillary lumen fibrillar material is distinctly seen in the lumen in the upper part of the fiēld, while in the lower half, the dark material is granular and amorphous. (Case 14; x 26,000).

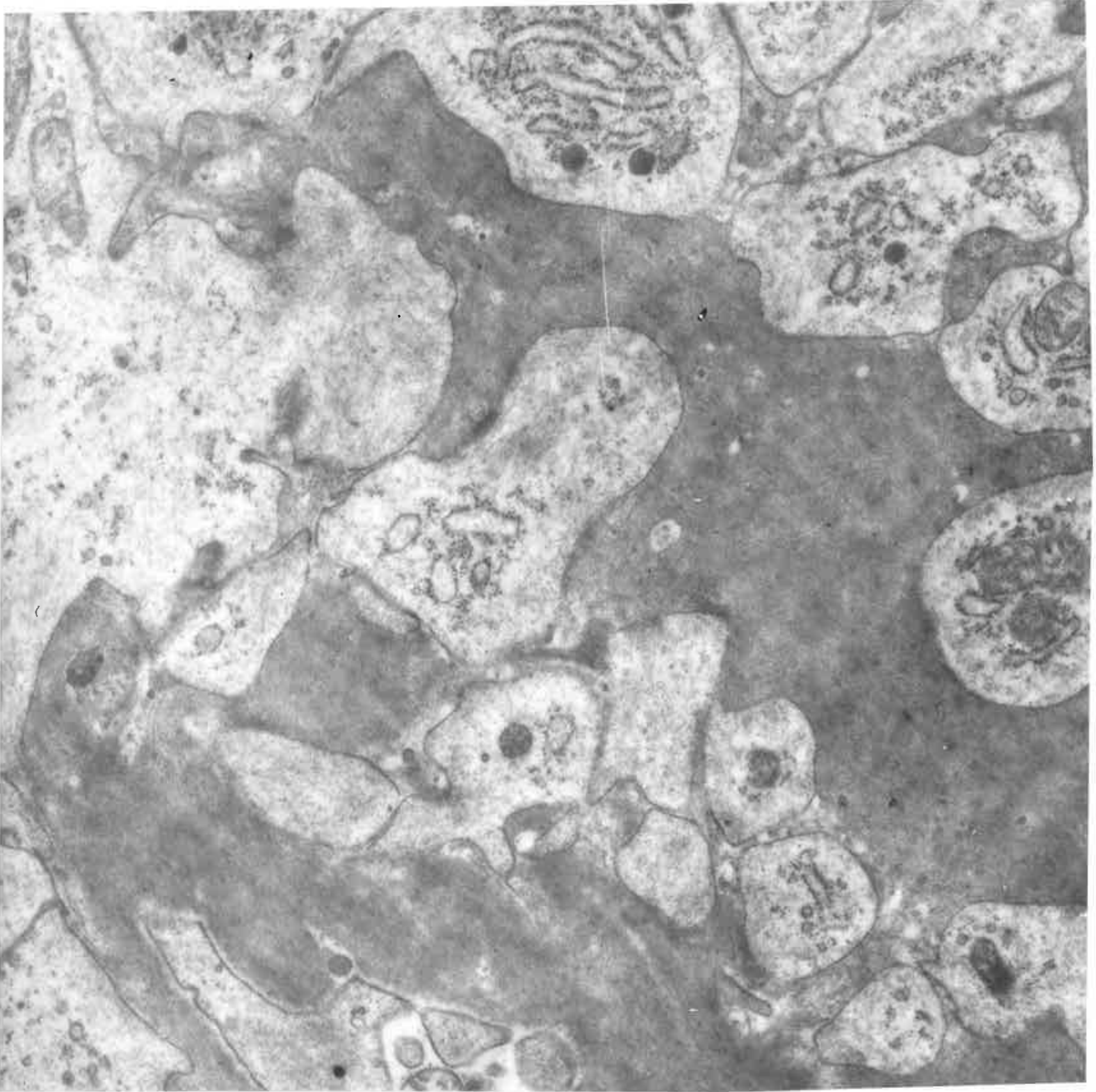


Fig. 31. Well defined rounded cytoplasmic masses in a capillary lumen, some possessing rough-surfaced endoplasmic reticulum, are separated by dark, granular material. (Case 15; x 20,000)

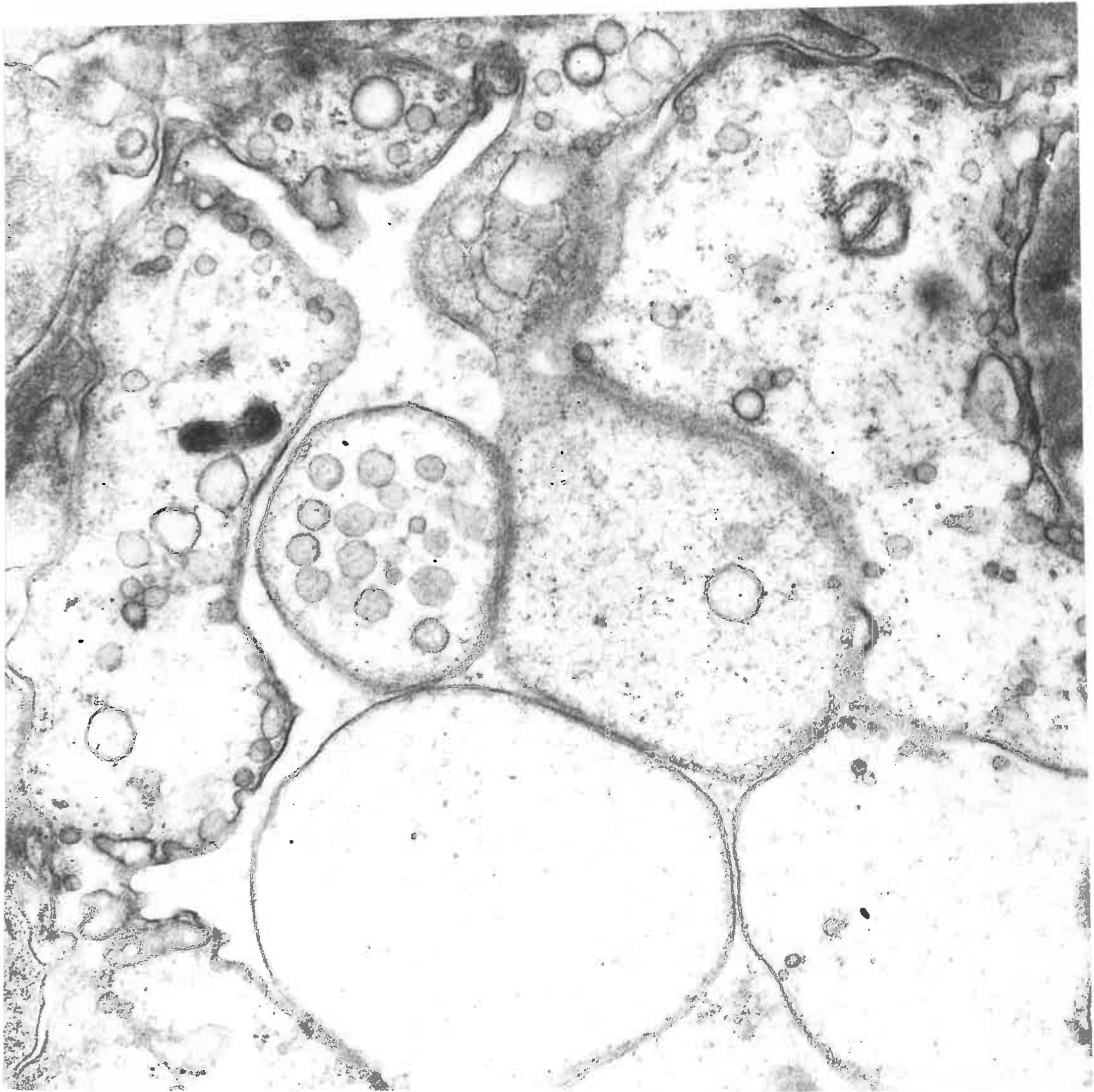


Fig. 32. This capillary lumen contains rounded vacuoles or cytoplasmic structures; they may be endothelial in origin, but some may be degranulated platelets. (Case 9; x 30,000)

vacuoles, and these occasionally exhibited small dark granules or fibrils at their periphery, producing an appearance similar to that reported by Brown, Stalker and Hall (1969) in rabbits injected intra-venously with liquoid, with resultant deposition of fibrin in glomerular capillaries (Fig. 33).

In some cases where the fully developed occlusive condition was not seen, occasional capillaries contained groups of dark fibrils or short strands of fibrin (Fig. 34). In others no significant fibrin deposition was demonstrated, but in these the capillaries showed considerable narrowing and sometimes disappearance of their lumina, usually associated with some endothelial cell swelling (Fig. 35). Occasionally red blood cells were seen embedded in the narrowed lumen and closely surrounded by thickened endothelium (Fig. 36). These appearances were thought to be compatible with obstruction to the lumen either proximal or distal to the site of the examined glomerular capillary, with subsequent impairment of filling or stagnation of blood flow in these vessels. In all cases some glomerular capillaries were filled with a granular material which, although unremarkable structurally, was dense and abundant. Rounded vacuoles, some of which had a peripheral thin membrane, were sometimes seen in the midst of this material and occasionally red blood cells were also present.

In addition, in most cases, though not all, platelets were seen more commonly and in greater numbers than usual within the capillary lumina (Fig. 37) often in association with red blood cells. This estimate of abnormal numbers of platelets is entirely subjective but

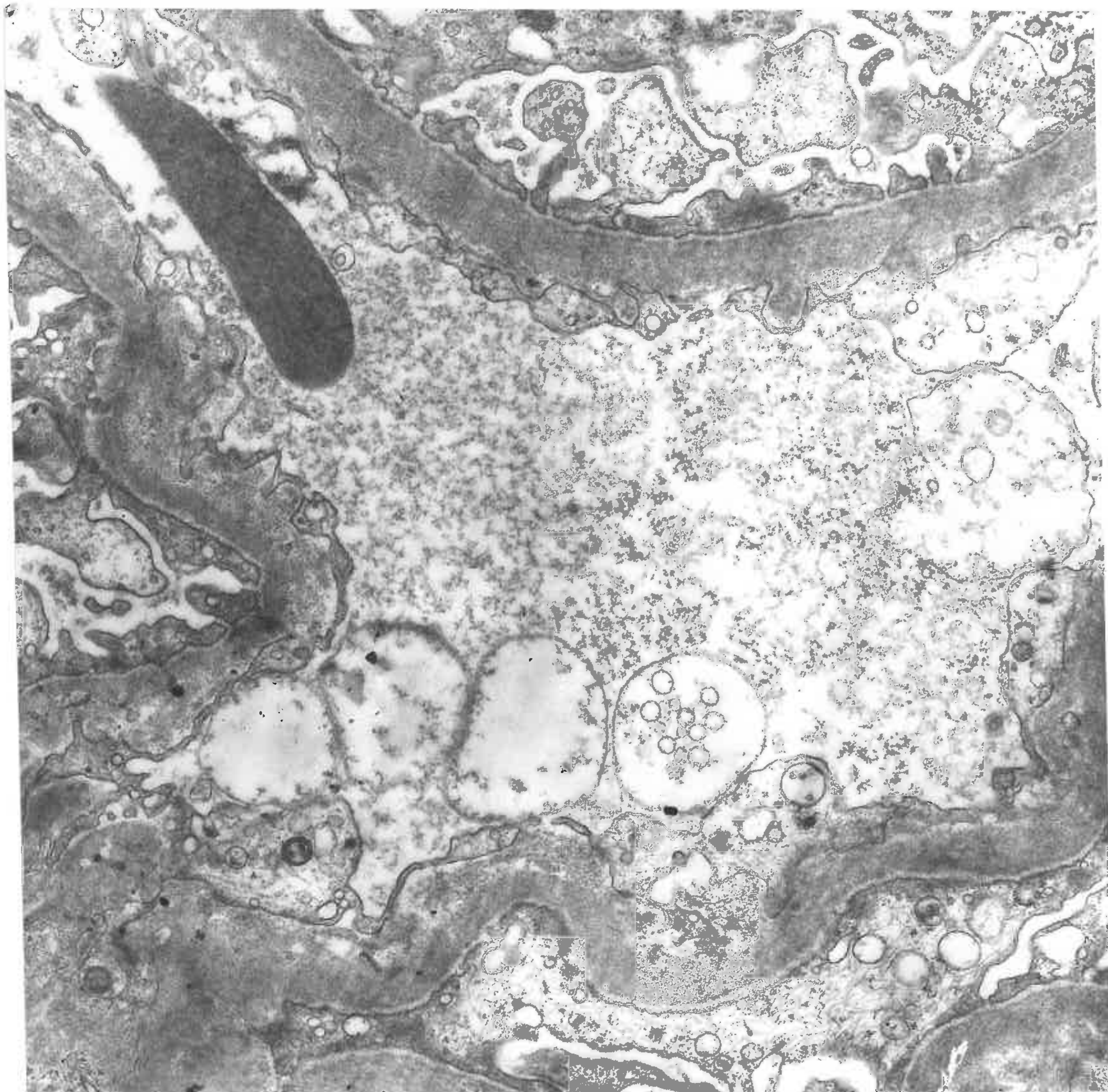


Fig. 33. Several rounded vacuoles are seen lying in the granular material within the lumen of this capillary; small dark particles adhere to their peripheral limiting membranes. (Case 10; x 16,000)

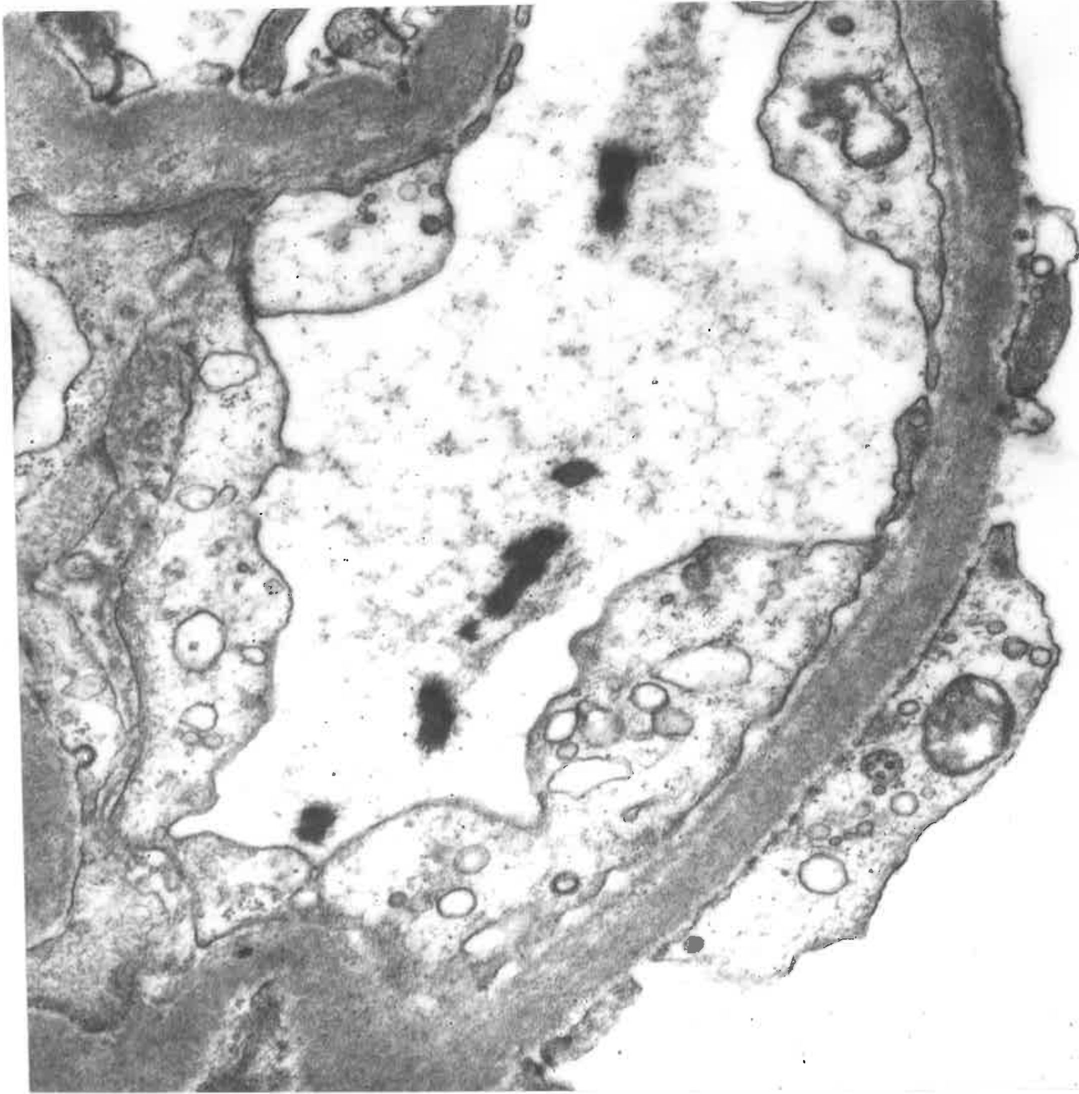


Fig. 34. This capillary shows some endothelial swelling and several intraluminal masses of fibrin. (Case 9; x 32,000)

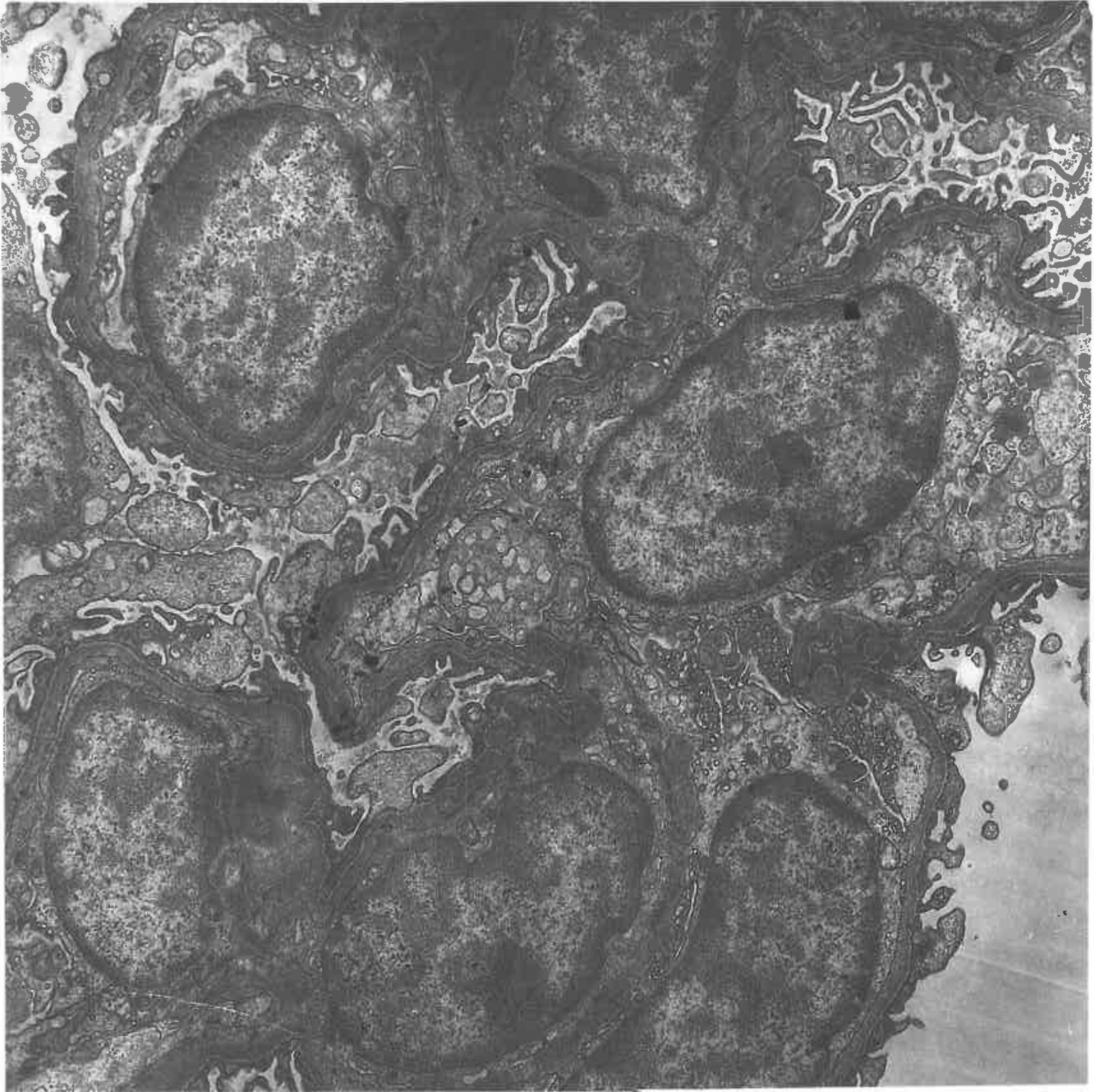


Fig. 35. This field shows marked narrowing of the capillaries with, however, only minor fibrin deposition. One rounded body which may be a platelet is seen about the middle of the picture. (Case 9; x 5,625)

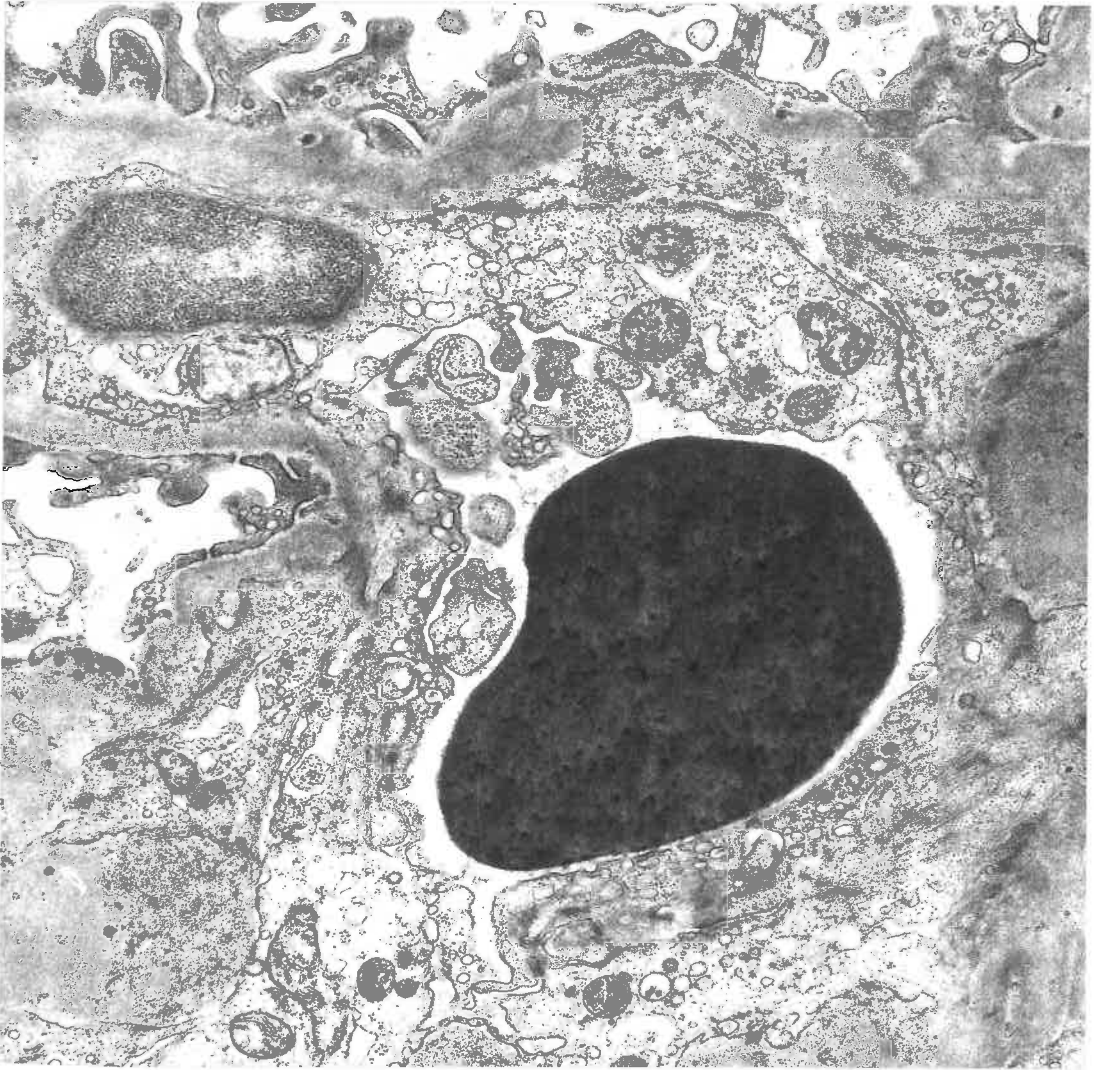


Fig. 36. This glomerular capillary exhibits endothelial swelling, with some subendothelial fibrin deposition, and a red blood cell embedded in the narrow lumen. (Case 9; x 18,000)

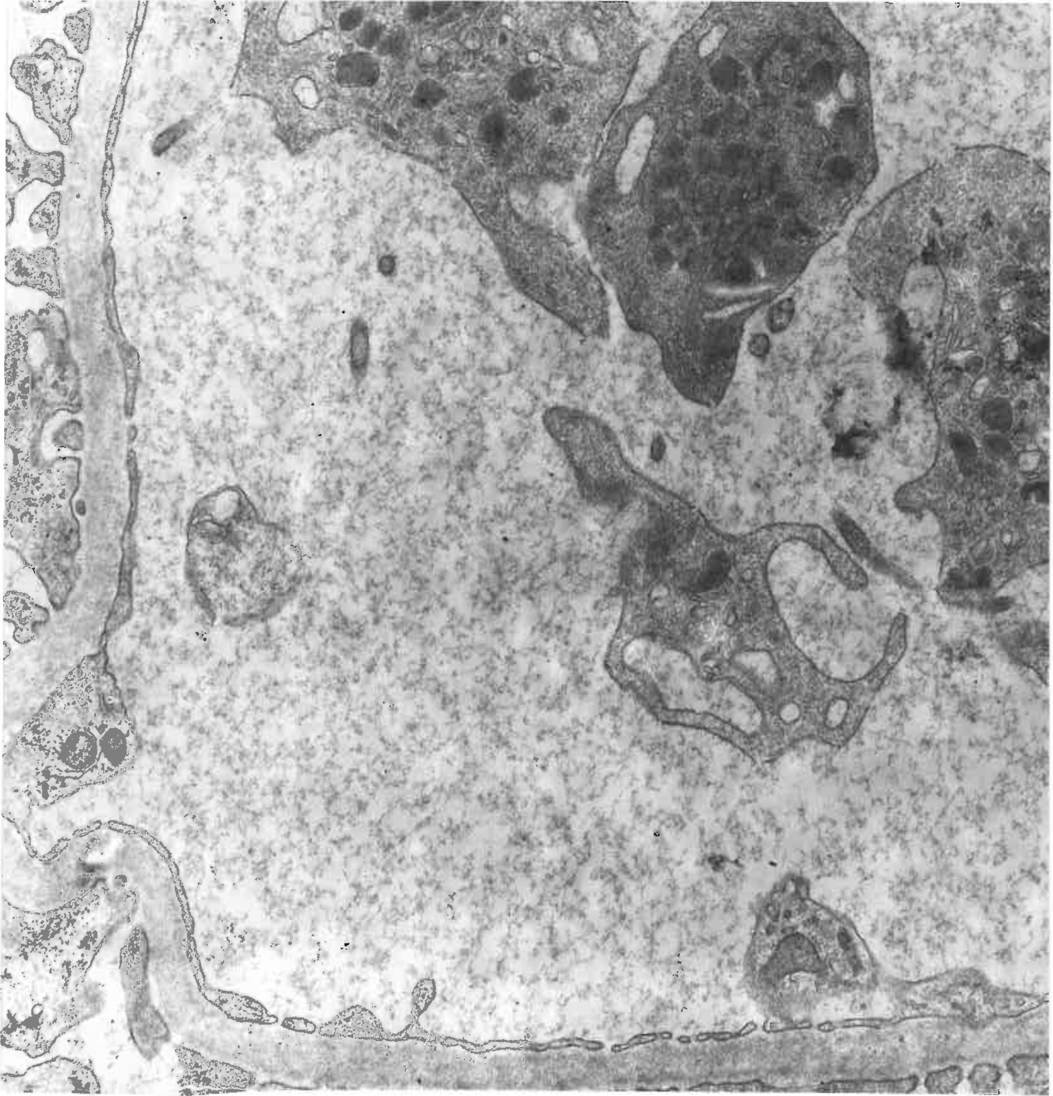


Fig. 37. Several platelets are seen in the lumen of this capillary. (Case 7; x 20,000)

the point seemed so definite as to be unequivocal. Capillaries were often distended with red blood cells and again the degree of red cell packing seemed excessive.

The epithelial foot processes were, on the whole, well preserved, although foci of podicel loss were seen. Many epithelial cell bodies showed changes suggestive of an ischaemic state, possibly amounting to necrosis (Fig. 38).

In several cases fibrillar material resembling fibrin was seen in Bowman's space (Fig. 39).

In renal biopsies taken from patients during the recovery phase, these lesions were less prominent and seemed to involve a smaller percentage of glomerular capillaries. Later biopsies were normal.

3.1.5 DISCUSSION

Understanding of the pathogenesis of acute renal failure has not kept up with the advances achieved in its management. Although many cases follow shock, this is not a universal accompaniment, whilst the majority of shocked patients do not develop acute renal failure. Hardaway et al (1954) drew attention to defects in coagulation in four patients with acute renal failure, and later described alterations in blood coagulation in animals after incompatible blood transfusion (Hardaway and McKay, 1955), the injection of thrombin (Hardaway et al, 1960) and the experimental crush syndrome (McKay and Hardaway, 1959). Teschan et al (1955) after a study of acute renal failure developing in Korean war casualties, commented that some factor in addition to hypotension and a fall in renal blood flow was necessary to explain the

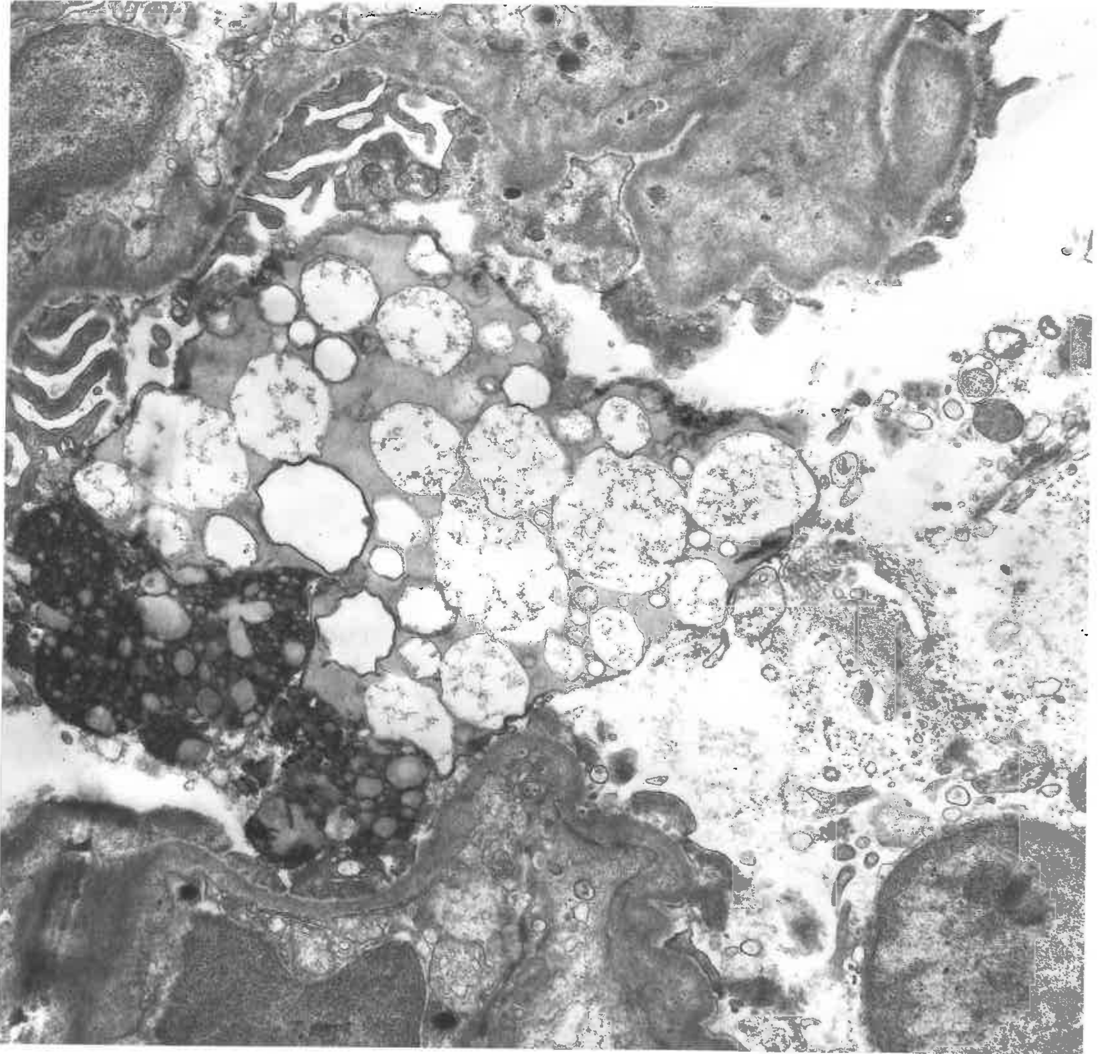


Fig. 38. The vacuolated structure is a degenerated epithelial cell; such appearances were seen fairly frequently. (Case 9; x 12,000)

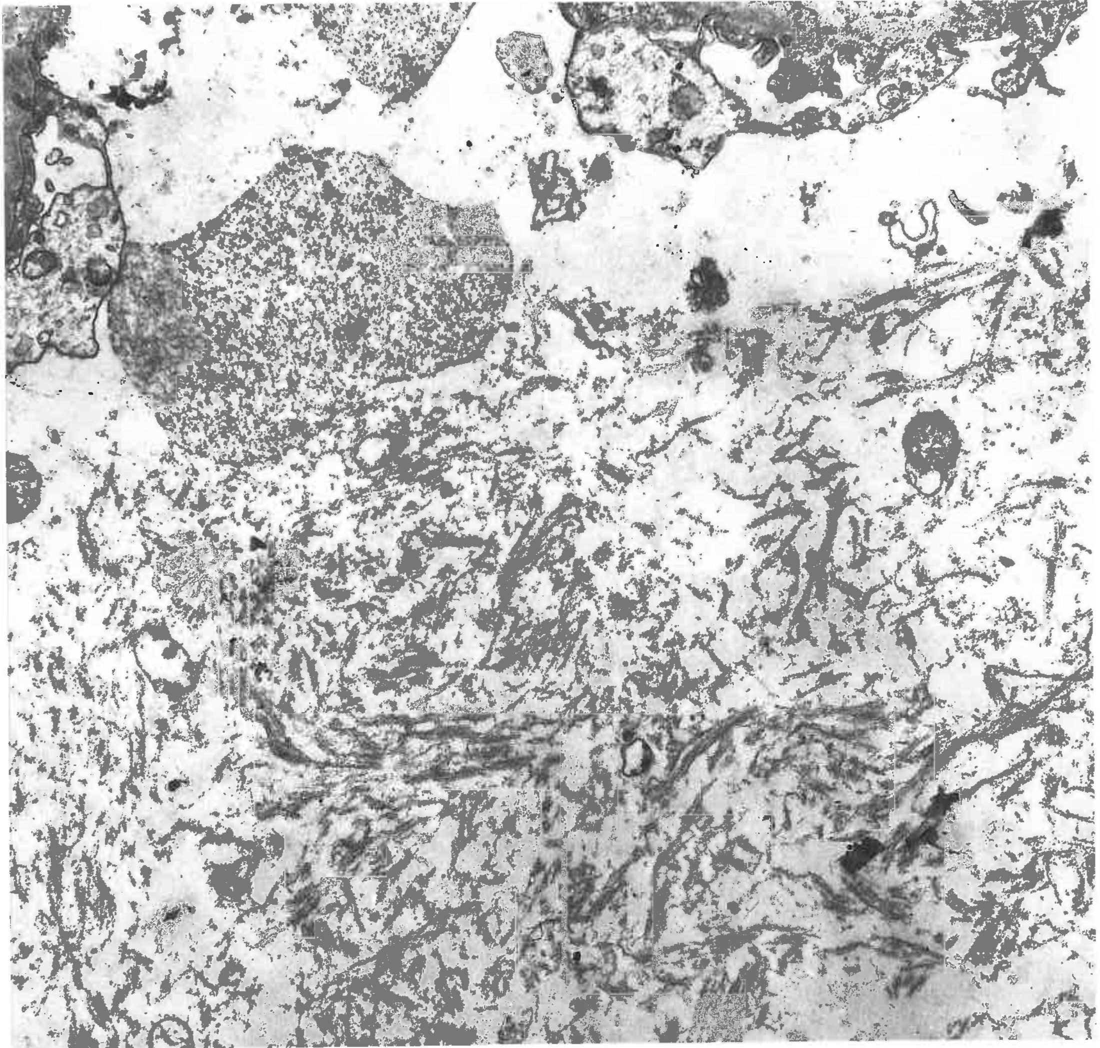


Fig. 39. This micrograph demonstrates the presence of fibrin in Bowman's space. (Case 8; x 7,000)

sequence of events in acute renal failure and Hardaway (1966) concluded that intravascular coagulation may be this additional factor.

However, lack of morphological evidence and of abnormality in coagulation as determined by routine methods in patients did not support this view. Furthermore, Dalgaard (1959, 1960) and Holden et al (1965) found no structural changes in the glomeruli by light or electron microscopy, although Steen-Olsen and Skjeldborg (1967) reported slight swelling of the epithelial cells in 2 patients and endothelial cell swelling in one of these. Handa (1970) concluded that "glomerular abnormalities in acute tubular necrosis remain vague and inadequately understood".

The 47 patients in this study all suffered from acute renal failure of what is presumed ischaemic origin. On light microscopy of renal biopsies, the glomeruli were found to be normal and histological lesions in the tubules characteristic of acute tubular necrosis were seen in all cases.

When the electron microscopic, coagulation and fibrinolytic studies are considered together, however, some new light is cast on the pathogenesis and natural history of this condition. Electron microscopic examination of the glomeruli revealed abnormalities hitherto undescribed. These consisted of the presence in capillary loops of abnormal amounts of granular material, excess platelets and red blood cells and evidence in many cases of fibrin and established thrombosis. Fibrin was also seen in Bowman's space. Comparative studies of the electron microscopic and immuno-fluorescent features of glomerular fibrin deposition (Vassalli

et al, 1963; Vassalli and McCluskey, 1964) and scanning electron micrographs of fibrin precipitated in the presence of its degradation products (Stewart, 1970) indicate that the characteristic periodicity is not always found in unequivocal fibrin fibrils. Moreover, the granular and amorphous material seen in the cases studied almost certainly represent products of the enzymatic breakdown of fibrin and/or fibrinogen. The findings of fibrin and F.D.P. in those glomerular capillaries not exhibiting complete blockage, together with abnormal numbers of platelets and red cells, are consistent with coagulation taking place at these sites, and suggest stasis and incipient or resolving thrombosis within the capillary lumina. Such changes, demonstrable in a large number of cases of varied aetiology in biopsies taken at different periods in the course of the process, including that of recovering renal function, and failure to observe similar abnormalities either after recovery or in normal glomeruli, indicate that the findings are likely to have functional significance.

These ultrastructural findings are substantiated by the observations of changes in coagulation and fibrinolysis during the phase of renal failure which revert to normal during recovery. Consumption of coagulation factors, thrombocytopenia and evidence of microangiopathic haemolytic anaemia reflect a severe degree of intra-vascular coagulation and very high concentrations of serum F.D.P. are found in these circumstances (Merskey et al, 1967). Elevation in serum F.D.P. to a lesser extent was found in all cases examined during the phase of renal failure and was followed by a fall to control values with the onset

of diuresis. The abnormal elevation of plasma fibrinogen during the early phase is also of interest and although it possibly represents a non-specific rise in response to the stress of the illness, may also in part reflect the stimulatory effect of prolonged elevation of serum F.D.P. on fibrinogen synthesis (Young and Koleman, 1970). The products of fibrin degradation are normally removed from the circulation by the reticulo-endothelial system and under certain circumstances in the urine. In the light of the presence of fibrin in Bowman's space, it was not surprising that F.D.P. were detectable in the urine. High concentrations were found in the urine secreted during the oliguric period, but the sharp increase in excretion coinciding with the onset of the recovery phase is of relevance in that it may indicate that blood flow is restored to many glomeruli at this time. Continued excretion of F.D.P. well after clinical improvement suggests that full recovery is gradual. In support of this contention is the persistence of some of the typical ultrastructural lesions during this period. As no significant chqnges were observed in the small group of patients with renal failure following urinary tract obstruction, it is assumed that the abnormalities are specific for the intrinsic renal disease.

The simultaneous elevation of PF_4 and S.F.M.C. in the phase of renal failure provides further evidence of a continuous coagulation process during this period, and may offer a clue to the intense vasoconstriction reported in the afferent arterioles of the glomeruli. Potent vasoactive chemicals are liberated along with PF_4 during platelet aggregation and their action, localized to the site of release, may be

sufficient to cause cessation of glomerular perfusion. The persistent elevation of plasma S.F.M.C. indicates the presence of free circulating thrombin, as the formation of these complexes only occurs after the removal of fibrinopeptides from the parent fibrinogen molecule. Hyperthrombinaemia causes platelet aggregation, peripheral vasodilatation and selective pulmonary and renal vasoconstriction in animals. This increased tone in the renal vasculature can be prevented by drugs which block either platelet aggregation, e.g. aspirin (Olsson et al, 1970) or α-adrenergic receptor sites, e.g. phenoxybenzamine (Grandchamp et al, 1971). Platelet factor 4 also is a powerful paracoagulating agent and its presence in high concentration may in part explain the deposition within the glomeruli of fibrin and its degradation products.

The abnormal depression of plasma plasminogen activator content throughout the oliguric phase is difficult to explain, although a fibrinolytic "shut-down" is known to occur following many situations of acute stress. Two possibilities exist, however. The decrease perhaps reflects increased utilisation in the lysis of extensive fibrin deposits, or it may indicate decreased release from the usual source, the endothelium of small vessels and veins.

While these abnormalities of coagulation and fibrinolysis in acute ischaemic renal failure are unequivocal, the mechanism of their activation remains obscure. The question also arises concerning the specificity of these disturbances to the kidney, as it seems unlikely that they reflect a process which is confined to this organ. The activities of

certain serum enzymes are elevated throughout the oliguric phase of acute renal failure and return to normal at or about the onset of diuresis (Martin et al, 1971). These include serum hydroxybutyric dehydrogenase (H.B.D.), lactic dehydrogenase (L.D.H.), creatine phosphokinase (C.P.K.) and alanine (G.P.T.) and aspartate transaminases (G.O.T.). The origins of these enzymes is diverse, and although the high concentrations of H.B.D. and L.D.H. may in part be due to intrinsic renal damage, the elevations of other enzymes would seem to indicate damage to other organs, particularly the liver and skeletal muscle. In the poisoned patients studied as controls the striking parallel between the timing of enzyme and coagulation changes may indicate a cause and effect relationship which may also exist in acute renal failure. Although further work is necessary to determine whether organs apart from the kidney are damaged in acute renal failure, data from one of the subsequently described cases is suggestive.

The evidence available from the study of two patients during the onset phase suggested that abnormal coagulation may also play a part in the development of the oliguria. Serum F.D.P. rose during the onset of oliguria and there was a sudden increase in urine F.D.P. content at this stage (Figs. 19 and 20). Extended study of one of these patients (Case 47), a 28 year-old woman whose renal failure was precipitated by self poisoning with paracetamol, disclosed that serum enzyme activities also rose precipitously at this stage; indicating, perhaps, that the kidney was not the only organ involved in this process (Fig. 40). Of additional interest in this case was the observation that

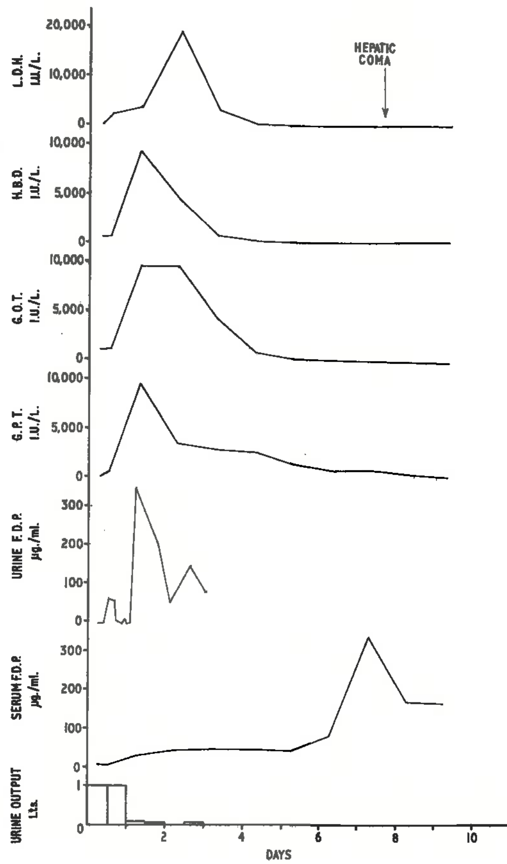


Fig. 40. Relation between rise in serum activities and F.D.P. concentration and the onset and course of acute renal failure in Case 47. The onset of hepatic coma was associated with further abnormalities in serum F.D.P. and liver enzymes.

the onset of hepatic coma was associated with a marked rise in serum F.D.P. concentration to levels much higher than commonly observed during the oliguric phase, thereby implicating the coagulation process in the development of acute hepatic necrosis of which this woman died.

Data from each of the groups of control patients studied after a stressful event, suggest that changes in coagulation and fibrinolytic indices occur which possibly render the blood hypercoagulable. These changes, which include elevation of plasma fibrinogen, S.M.C.F., PF_4, serum F.D.P. and depression of plasmaactivator content, are maximal between the second and fifth days after the stress, and last as long as two weeks. Similar changes have been described in other situations of acute stress such as myocardial infarction, severe trauma, burns and electropexy, and probably represent a non-specific response, the extent of which depends partly on the degree of stress and partly on the individual's ability to cope with it. It is in precisely these circumstances that acute renal failure most commonly develops, and it is tempting to postulate that the addition of a further factor favouring coagulation or decreasing fibrinolysis may precipitate acute renal failure. Such factors include infection, hypotension, haemorrhage, hypovolaemia, haemolysis and hypoxia.

The theories proposed to explain the oliguria of acute ischaemic renal failure in man need to be revised to take account of the ultrastructural and biochemical findings described. Intraglomerular coagulation may be an important factor which contributes to the prolonged depression of glomerular filtration rate in experimental models of acute

renal failure and for the continued modest depression in renal blood flow which in man extends far beyond the time when restoration of blood volume and blood pressure have been achieved. Of course, such a view does not preclude the possibility that tubular necrosis itself also contributes to the renal dysfunction as has been suggested in the past by several workers. Indeed glomerular thrombosis may be partly responsible for this ischaemic lesion, because much of the tubular blood supply is derived from post-glomerular sources. In view of the portal nature of the cortical tubular blood supply, and the variability of anastomotic channels between afferent arterioles and the tubular capillary network, it is not surprising to find that the tubular lesions are patchy. The degree of damage might be determined, on the one hand, by the number of glomeruli involved, and on the other by the extent and patency of anastomotic vessels bypassing the glomerulus. Moreover, because of the free communications of post-glomerular vessels with adjacent nephrons, blockage of one glomerulus may affect more than one tubule. A similar mechanism may also explain structural changes of acute tubular necrosis often present in severe acute proliferative glomerulonephritis and the haemolytic uraemic syndrome. In these states, glomerular damage is often sufficiently severe to cause total occlusion of glomerular blood flow by either extensive cellular proliferation or thrombosis.

Nor do these findings necessarily invalidate other theories as to the causation or maintenance of the oliguria. At the present time, it is probably wise to regard abnormal coagulation and fibrinolysis

as contributing factors in the pathogenesis of a condition in which many other physiological and pathological mechanisms play a role. It is pertinent to point out, however, the integral part played by coagulation and fibrinolysis in the onset, oliguric and recovery phases of acute ischaemic renal failure. While intraglomerular coagulation may have serious consequences in the short term, it may also represent a process which is essential to the recovery of full renal function. If glomerular filtration occurred unimpeded after renal shutdown, the tubular cells would be unable to handle the vast amount of glomerular filtrate, and homeostasis would be destroyed within hours. Thus the protection supplied by physical blockage of the glomeruli by thrombi allows regeneration of tubular cells which are capable of near normal function on recovery of the glomerular lesions. That the timing of glomerular and tubular recovery does not always coincide is evident from the massive solute and water diuresis which sometimes occurs during the early recovery phase.

3.2. THE HAEMOLYTIC URAEMIC SYNDROME

As a cause of acute renal disease, the haemolytic uraemic syndrome is uncommon, but its presentation, usually as acute renal failure, is dramatic, and sufficiently distinctive for it to be considered separately from classical acute ischaemic renal failure. Although Hensley recognized the association between severe haemolytic anaemia and acute renal failure in 1952, the original description of the haemolytic uraemic syndrome as a distinct clinical entity is attributed to Gasser et al

(1955). Since this time there have been many reports of the syndrome as a disease affecting infants and young children and only recently has its occurrence in adults been widely recognised. It is characterized by renal failure of acute onset, microangiopathic haemolytic anaemia, and typical renal histopathological changes. The syndrome has masqueraded under several titles of confusing diversity and the grouping of these cases under a single heading "the haemolytic uraemic syndrome" (H.U.S.) is perhaps controversial, as many possess features in common with thrombotic thrombocytopenic purpura (Moschcowitz, 1925). The invariable involvement of the kidneys serves, however, to isolate these patients, and H.U.S. is an apt yet not a comprehensively descriptive term. Review of the available literature indicates that all cases have uniform renal histopathological changes characterised by thrombosis of afferent arterioles and glomerular capillaries and variable degrees of ischaemic cortical damage. Extensive study of the haemolytic anaemia has shown that the red cell damage is caused by mechanical distortion and fragmentation in small blood vessels narrowed by the deposition of thrombotic material (Brain et al, 1962; Rubenberg et al, 1968). The pathogenesis of the H.U.S. has not been established and indeed all cases may not arise from the same pathogenetic mechanisms. The occurrence together of microangiopathic haemolytic anaemia and thrombotic lesions within the kidney strongly suggest, however, that intravascular coagulation may play a fundamental role. For this reason there follows a detailed description of the clinical and pathological features of 22 cases personally encountered. In seven of these, detailed studies of coagulation and fibrinolysis have been performed.

3.2.1 CASE SELECTION

All patients presented with a relatively rapid onset of renal failure accompanied by severe microangiopathic haemolytic anaemia and biopsy or autopsy revealed characteristic renal pathology. Sixteen of the 22 patients were females and of these 6 had recently been pregnant and another three were taking the contraceptive "pill". The ages of the patients ranged from 12 to 65 years. Recovery occurred in only five patients and in each the onset of the illness was abrupt and the process short-lived (Table 5).

3.2.2 CLINICAL FEATURES

PRODROME

There was a prodromal period of ill health in each subject lasting from one to approximately 100 days (Table 6). Symptoms of upper respiratory tract infection initiated the prodrome in 12 cases and in 9 abdominal pain, vomiting and diarrhoea were the first signs of ill health. Deterioration of health occurred at variable speed, a calamitous course developing in cases 7, 10, 12, 15, 16 and 22, and a more gradual decline in the others. During this time a bleeding disorder became apparent in fifteen patients. In the post-partum patients and those on the "pill" increased vaginal blood loss, and in Case 8, menorrhagia occurred, while subarachnoid haemorrhage developed in Case 15. A generalized bruising tendency often with petechial haemorrhages was noted in Cases 6, 9, 11, 13, 14, 18, 20 and 22. Weight loss, anorexia, and pallor were common. Severe dyspnoea, progressive oedema, and

Table 5.

Details of Age, Sex, Previous History and Outcome in the Patients Studied

Case No.	1	2	3	4	5	6	7	8	9	10	11
Age	21	23	24	61	59	12	26	38	24	15	32
Sex	F	M	F	F	F	M	M	F	F	M	F
Past History	Post partum	Osteo-myelitis	Post partum				Tonsil-lectomy		Post partum	S.L.E. steroids	
Outcome	D	D	D	A	D	D*	A	D*	D*	D	D*

Case No.	12	13	14	15	16	17	18	19	20	21	22
Age	25	33	59	16	48	56	47	65	27	39	25
Sex	F	M	F	M	F	F	F	F	F	F	F
Past History	Septic abortion	Portal vein thrombosis					"Pill"	"Pill"	Post partum	"Pill" migraine	Post partum
Outcome	A*	D	D*	D	A*	D*	D	D*	D*	D*	A*

* Heparin therapy

Table 6.

Prodomal Period: Duration, Initial Symptoms, Type of Bleeding

Case No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Length (Days)	6	10	15	20	20	21	1	100	21	2	30	1	30	100	1	4	7	35	30	15	35	6
Initial Symptoms																						
Upper Respiratory	+		+		+	+			+		+		+		+	+			+	+		+
Vomiting and Diarrhoea		+		+				+						+			+	+				+
Abdominal Pain				+						+							+			+		
On Admission																						
Bleeding	V		V		S.A.	P		V	V,P		G			P	P		V,R	G	V	V,P	V,G	V,P
Weight Loss						+		+			+		+	+								
Oedema			+		+	+		+	+		+		+	+		+			+	+	+	+
Dyspnoea	+	+	+			+		+	+		+		+	+				+	+	+	+	+

V = vaginal S.A. = Subarachnoid P = Petechial R = Rectal G = Generalized

oliguria heralded hospital admission, by which time each patient was seriously ill with acute renal failure and severe anaemia.

HOSPITAL COURSE

Prominent features on physical examination are shown in Table 7. Initial urinalysis revealed heavy proteinuria and the urinary sediment contained many red cells and red cell casts. Although prior hypertension was not recorded in any patient, hypotensive therapy was needed in 11 cases. Retinal changes consisted of fresh haemorrhages and soft exudates suggesting a recent rise in blood pressure. Pulmonary oedema present on admission in 9 patients disappeared with correction of fluid overload, but gallop rhythm and variable cardiac murmurs persisted in several. Hepatomegaly was a consistent finding and the spleen was palpable in 2. Seven patients suffered epileptiform convulsions unrelated to azotemia or to rapid fluid and electrolyte shift during dialysis. In 5 cases episodes of ventricular tachycardia occurred and in Case 9 there was electrocardiographic evidence of anterior myocardial infarction. A peculiar localized, tender pitting oedema lasting several days was seen in Cases 6 and 7. In the former a swelling over the right shoulder was aspirated because of suspected abscess formation but no fluid was obtained and needle culture was sterile. Pyrexia up to 40° was present in 16 cases.

Table 7.

Clinical Features: Hospital Course

Case No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Blood Pressure (admission)	100/70	240/130	155/100	170/100	220/140	160/120	60/20	230/140	220/125	90/70	220/130	60/30	230/140	220/120	80/60	130/90	100/60	120/70	160/80	190/120	210/120	110/65
Retinal Changes (K-W grade)	0	0	3	0	1	0	0	4	3	0	3	0	3	3	0	0	3	3	1	3	4	0
Pulmonary Oedema	+	+	+			+		+	+		+		+	+			+			+		
Fever	+	+	+			+		+	+		+	+	+	+		+	+	+	+	+	+	
Hepatomegaly	+	+	+		+	+		+	+	+	+		+	+		+		+	+		+	+
Splenomegaly			+						+													
Pulmonary infarction					+	+*		+*	+*		+*			+				+	+*	+*		+*
Epileptiform convulsions	+		+			+			+								+	+				
Local Oedema						+	+															

* Pulmonary infarction occurred despite anticoagulant therapy.

3.2.3 LABORATORY FEATURES

HAEMATOLOGICAL

The results of blood-film examination on admission to hospital are shown in Table 8. A haemolytic anaemia was present in each patient, initial haemoglobin values varying from 3.1 to 11.5G/100ml and the reticulocyte count from 3 to 18%. Normoblasts were frequently seen. In no patient were L.E. cells found or the Coombs' test positive. Circulating free haemoglobin was detected spectroscopically in 12 cases. White-cell counts varied from normal to high values, myelocytes and metamyelocytes were often found, while in each case there were numerous fragmented and burr cells (schistocytes) together with microspherocytes.

BIOCHEMICAL

Severe oliguric renal failure was present in each case and azotemia persisted despite dialysis in 17 patients with no significant return of renal function before death. In 5, progressive improvement to normal function occurred after oliguric periods ranging between 5 and 21 days. Hyperbilirubinaemia was present in 12 of the 22 patients, the greater proportion being due to conjugated bilirubin in each case. Alanine and aspartate transaminase activities were elevated in the acute phases of the illness indicating hepatic damage, but serum alkaline phosphatase was raised only in Case 7, and serum amylase in Cases 6, 7 and 17.

Table 8.

Haematological data in patients with haemolytic uraemic syndrome on admission to hospital. Severe microangiopathic haemolytic anaemia with a variable leukocytosis and a leuko-erythroblastic picture.

Case No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Haemoglobin (G/100ml)	6.9	7.3	6.3	8.0	8.5	3.8	11.5	7.2	9.2	9.5	8.7	11.2	10.6	7.5	10.6	9.1	9.3	6.5	9.5	7.6	3.1	7.8
Reticulocytes (%)	18	17	15	8	7	8	9	15	12	10	15	9	13	17	9	3	4	5	7	15	8	10
Normoblasts (per 100W.B.C.)	6	2	2	2	0	3	3	2	2	11	2	1	4	3	3	1	2	3	4	1	9	2
Leukocytes ($10^3/ml^3$)	32	8.4	9.3	7.0	15	8.6	24	15	9.5	17.5	15	13	9.5	19	21	11.7	14.6	21.9	10.2	17.4	9	15.6
(polymorphs %)	88	85	77	72	86	46	65	84	79	87	92	91	85	83	90	81	91	88	94	88	91	85
(precursors %)	7	3	2	2	1	3	6	3	3	22	1	1	3	1	3	2	2	5	1	3	2	2
Microangio-pathic changes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

3.2.4 COAGULATION STUDIES

Routine study of coagulation was performed at least once in 20 of the 22 patients. Results were variable depending on the timing of the tests in relation to the previous length of illness. In general, the grossest abnormalities were detected early in the course of the illness and included thrombocytopenia, hypofibrinogenaemia, prolonged prothrombin time, elevated serum F.D.P. concentration, and decreased concentrations of Factors V and VIII. Screening tests for intrinsic system abnormality were, however, normal, and only in 4 cases was the bleeding time (DUKE) prolonged, while the clotting time in glass was lengthened in three. Later in the illness, platelet count, plasma fibrinogen and Factors V and VIII concentrations often rose to levels far in excess of normal. More detailed studies in the later cases revealed abnormalities which are of significance in the understanding of the natural history of the process.

Serum F.D.P. concentrations were grossly elevated to over 2,000 μg per ml early in the disease in 4 of the 7 cases examined serially. In the 2 patients who recovered (Cases 16 and 22) a progressive fall in concentration occurred until normal values were attained at the time of onset of diuresis. The similarity in this pattern with that occurring in acute ischaemic renal failure is striking, although maximal values are significantly higher. Both of these patients were treated with heparin (Fig. 41). When recovery did not take place, serum F.D.P. concentrations fluctuated throughout the illness suggesting that, by contrast to the patients who recovered, the process was a recurring

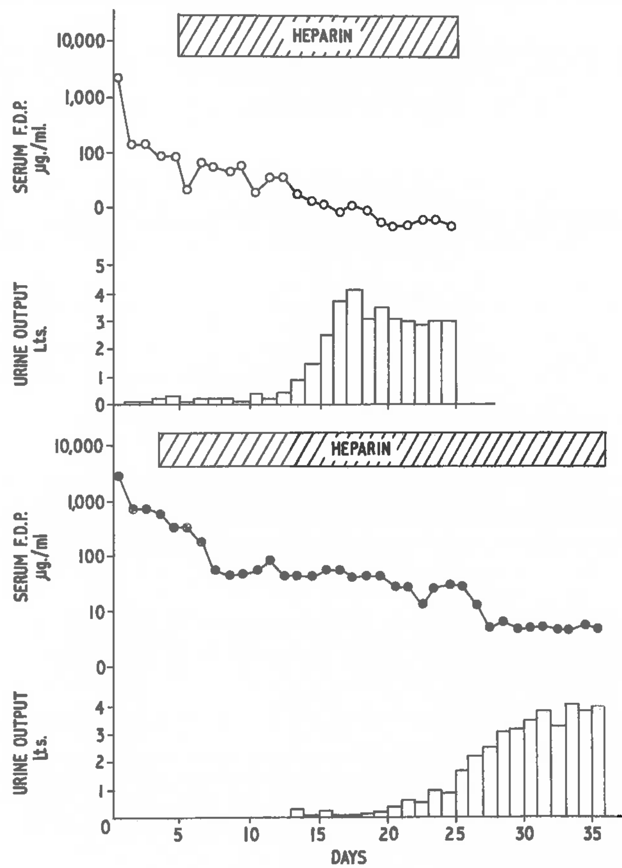


Fig. 41. Serum F.D.P. concentration (logarithmic scale) in relation to urinary output in 2 patients who completely recovered from the haemolytic uraemic syndrome. Case 16 (lower) and Case 22 (upper). There is a striking similarity between the pattern observed and that seen in acute ischaemic renal failure.

one. This pattern was present in patients treated (Fig. 42) with heparin as well as those untreated (Fig. 43), although generally serum F.D.P. levels fell during heparin therapy. F.D.P. were found in very high concentrations in available urine throughout the illness, and these levels again did not seem to be influenced by heparin therapy (Fig. 42 and Fig. 44).

Plasma fibrinogen levels were elevated at periodic intervals throughout the illness (Fig. 43) and these elevations appeared to coincide with abnormalities in other parameters studied. Similar changes were observed in plasma S.F.M.C. in all cases examined. Euglobulin lysis time was prolonged indicating decreased plasma activator content early in the illness and then gradually rose only to fall again at a time coinciding with increased abnormalities of other factors. In one closely studied patient (Case 18) a marked decrease in activator activity was found a few hours prior to cardiac arrest (Fig. 45).

Plasma PF_4 could be measured only in those patients not treated with heparin. Three distinct peaks of increased concentration were observed in one such patient (Fig. 43) and were associated with a significantly diminished platelet count, indicating a consumption or using up of platelets in these episodes of coagulation. Serial platelet counts in treated patients revealed similar fluctuations, and thrombocytopenia of less than 50,000 per cubic mm were found in all patients whose platelet count was measured daily. When only random measurements are made, normal, or high platelet counts may be found and provide no clue to the underlying illness.

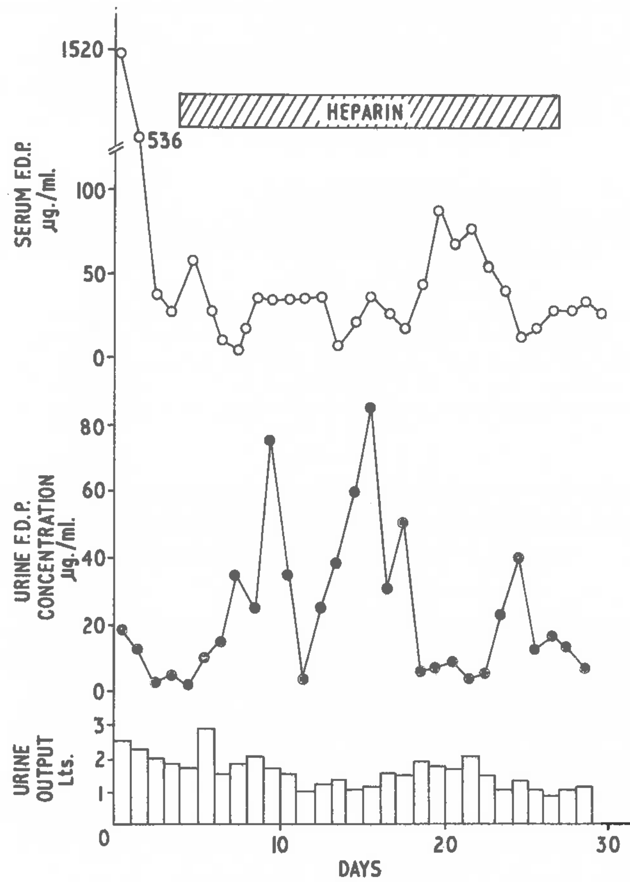


Fig. 42. Continued elevation of serum and urinary F.D.P. concentrations despite heparin therapy in Case 20, a woman with post partum renal failure who died from chronic renal failure.

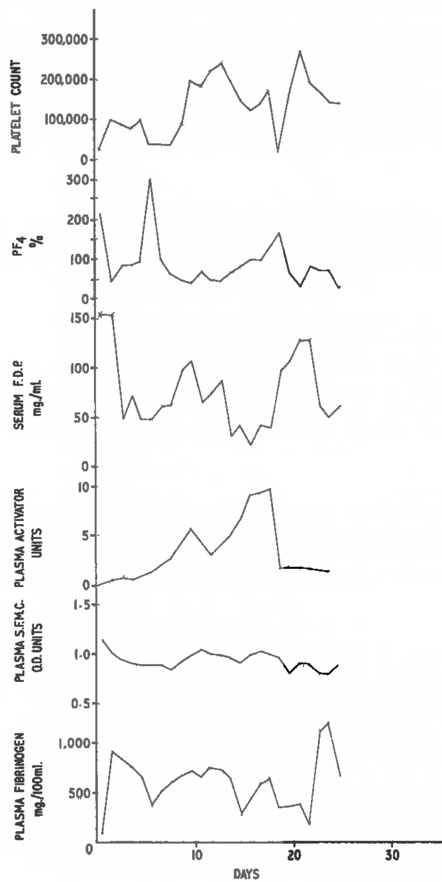


Fig. 43. Case 18. Serial studies of coagulation and fibrinolysis in this untreated patient demonstrate unequivocal evidence of pe riodic episodes of coagulation. She remained anuric during hospitalization.

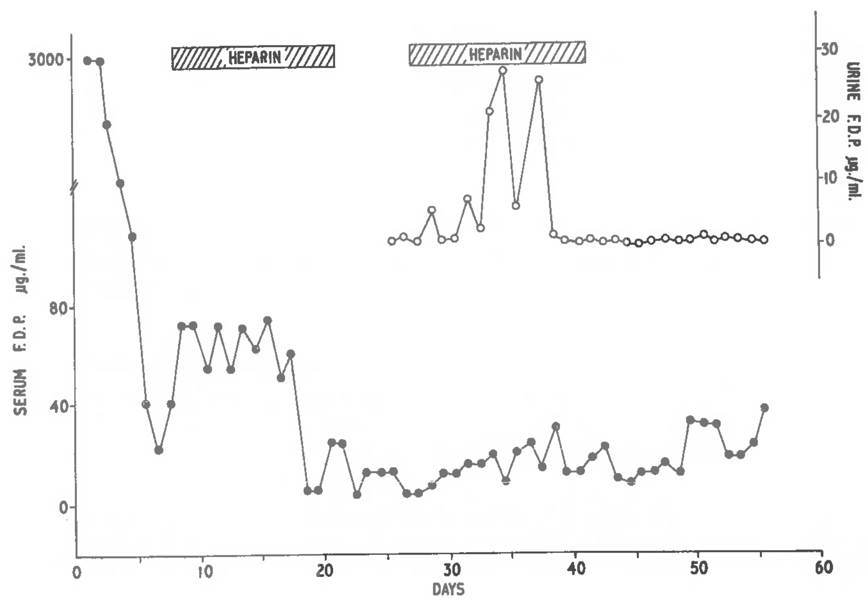


Fig. 44. Case 19. The response of serum and urinary F.D.P. concentrations to 2 separate courses of heparin. Periodic episodes of coagulation have seemingly occurred despite anticoagulation.

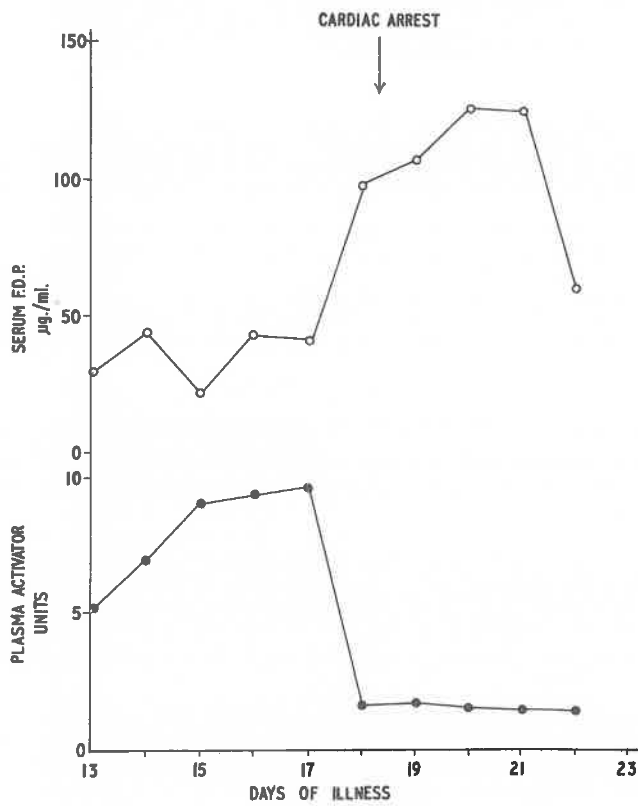


Fig. 45. Serial changes in serum F.D.P. and plasma plasminogen activator concentrations in Case 18. Elevation in F.D.P. and decrease in activator occurred just prior to an episode of cardiac arrest.

3.2.5 HISTOPATHOLOGY

THE KIDNEY

Kidneys were of normal size or even enlarged in those who died early in the illness, but reduction in size in others corresponded to the length of maintenance of life. The external surfaces of ten exhibited a "flea bitten" appearance whilst the cut cortical surfaces were mottled and haemorrhagic. Patchy cortical infarcts seen in four coalesced into bilateral renal cortical necrosis in Cases 10 and 15.

The most typical feature of the renal histology was occlusive thrombus formation in afferent arterioles and glomerular capillaries (Fig. 46 and Fig. 47). Often, direct continuity of the thrombus could be traced from afferent arterioles to glomerular capillaries (Fig. 48). The visible thrombosis was confined to the glomerular capillaries in Cases 16 and 22, both of whom recovered, but in all patients where the outcome was fatal, extension of thrombosis to afferent arterioles and often interlobular arteries was a feature. The thrombus itself most commonly presented a bland appearance by conventional staining, but red cells and platelets were seen entrapped in several. Special stains confirmed the presence of fibrin both in the lumen and incorporated in the wall of afferent arterioles and interlobular arteries (Fig. 49, 50 and 51). There was no inflammatory reaction in the vascular cells which was of sufficient degree to cause occlusion of the lumen in long-standing cases (Fig. 52 and 53). The internal elastic lamina was disrupted in several cases and the appearances were similar to those seen in the malignant phase of hypertension. Occasionally a picture consistent with recanalisation of thrombus was seen in afferent arterioles (Fig. 54).

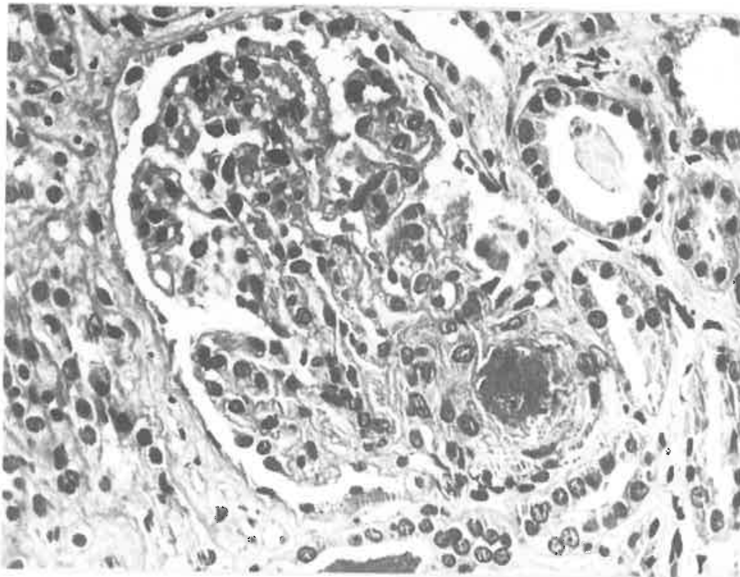


Fig. 46. Case 9. A thrombotic lesion is demonstrated at the glomerular hilum. (H and E; x 360)

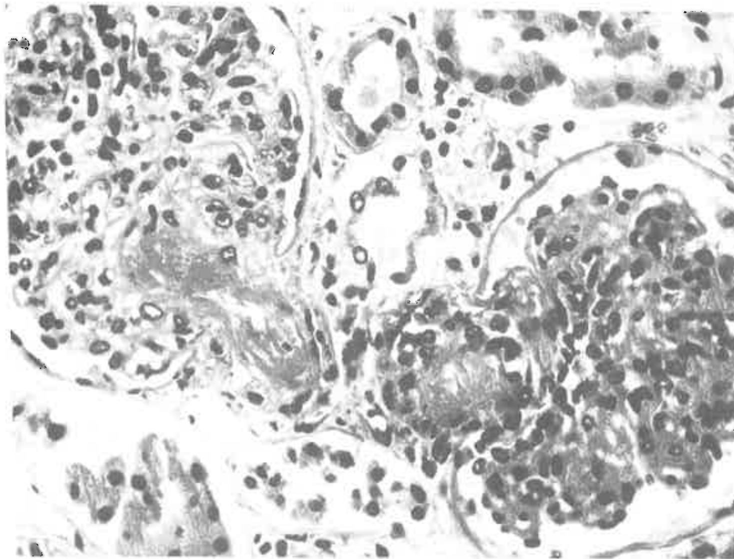


Fig. 47. Case 1. Afferent arteriolar thrombosis affecting adjacent glomeruli (H and E; x 360)

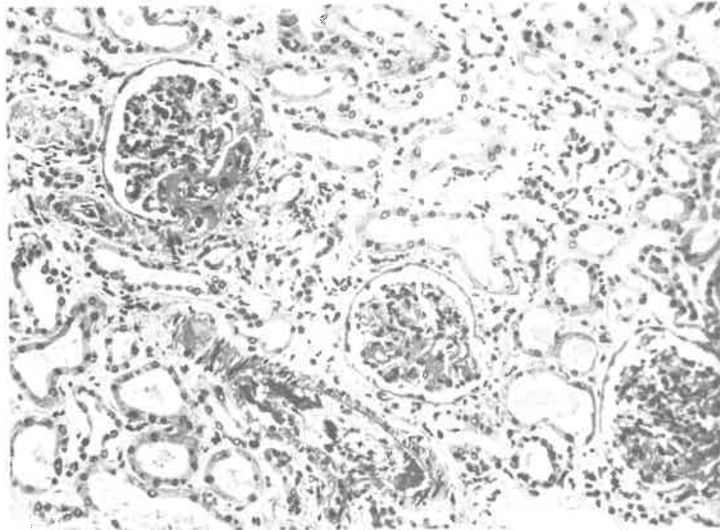


Fig. 48. A low power view demonstrating the continuity of thrombosis from afferent arteriole to glomerular capillary. (Case 1; H and E; x 150)

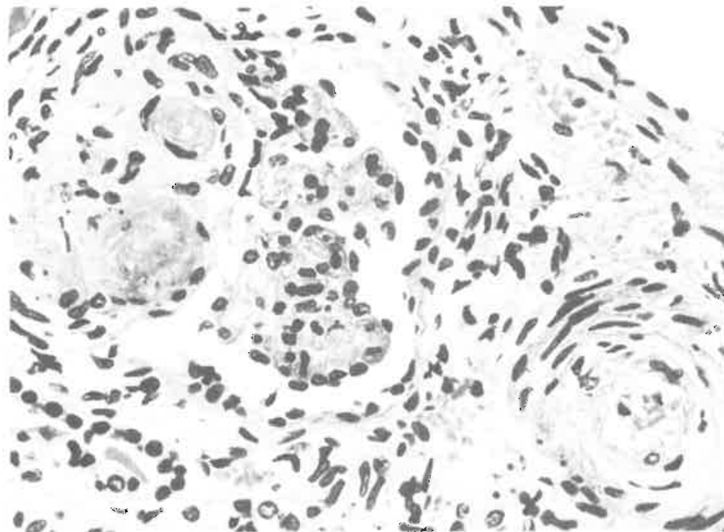


Fig. 49. Thrombosis of afferent arteriole and glomerular capillary in case 8. An adjacent interlobular artery shows the typical hyperplastic intimal changes. (H and E; x 360)

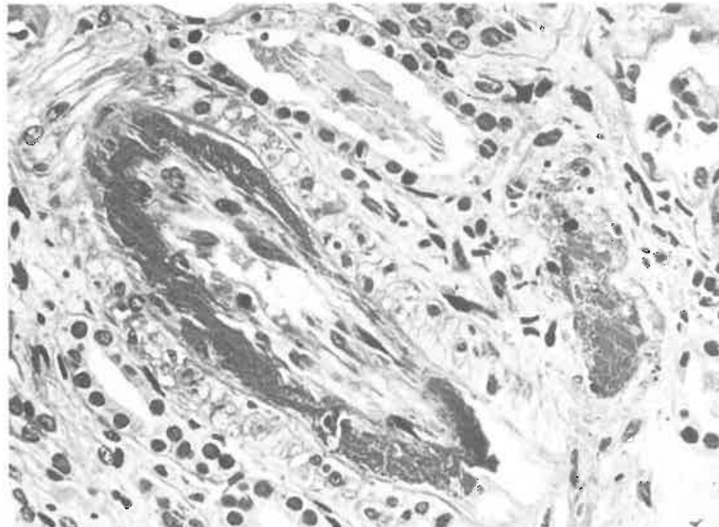


Fig. 50. Interlobular artery from Case 3 showing "fibrinoid" deposited between a hyperplastic intima and the internal elastic lamina. (P.A.H.; x 360)

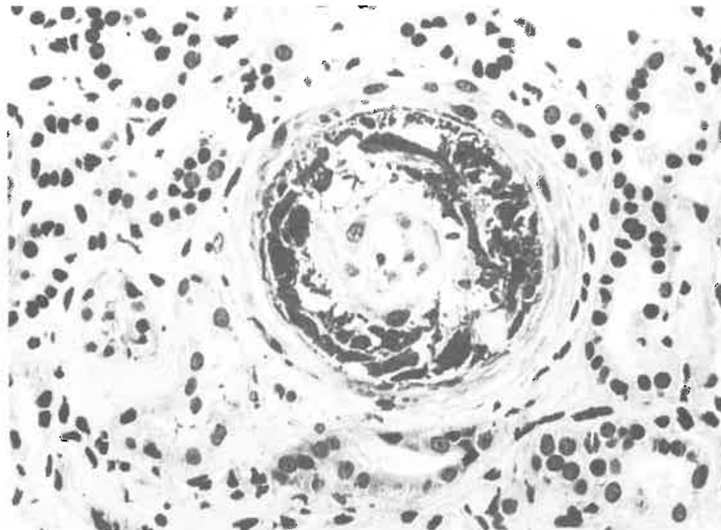


Fig. 51. A further example of fibrinoid deposition within the wall of an interlobular artery. The arterial lumen is markedly narrowed. (Phloxine Tartrazine; x 360)

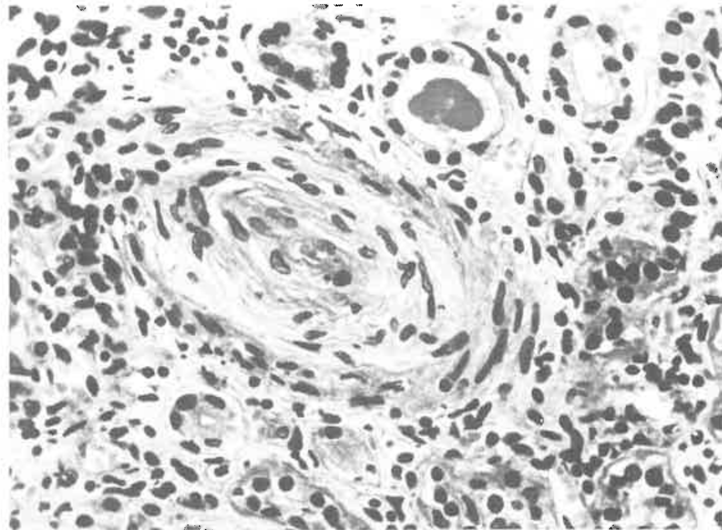


Fig. 52. Interlobular artery obtained at autopsy from case 11. The marked intimal proliferation has caused complete obliteration of the vascular lumen. (H and E; x 360)

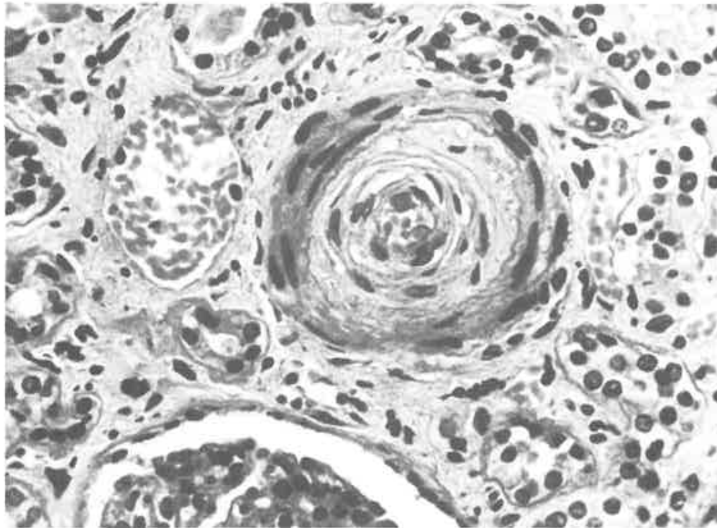


Fig. 53. Case 9. Autopsy specimen. Changes similar to those seen in Fig. 52 are illustrated, which are identical to those observed in malignant hypertension. (H and E; x 360)

Glomerular appearances were variable. When afferent arterioles were occluded, the tuft appeared bloodless, and relatively hypocellular (Fig. 55) although occasional glomeruli were filled with red cells, suggesting a form of recent infarction (Fig. 56). In others, endothelial cell proliferation, leukocyte infiltration and sometimes epithelial cell "reactivity" enlarged the glomerulus in a similar fashion to that seen in acute proliferative forms of glomerulonephritis (Fig. 57). Endothelial cell proliferation was more commonly present in local areas of the tuft and surrounded deposits of fibrin (Fig. 46). Rarely, cells with large foamy cytoplasm were prominent (Fig. 58). Such appearances have been attributed to heparin therapy (Hall et al, 1971), which activates the lipoprotein lipase system, releasing free fatty acids into the plasma. In the presence of vascular damage this free fatty acid may be incorporated into the vessel walls in excessive amounts. However, similar histological changes were observed in treated and untreated cases.

Electron microscopic appearances of the glomeruli differed from those seen in acute renal failure only in their extent, severity and uniformity. There was marked reduction in the calibre of the glomerular capillary lumina leaving only very small channels through which blood might flow (Fig. 59). The basement membrane appeared in general to be of normal density and thickness, but between it and the proliferating endothelial cells, was an irregular often rarefied layer sufficiently thick to cause much of the capillary obstruction. Islands of endothelial cytoplasm and platelets (Fig. 60) often appeared isolated

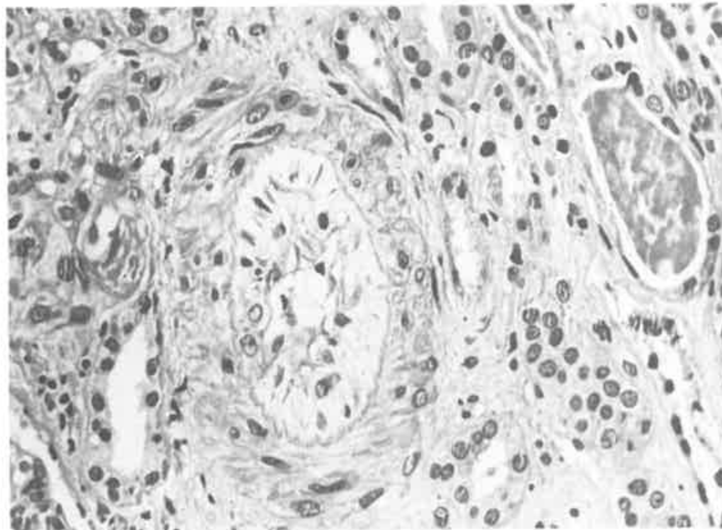


Fig. 54. Case 6. The appearance of the interlobular artery is similar to that observed after recanalization of a luminal thrombus. (H and E; x 360)

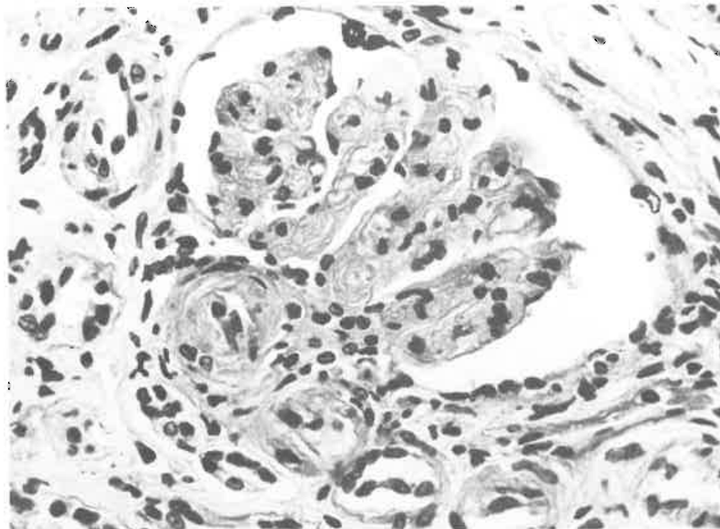


Fig. 55. As a consequence of afferent arteriolar thrombosis, this glomerular tuft appears bloodless and hypocellular. (Case 9; H and E; x 360)

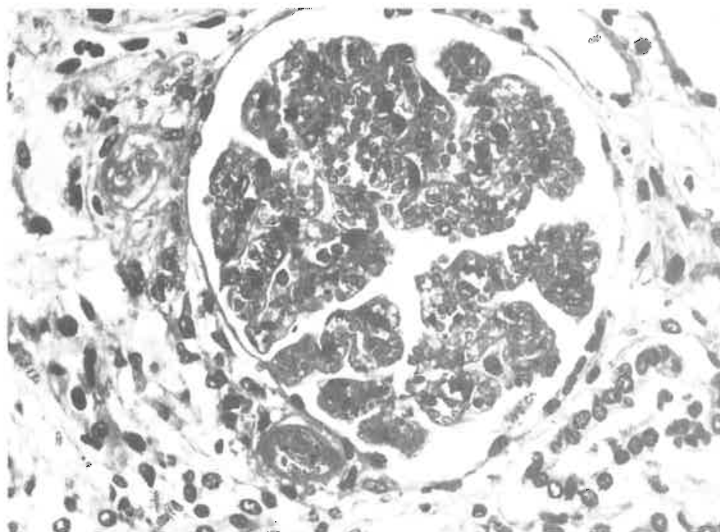


Fig. 56. The filling of the capillaries in this glomerulus with red blood cells, suggests recent infarct necrosis (Case 1; H and E; x 360)

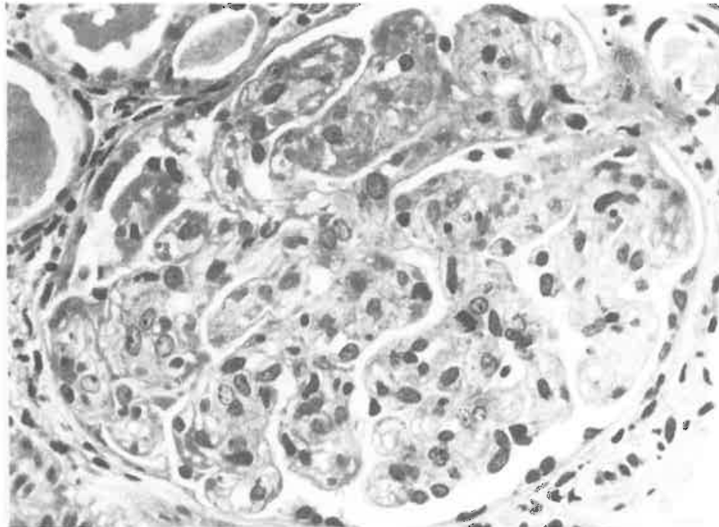


Fig. 57. Case 9. This glomerulus is swollen and hypercellular. The hypercellularity appears to be caused by proliferation of glomerular capillary endothelial cells. The basement membrane appears thick. (H and E; x 360)

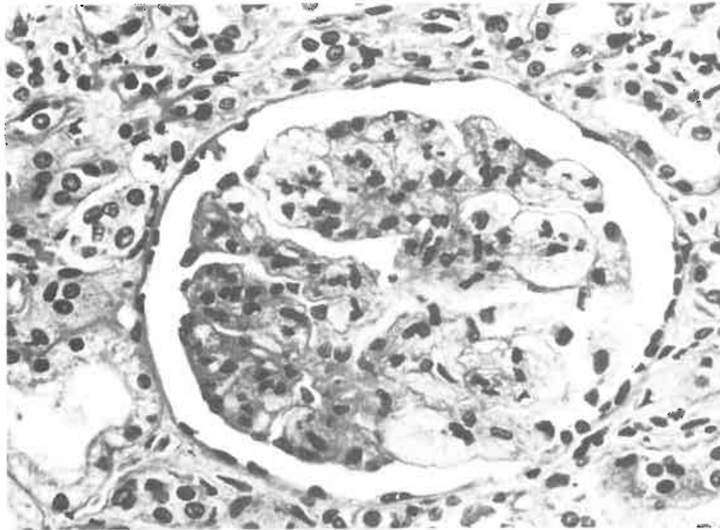


Fig. 58. In this glomerulus, cells with large foamy cytoplasm are seen. Although this appearance has been attributed to heparin therapy it was equally common in both treated and untreated patients. (Case 9; H and E; x 360)

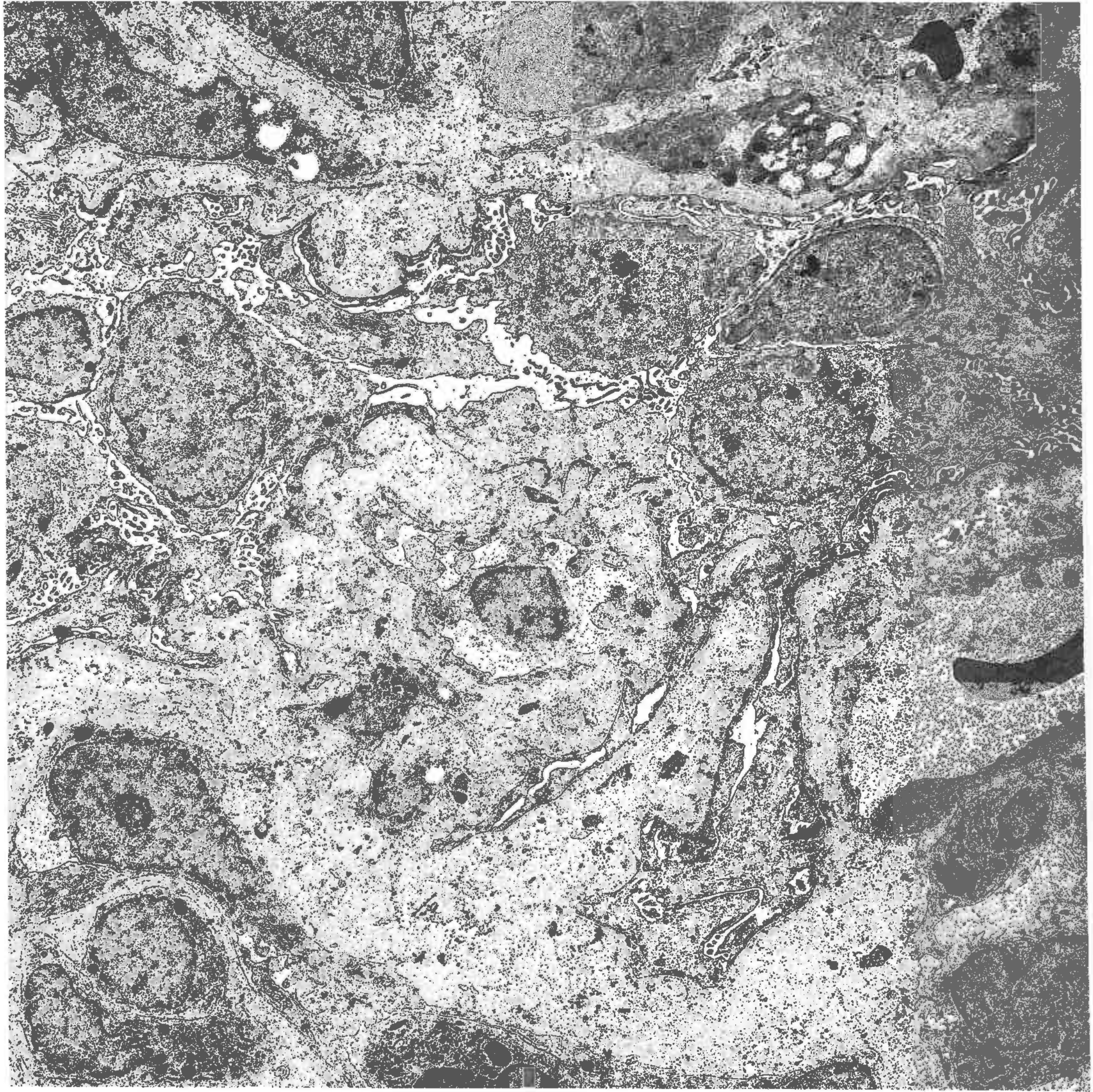


Fig. 59. This electron micrograph shows a low power view of an affected glomerulus. Capillary lumina are severely narrowed, and this can be seen to be due to endothelial thickening and the presence of a pale zone of varying thickness between the basement membrane and endothelium. (x 1,500)

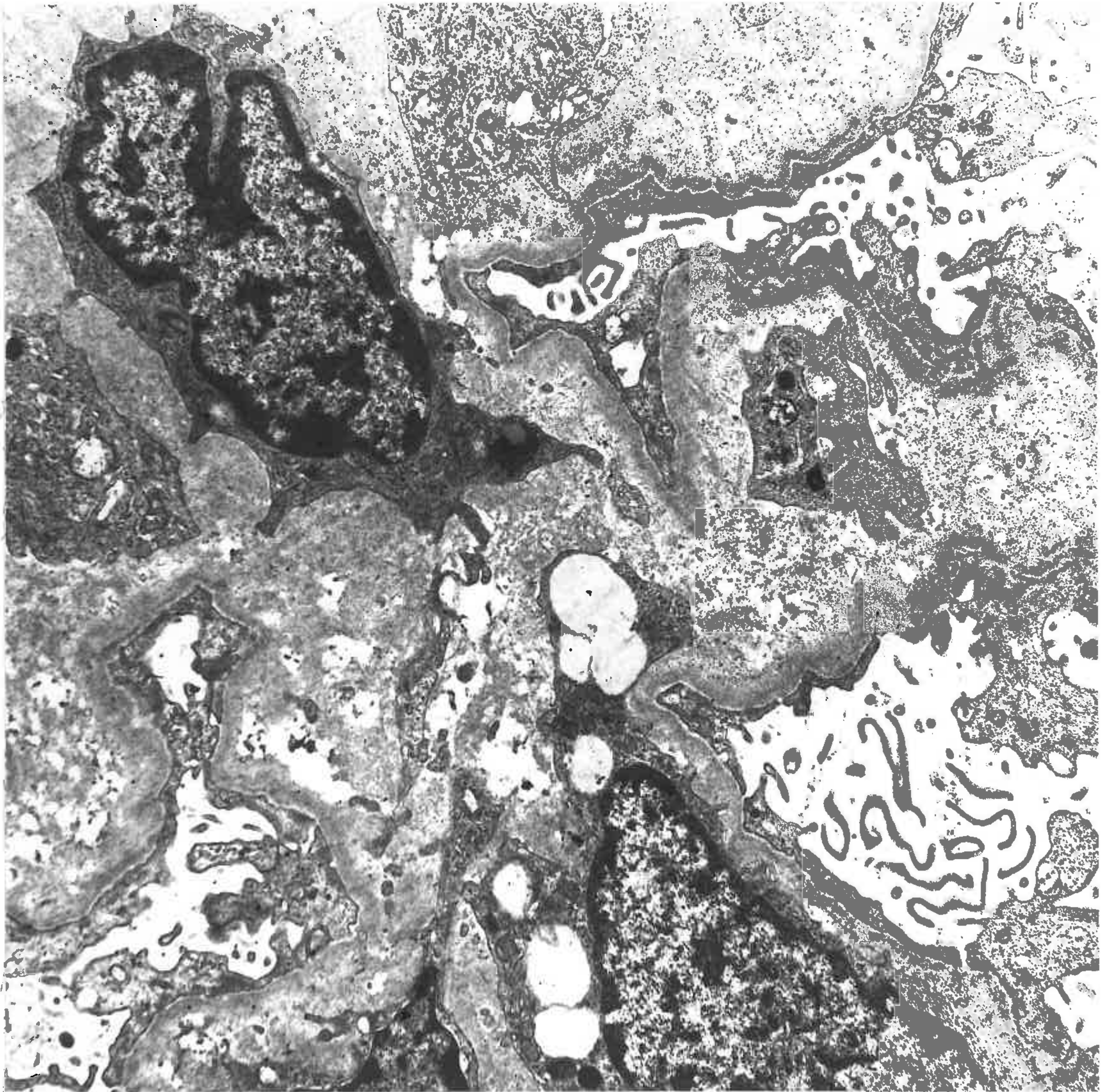


Fig. 60. This electron micrograph shows the narrowing of the capillary lumina by enlargement of endothelial cells and the presence of a pale zone of granular material between the basement membrane and endothelium. Islands of endothelial cytoplasm and on the right of the picture a platelet, appear caught within the lumen. (x 8,000)

in this material which sometimes occluded the entire capillary lumen. In many places this material had a fibrillar structure, but more commonly was amorphous or granular, and although no periodicity was observed, the appearances were those of fibrin and its degradation products (Fig. 61). The epithelial foot processes were fused, and the epithelial cell cytoplasm lay in a continuous layer over the basement membrane (Fig. 62).

Immuno-fluorescence studies revealed the presence of large amounts of fibrin within the glomerular capillaries, afferent arterioles and interlobular arteries (Figs.63). Complement and γ-globulin were not detected.

Interstitial oedema and irregular areas of tubular necrosis were seen in biopsy and early autopsy specimens. Peritubular capillaries were often thrombosed. In those with a prolonged clinical course, interstitial fibrosis, tubular atrophy and dilatation were prominent.

LIVER

Liver biopsy was performed in Cases 5 and 11, and autopsy material was available in 11 others. The liver was moderately enlarged but generally showed no distinctive gross changes. In one patient there were many small shrunken red areas and the liver was soft. The major vessels and extrahepatic biliary tree were normal. Microscopic hepatic cell necrosis was present in all cases, the extent of necrosis varying from individual cells and small groups of cells to large portions of the lobule. Some small foci contained polymorphs or mononuclear cells, but there was usually minimal inflammatory infiltration in portal tracts

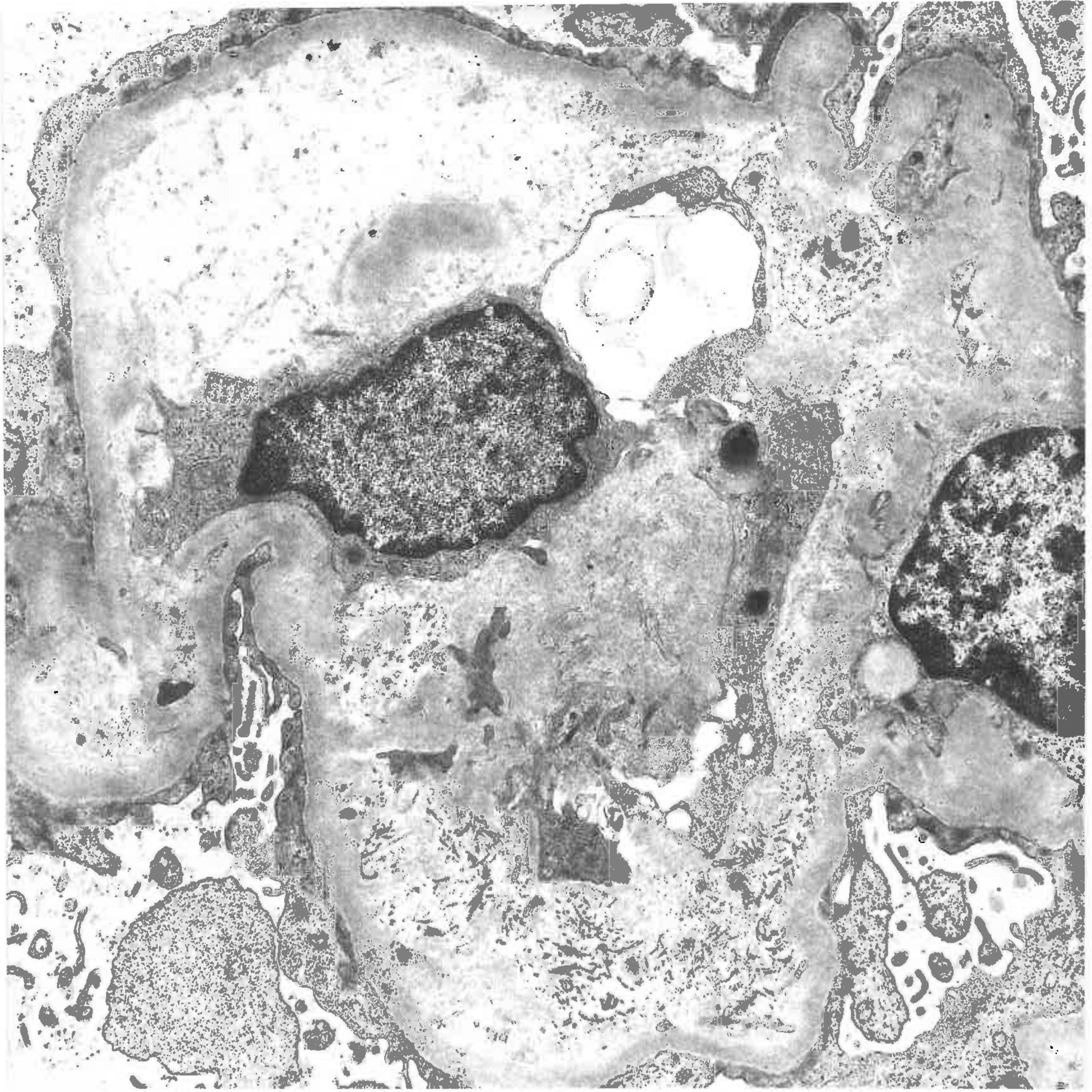


Fig. 61. An affected glomerular capillary with a very narrow lumen. Endothelial cells seem to have grown over the large rarefied area which in this case contains distinct fibrillar material. Epithelial foot processes are fused. (x 8,000)

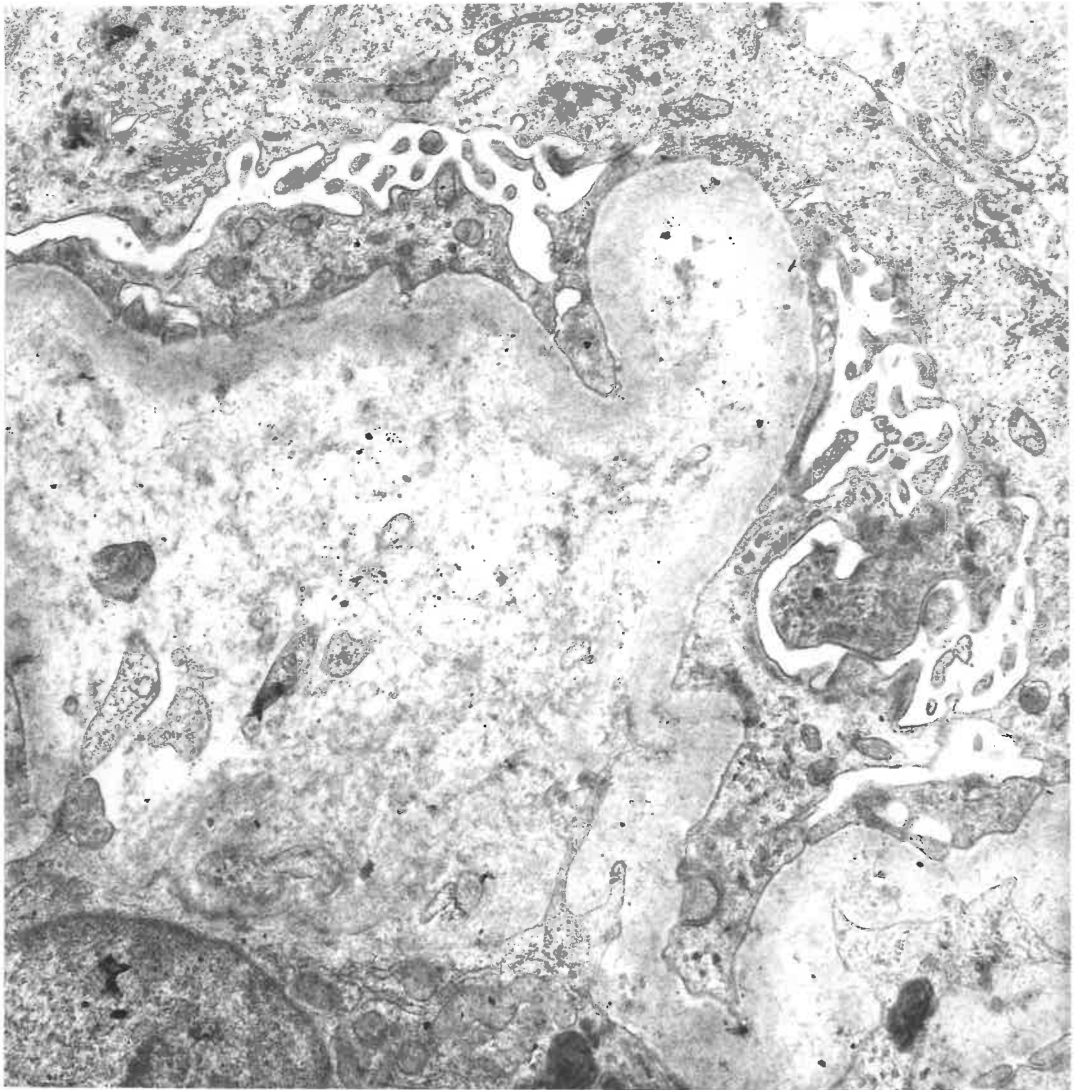


Fig. 62. This picture shows part of a glomerular capillary wall exhibiting a thick layer of rarefied material between the basement membrane and endothelium: in it are seen several small islands of cytoplasm. Loss of epithelial pedicel structure is apparent. (x 16,000)

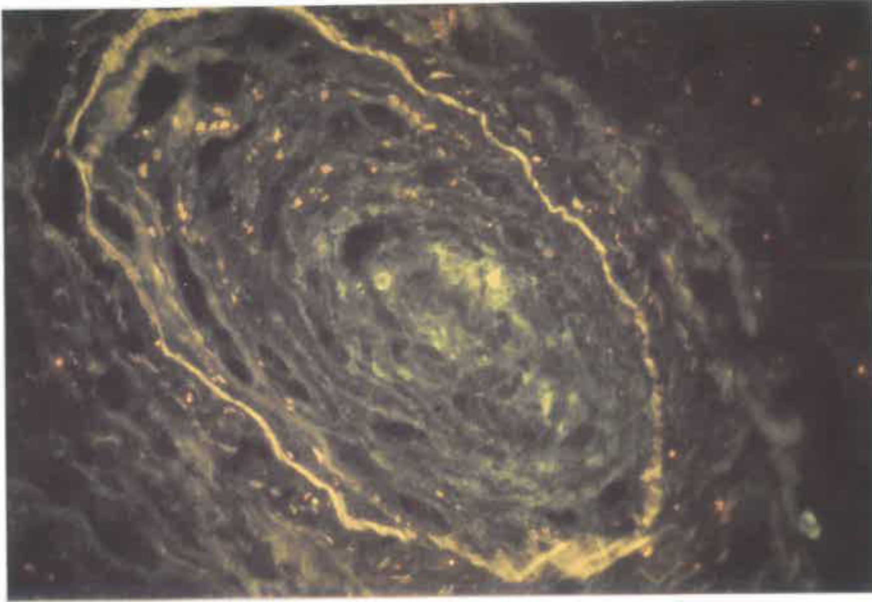


Fig. 63. Immunofluorescence micrograph of an interlobular artery in Case 19. Positive fluorescence is seen within the lumen and between proliferating intimal cells. Antifibrinogen serum. (x 960)

or lobules. Binucleate liver cells were frequent and nuclear pleomorphism invariable, but mitoses were rare. Fibrin thrombi were present in sinusoids in most patients and prominent intimal thickening of portal arterioles were seen in two autopsy specimens.

HEART

Changes were seen in the heart in 5 cases. In three there was verrucous endocarditis, with fibrin thrombi attached to the free surfaces of the mitral and/or aortic valves (Fig. 64). Small wedge-shaped infarcts were present in the myocardium in one of these (Case 9) and in Cases 6 and 18 myocardial necrosis was associated with thrombosis of coronary arterioles and venules.

LUNGS

Hyaline membrane was apparent in many alveoli of Case 1 while pulmonary infarction was seen in 6 including 4 cases on constant anticoagulation prior to death.

BRAIN

Small areas of haemorrhagic infarction of the brain were seen in Cases 1, 3, 5, 15, 17 and 18, and subarachnoid haemorrhage was also present in Cases 5 and 17.

PANCREAS, ADRENAL, PITUITARY, BOWEL

Necrosis was present in the pancreas (Cases 6, 11, 15, 17 and 18) adrenal (Cases 11 and 15), anterior pituitary (Case 15) and both small and large bowel (Case 8). Careful search revealed occasional small-vessel thrombosis in relation to these areas of necrosis.

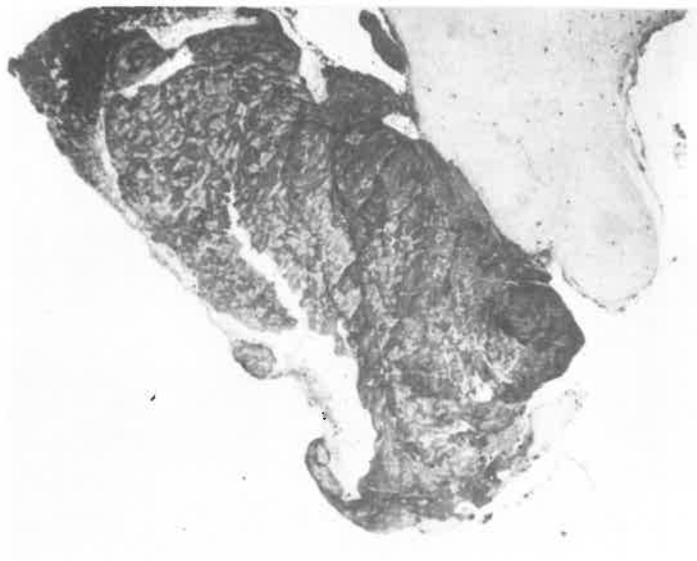


Fig. 64. Vegetation on a heart valve found at autopsy of Case 9. The appearancesare of organizing fibrin without apparent bacteria. (H and E; x 50)

3.2.6 PROGRESS AND MANAGEMENT

Dialysis was undertaken in most patients as treatment for the acute uraemia. Chronic haemodialysis was maintained for 7 months prior to renal homotransplantation in Case 8. Drug therapy is detailed in Table 9. In the 3 patients receiving corticosteroids and 2 azathioprine, no benefit was derived from this therapy. In 12 patients, anticoagulation with intravenous heparin was used for periods ranging between one and 6 weeks (Table 10). Dramatic improvement in urinary output and renal function as described by Kincaid-Smith et al (1960) in 2 patients with thrombotic thrombocytopenic purpura, occurred in only one of the patients so treated (Case 12). In this patient, clinical recovery, restoration of renal and hepatic function, improvement of microangiopathic haemolytic anaemia and in clotting studies (Fig. 65 and 66) were observed soon after commencing heparin one day after commencement of the disease. Of interest, moreover, was the sudden cut-back in urinary output associated with treatment of massive vaginal bleeding with epsilon amino caproic acid (E.A.C.A.) despite concurrent anticoagulation (Fig. 67). The diuresis and full recovery of renal function which commenced between 2 and 3 weeks after the initiation of heparin in Cases 16 and 22 may have been related to treatment, but as similar recovery occurred in Cases 4 and 7 who were untreated, this deduction may be invalid. Recovery, rather than being related to heparin therapy was associated with a short prodrome, rapid diagnosis and confinement of the histological evidence of thrombosis to the glomerular capillaries. In this series, no patient with thrombosis of afferent arterioles or interlobular arteries recovered renal function, and all died. Included

Table 9.

Progress, Management and Outcome in the 22 Patients Studied

Case No.	1	2	3	4	5	6	7	8*	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Length of Illness	10	14	23	42	34	62	38	350	94	10	69	19	44	110	2	35	18	50	80	25	70	30
Heparin						+		+	+		+	+		+		+	+		+	+	+	+
Corticosteroid									+	+	+											
Azathioprine									+	+												
Hypotensive Drugs					+	+		+	+		+		+	+			+	+	+	+	+	
Outcome	D	D	D	A	D	D	A	D	D	D	D	A	D	D	D	A	D	D	D	D	D	A

* Transplantation

Table 10.

Details of Anticoagulant Therapy and Complications in the 12 Patients Who Received it

Case No.	6	8	9	11	12	14	16	17	19	20	21	22
Heparin	+	+	+	+	+	+	+	+	+	+	+	+
Length of illness before therapy	28	100	25	48	1	100	7	10	25	16	36	10
Length of treatment (days)	28	28	42	39	12	12	28	6	24	28	6	20
Complications of therapy		Haemorrhage a. Biopsy site b. Intra-peritoneal	Haemorrhage Intra-peritoneal	Haemorrhage Intra-peritoneal	Haemorrhage Vaginal	Haemorrhage Biopsy site		Haemorrhage a. Vaginal b. Cerebral	Haemorrhage Bladder		Haemorrhage Retro-peritoneal	
Outcome	Death	Death	Death	Death	Alive	Death	Alive	Death	Death	Death	Death	Alive

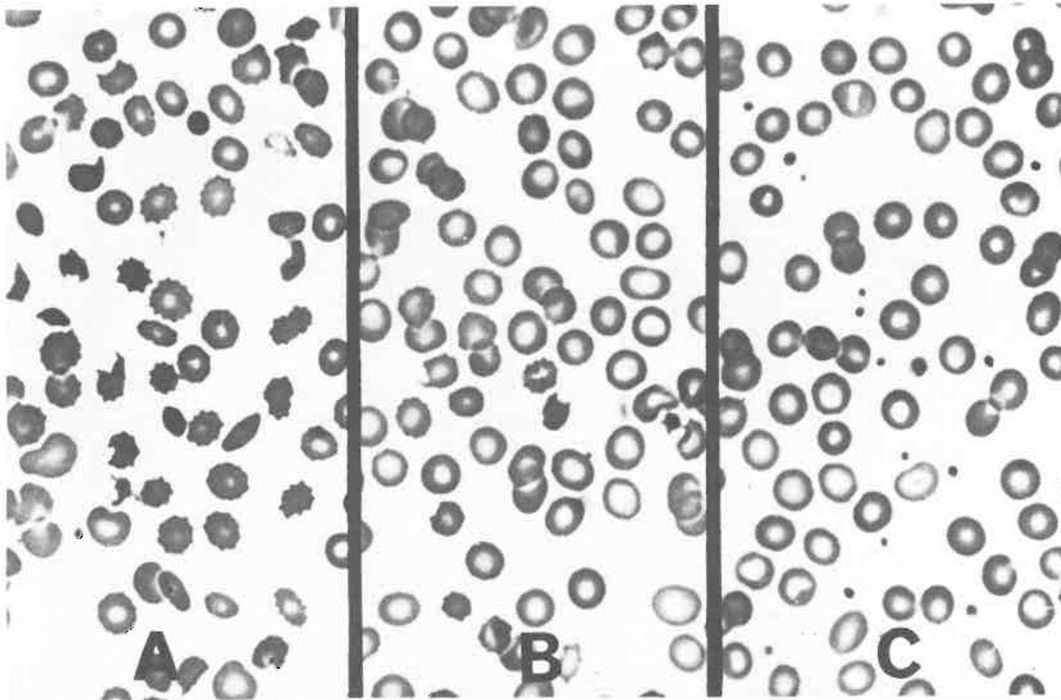


Fig. 65. Red cell morphology as shown by examination of the peripheral blood in Case 12.

A Day 1. There is marked red cell distortion and fragmentation. Spherocytes are seen and thrombocytopaenia is apparent.

B Day 4. There is diminution in the number of distorted cells but thrombocytopaenia persists.

C Day 9. Normal red cell morphology with a slight thrombocytosis.

All slides stained with Leishman X 1,070.

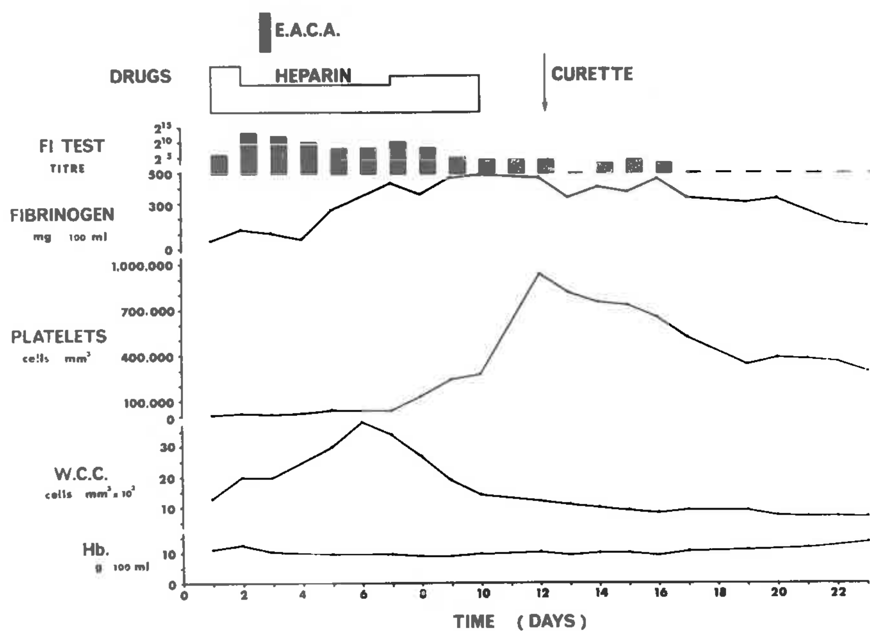


Fig. 66. Case 12. Serial changes in haemoglobin, white cell count, platelet count, plasma fibrinogen and F.D.P. (measured in this case with the Fi Test, Hyland Laboratories) in relation to therapy.

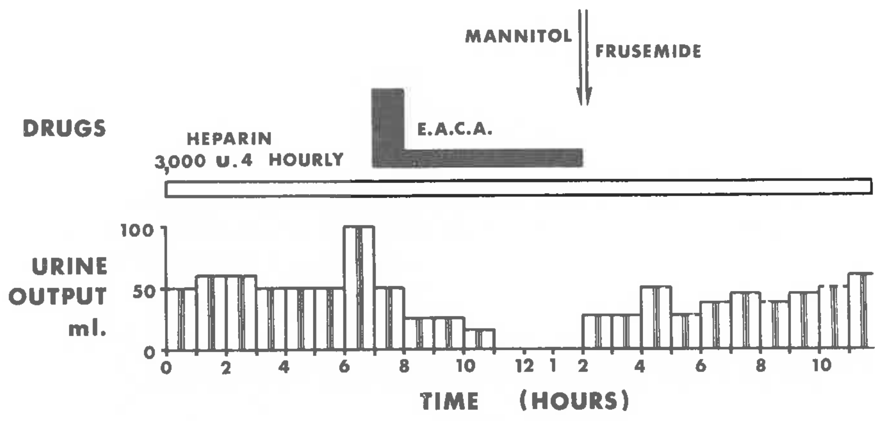


Fig. 67. Case 12. Urinary output during day 2. Progressive oliguria was associated with epsilon amino caproic acid therapy (E.A.C.A.), but urinary output was re-established with mannitol and frusemide.

in this group with a fatal outcome are 9 who were treated with heparin and 8 untreated. There seemed to be no advantage gained by treating these patients as there was no lengthening of life, no improvement of renal function and a significant increase in morbidity when compared with untreated patients. Serious and significant haemorrhage occurred in 8 of the 12 patients anticoagulated and may have contributed to death in Cases 14 and 17. A serious haemorrhagic complication occurred in only one untreated patient (subarachnoid haemorrhage in Case 5) and the deaths in the others resulted from cardiac failure in 2, intercurrent infection in 3 and untreated renal failure in 2.

BACTERIOLOGICAL AND VIRAL STUDIES

Blood culture grew Esch. Coli in Cases 1, 11, 12 and 19 but were sterile despite repeated routine testing in the remainder. A variety of pathogenic organisms were cultured from the nose, throat, sputum and urine. Viral agglutinin studies were unrewarding apart from in Case 8 where there was a high titre to mumps antigen.

3.2.7 DISCUSSION

Although the precise aetiology in these cases is not apparent and may be multifactorial, evidence derived from pathological, coagulation and haematological studies suggests that a common pathogenetic and pathological process occurs in all cases and that this process is disseminated intravascular coagulation. Demonstration of fibrin thrombi in kidney, liver, lung, heart, brain, pancreas, adrenal and bowel proves the disseminated nature of the pathology and confirms

the observations of Habib et al (1967) and Margaretten (1967) who reported a wide variety of seemingly unrelated lesions in patients coming to autopsy following the syndrome of disseminated intravascular coagulation. However, it is in contradistinction to the findings of Gilchrist et al (1969) who suggested that the syndrome in children may be an example of localised intravascular coagulation. Margaretten (1967) described lesions having the appearance of "infarct necrosis" caused by an obstruction to blood flow by fibrin thrombi and suggested that the type and distribution of the lesions were affected by the activity of the reticulo-endothelial system, local vascular factors and activation of fibrinolysis. The reason for the severe selective renal damage caused in the haemolytic uraemic syndrome is unknown, but may be due to the nature of the renal circulation, to decreased fibrinolysis especially in pregnant cases (Woodfield, Cole and Cash, 1968) and perhaps to rapid overwhelming intravascular coagulation in which renal damage is known to predominate.

Whatever the causes, the kidney is the site of lesions which are characteristic, and consist of thrombosis of afferent arterioles and glomerular capillaries. The extent of the lesions is probably underestimated by light microscopy, whereas electron microscopy and immunofluorescence demonstrates fibrin in most glomerular capillaries. The result in most cases is destruction of large but variable amounts of renal cortical tissue which, due to its nature and extent is unlikely to heal with restoration of normal renal function whatever therapy is employed. Careful analysis by immunofluorescence reveals that γ-globulin and complement do not play a role in the genesis of these

lesions, a finding in keeping with those of Rosenmann et al (1969), Koffler and Paronetto (1966) and Churg et al (1969) which further suggests that intravascular coagulation plays a key role in the genesis of the renal lesions. Aspects of the renal pathological lesions show certain similarities to the renal lesions in other conditions and raise the question of the role of intravascular coagulation in those conditions. For example the gross lesions in H.U.S. are akin to those seen in acute rejection of renal homotransplants, the vascular lesions are similar to those of malignant hypertension and scleroderma, and the glomerular changes resemble those seen in many varieties of proliferative glomerulonephritis.

Unequivocal evidence of a severe coagulation abnormality has been demonstrated in each patient in whom tests were performed. The changes were most marked early in the course of the illness and consisted of elevation of serum F.D.P. concentration, plasma PF_4 and S.F.M.C., thrombocytopenia, hypofibrinogenaemia, diminished plasma plasminogen activator and Factors V and VIII. These data are consistent with consumption of coagulation factors and the defibrination syndrome as described by Merskey et al (1967) and indicate that the abnormalities are the consequence of massive intravascular clotting. In patients who recovered, the episode seemed short-lived and did not recur, but where the outcome was fatal, periodic recurrence of these abnormalities was noted which seemed independent of anticoagulant therapy and suggested a persistent or recurring stimulus to coagulation. While the causes of these recurring episodes are unknown it is possible that they may result from thromboplastic material released from red cells during

haemolysis. A vicious cycle could thus be set up whereby intravascular coagulation causes microangiopathy which in turn damages red cells rendering them susceptible to haemolysis and release of thromboplastins.

Many of the clinical features of H.U.S. may be attributed to disseminated intravascular coagulation. Thus, besides the haemolysis and acute renal failure, coagulation may cause parenchymal necrosis of the liver and consequent abnormality of liver function, microscopic areas of infarction of the brain often associated with transient neurological deficits and epileptiform seizures, acute pancreatic necrosis with elevated serum amylase activity, and infarction of adrenals, pituitary and bowel. Involvement of myocardium and heart valves may in part explain the severe resistant cardiac failure which was a feature of several cases in this series and in the four patients described by Robson et al, (1968). The overt bleeding tendency manifest in most cases even before anticoagulation is explicable on the basis of thrombocytopenia, consumption of coagulation factors and the circulation of high concentrations of high molecular weight F.D.P. and S.F.M.C.

The striking similarity between the H.U.S. and the experimental generalised Shwartzman reaction (G.S.R.) in animals is readily apparent, and may provide some clue as to the aetiology of the human condition. In the G.S.R. the priming dose of gram negative endotoxin produces minor intravascular clotting, but causes the animal no harm unless previously "prepared". If the reticulo-endothelial system is incapable of clearing further endotoxin, the second or provoking injection causes

widespread intravascular coagulation which is most prominent in the kidneys where cortical necrosis develops. Accompanying the fibrin deposition, a bleeding tendency with features similar to that of H.U.S. (Corrigan et al, 1967) and the red cell changes of microangiopathic haemolytic anaemia develop (Brain et al, 1962). Tissue damage is due to ischaemia caused by widespread small-vessel thrombosis. The G.S.R. may be produced with but a single injection under the preparative circumstances of reticulo-endothelial blockade (Thomas, 1957; Lee, 1962) pregnancy (Apitz, 1934; McKay and Galton, 1963) immaturity (Smith and Thomas, 1954) corticosteroid therapy (Thomas and Good, 1952) and antigen-antibody reaction (Lee, 1963).

The most common "preparation" in this series was pregnancy or a pseudo-pregnancy state induced by the contraceptive pill in 9 of the 16 female patients. This striking association between H.U.S. and pregnancy is most commonly manifest in the post-partum period and has been reported by several authors (Scheer and Jones, 1967; Robson et al, 1968; Wagoner et al, 1968; Clarkson et al, 1969; Churg et al, 1969). Gram-negative septicaemia due to Esch. Coli was observed in four cases (Cases 1, 11, 12, and 19) while corticosteroid therapy (Cases 8, 10 and 11) and antigen-antibody reactions (Cases 8 and 11) may also have contributed. Apart from a rising titre to mumps antigen in Case 8 no abnormal virological tests were obtained and the precise aetiology in other cases remains unclear.

The analogy between H.U.S. and the G.S.R. can be carried further in terms of treatment. Anticoagulation prevents disseminated intravascular

coagulation caused by the provoking injection in the G.S.R. (Good and Thomas, 1952; Shapiro and McKay, 1958) and more recent reports suggest its efficacy in H.U.S. (Brain et al, 1968; Gilchrist et al, 1969; Timor-Tritsch et al, 1970; Luke et al, 1970). However, anticoagulation is valueless in the G.S.R. after the provoking dose has caused disseminated intravascular coagulation and end-organ necrosis. In the H.U.S., heparin administered after a long period of ill-health did not alter renal function or histological changes in 9 cases, and only in Case 12 where prompt recognition of the nature of the illness allowed early therapy did a convincing response occur. In Cases 16 and 22 where recovery also occurred, there was no convincing evidence that heparin was responsible. More likely, natural resolution of a short-lived and single episode of intravascular coagulation took place and diuresis similar to that seen in untreated Cases 4 and 7, and in acute ischaemic renal failure eventuated. There was no significant difference in the clinical courses of the 9 treated and 8 untreated patients who died, apart from the increased morbidity of bleeding in the treated patients. While this was usually easily recognized it may have contributed to the death of 2 patients.

Thus, although theoretically anticoagulation should be of benefit, in practice it has proved no more useful than simple conservative management in the majority of cases. Perhaps an entirely different approach to treatment such as bilateral nephrectomy and transplantation as suggested by Gironini and Laperrouza (1969) should be entertained once the histological diagnosis is confirmed and established thrombosis is demonstrated in afferent arterioles or interlobular arteries.

Section 4.

GLOMERULONEPHRITIS

4.1 INTRODUCTION

The term glomerulonephritis in its strictest sense denotes glomerular inflammation. As generally used, however, it encompasses many distinct disease entities in which glomerular involvement is primary or secondary to some systemic disorder, and the features of glomerular inflammation may or may not be present. Most types of experimental glomerulonephritis have an immunological basis (Dixon, 1968) and such an association, although not proven in all is tacitly assumed in the majority of human cases. For years this concept was based on clinical experience but over the last 20 years laboratory experimentation has helped to define two distinct mechanisms whereby the antibody response of the host may cause glomerulonephritis. The same two pathogenetic mechanisms are operative in the human diseases and may account for most cases. In both processes antigen-antibody reactions are concentrated within the glomeruli in which inflammation occurs mediated by such factors as complement activation, leukocyte infiltration, platelet aggregation, coagulation initiation and cellular hypersensitivity.

4.1.1 GLOMERULONEPHRITIS CAUSED BY ANTI-GLOMERULAR BASEMENT MEMBRANE (anti-GBM) ANTIBODIES

The critical antigen in Masugi nephritis has been shown by Krakower and Greenspon (1951) to reside in the glomerular basement membrane (GBM). In 1962 Steblay demonstrated that glomerulonephritis developed in animals immunised with GBM derived from other animals of the same species or from the contralateral kidney of the same animal. Later,

GBM antigens extracted from the urine of normal animals were found to cause glomerulonephritis similar to that observed after injection of GBM (Dixon, 1968). More recently anti-GBM antibodies have been isolated from the serum of human patients with glomerulonephritis (Lerner, Glassock and Dixon, 1967) and were demonstrated by elution studies to be present in the kidney. Thus, there seems no doubt, that this mechanism is operative in human glomerulonephritis and perhaps the clearest demonstration of it is by immunofluorescent staining of renal biopsies taken early in the course of the disease. Immunoglobulin and complement are seen in a distinct linear pattern along the basement membrane. A similar pattern may also be observed under the electron microscope. However, it probably accounts for only a small percentage of cases of glomerulonephritis such as Goodpasture's syndrome, and some forms of rapidly progressive disease.

4.1.2 GLOMERULONEPHRITIS CAUSED BY NON-GLOMERULAR ANTIGEN-ANTIBODY COMPLEXES

Antigen-antibody complexes are now known to be the "toxic compound" described by von Pirquet (1910) responsible for the clinical manifestations of serum sickness. In this disease antigen disappears from the circulation just before the appearance of free antibody. The complement system is activated and the complexed antigen and antibody interact with circulating cells. Dependent on the size of the complexes and, perhaps, the mediator-induced damage to capillary walls, deposition of complexes concentrates the inflammatory reaction within glomerular

capillaries although vessels throughout the body are not uncommonly involved during such experimental and human diseases. In man, the "toxic complex" mechanism of pathogenesis is readily established by demonstration of the characteristic "lumpy-bumpy" pattern of immunofluorescence which is also seen by electron microscopy where discrete antigen-antibody complexes are deposited within or on the epimembranous aspect of the glomerular capillary basement membrane. The glomerulonephritides occurring in systemic lupus erythematosus, malaria, after streptococcal infection and associated with mixed cryoglobulinaemia display such abnormalities.

4.1.3 MEDIATORS OF THE INFLAMMATORY REACTION

THE COMPLEMENT SYSTEM

The human complement system consists of nine plasma protein components termed C'1-9 and at least two inhibitors, those of C'1 esterase and C'3. Antigen-antibody complexes bind the first component (C'1) of complement which comprises three subunits C'1q, C'1r and C'1s. C'1q binds on to immune aggregates while the C'1s portion is a proesterase which is converted by the complexes to an active esterase which acts on C'4 and C'2 which in turn splits C'3 into fragments of smaller size. After completion of this step complexes react in immune adherence, undergo enhanced phagocytosis and are associated with the generation of anaphylotoxin, a potent histamine liberator, and increased vascular permeability. Activation of the further factors, the sequence of which is not characterised, promotes the chemotaxis of polymorphs, lysis of red cells and bacteria.

Total haemolytic complement concentrations are depressed in patients with immune complex diseases such as S.L.E., post-streptococcal, mixed cryoglobulinaemic, and subacute bacterial endocarditic forms of glomerulonephritis. This depression, most conveniently measured by determining β1A (the stable product of C'3) concentrations of plasma, has been shown to parallel the severity and activity of these diseases and is thought to be due to utilization in the immune reaction. However, in membranoproliferative glomerulonephritis there is a continued depression of C'3 which is unrelated to disease activity, complement utilisation in immune reactions and urinary excretion (Cameron et al, 1970). The role of the properdin system especially in relation to C'3 inactivation is as yet unknown although it is deposited with complement in acute post-streptococcal and membranoproliferative glomerulonephritis (Westberg et al, 1971).

POLYMORPHONUCLEAR LEUKOCYTES

Chemotaxis of polymorphs plays an important role in inflammation by bringing to the locality scavenger cells which phagocytose and remove offending material. A complex of C'5, C'6, C'7 induces chemotaxis by activation of a cell-bound serine esterase (Becker, 1969) and it is probable that kallikrein, lymph node permeability factor and the slow reacting substance of anaphylaxis are either serine esterases or involve the activation of it. Fibrin also possesses leukotaxic properties (Barnhart and Cress, 1967). Involvement of polymorphs is potentially damaging as they release lysozymes and hydrolizing enzymes capable of tissue proteolysis.

CELLULAR HYPERSENSITIVITY

During the last 3-4 years, it has become possible to confirm the role of cellular immunity in various diseases by *in vitro* testing where previously it had been only suspected because of the presence of lymphocytes and plasma cells in histological sections. Sensitisation of the small lymphocyte can be demonstrated by tests such as lymphocyte-transformation to blast-cell forms, and the inhibition of macrophage migration when the sensitised lymphocyte is exposed to the specific antigen. For example Rocklin et al (1970) demonstrated sensitised lymphocytes by macrophage migration inhibition in 75% of patients with anti-GBM type glomerulonephritis, but not in patients with other forms of glomerulonephritis. Considerable objection may be raised to the suggestion that glomerulonephritis results solely from a delayed-hypersensitivity reaction as the inflammatory exudate in the glomerulus is different from that seen in the tuberculin response in which mononuclear cells predominate. Humoral mechanisms must therefore play an important role.

THE COAGULATION SYSTEM

Much of the discussion in the early part of this thesis dealt with mechanisms by which immune reactions might activate coagulation, fibrinolysis and platelet aggregation. Interrelation between these systems was emphasized and attention was drawn to the possible interplay between them and the complement and kinin systems. The role of coagulation, fibrinolysis and platelet aggregation in the pathogenesis of glomerulonephritis has attracted much attention recently, but has

been assumed secondary, for endothelial and basement membrane damage are thought to be prerequisite. Evidence is accumulating, however, that in certain circumstances platelet aggregation may be induced without prior complement activation (Henson and Cochrane, 1971) and in these situations coagulation may play a primary role.

In this section a description follows of a large-scale investigation of many forms of human glomerulonephritis. The primary aim of this work was to elucidate the disorders in which abnormalities of coagulation and fibrinolysis existed. It was soon apparent, however, that the technology used was insufficiently sensitive to detect changes in the systemic circulation which could be reliably interpreted as reflecting renal events. In particular, changes in plasma fibrinogen, S.F.M.C., PF_4 and plasminogen activator, and in many cases serum F.D.P. could not be regarded as specific for the renal diseases under study. Moreover, the large population of patients with glomerulonephritis precluded investigation with these time consuming techniques, particularly when serial study was deemed necessary. There remained 2 possible alternatives. The first of these, the examination of renal arterio-venous differences of coagulation and fibrinolytic factors was impractical on a large scale and therefore not considered. Examination of the urine, however, provided sensitive and specific information with little inconvenience to the patient, and allowed the investigation to be carried out on a large scale both on an inpatient and outpatient basis. The basic investigative tool used was the tanned red cell haemagglutination inhibition immuno-assay for measuring

F.D.P. concentrations in serum and urine, and correlation of these findings with clinical, pathological and therapeutic data was performed in all cases.

4.1.4 CLASSIFICATION OF GLOMERULONEPHRITIS

Modern classifications of glomerulonephritis have been derived from clinico-pathological correlations based on light microscopy of renal biopsies, and usually differentiate between primary and secondary glomerular lesions. In the light of current concepts of pathogenesis, however, this distinction is arbitrary and has been disregarded in the formulation of Table 11.

Proliferative forms of glomerulonephritis were diagnosed when histological examination showed diffuse or focal proliferation of endothelial and mesangial cells. Further evidence of this diagnosis was obtained by electron microscopy under which minor proliferative changes were magnified, and material with the appearance of fibrin or its degradation products was identified. In our experience, variable degrees of endothelial and mesangial cell proliferation were almost always seen when fibrin was present. Epithelial crescents were observed in a small number of acute and rapidly progressive cases (acute necrotizing glomerulonephritis). Other subgroups identified histologically included polyarteritis nodosa affecting medium sized arteries and smaller ("microscopic") vessels, and membranoproliferative glomerulonephritis (Cameron et al, 1970; Bariety et al, 1971). On clinical grounds, further subdivision is possible into acute post

Table 11.

CLASSIFICATION OF GLOMERULONEPHRITIS

Minimal Lesion Glomerulonephritis

Membranous Glomerulonephritis

Membrano-Proliferative Glomerulonephritis

Proliferative Glomerulonephritis:

- Acute post-streptococcal glomerulonephritis
- Mesangial proliferative glomerulonephritis
- Systemic lupus erythematosus
- Polyarteritis nodosa
- Wegener's granulomatosis
- Henoch Schönlein (anaphylactoid) purpura
- Systemic sclerosis (scleroderma)
- Subacute bacterial endocarditis
- Cryoglobulinaemic glomerulonephritis
- Malaria
- Acute necrotising (rapidly progressive) glomerulonephritis
- Benign focal glomerulonephritis
- Focal sclerosing glomerulonephritis
- Alport's syndrome
- Fabrey's disease (angiokeratoma corporis diffusum)
- Total and partial lipodystrophy
- Others

streptococcal glomerulonephritis, glomerulonephritis associated with Henoch Schönlein purpura, Goodpasture's syndrome, Wegener's granulomatosis, malaria, cryoglobulinaemia, subacute bacterial endocarditis, Alport's syndrome (hereditary glomerulonephritis associated with nerve deafness), Fabrey's disease, and the lipodystrophies. There remain a large proportion of cases diagnosed histologically as proliferative glomerulonephritis in which no definite clinical association is obvious. Positive antinuclear factor and the presence of L.E. cells, together with the typical histological and ultrastructural abnormalities (Meulmrcke et al, 1957; Baldwin et al, 1970) distinguished systemic lupus erythematosus (S.L.E.) glomerulonephritis from other proliferative forms.

The diagnosis of membranous and minimal lesion glomerulonephritis was based on their histological and ultrastructural appearances. In membranous glomerulonephritis glomerular abnormalities are largely confined to the basement membrane which is diffusely thickened and contains deposits of immune material in its substance or projecting from its epithelial (epimembranous) aspect. It is very unusual to observe cellular proliferation and fibrin in these cases. The glomeruli in minimal lesion appear normal under the conventional microscope. Electron microscopy, however, reveals fusion of epithelial foot processes which is present as a result of the proteinuria. In our experience the presence of platelets and fibrin as observed by Duffy et al (1970) has not been confirmed.

4.2 PATIENTS AND METHODS

PATIENTS STUDIED

Two hundred and eleven patients with glomerulonephritis have been studied and in each case the diagnosis was made on the basis of clinical, haematological and biochemical findings and on the light and electron microscopic appearances of material obtained by needle renal biopsy. The number of patients in each group is shown in Table 12.

F.D.P.

Serum and urinary F.D.P. were estimated in all patients by the T.R.C.H.I.I. Serum specimens were obtained daily during hospital admissions and periodically during outpatient clinics. Whenever possible, consecutive daily urine samples were obtained which were either in the form of aliquots of 24 hour urine samples (inpatients) or early morning specimens posted to the laboratory (outpatients). For inclusion in this study the minimum period of serum and urine collection was fixed at three days, and urine F.D.P. in the majority of patients were studied much longer. Seventy patients were studied for between 3 and 7 consecutive days and 140 patients for 7-250 days. In this latter group, a continuous study of urine F.D.P. was performed in 41 patients comprising all histological types of glomerulonephritis, for periods greater than 60 days (2 months).

For purposes of comparison, urine F.D.P. concentrations were measured daily for 7-23 days in five patients whose polycystic kidney disease was discovered by routine investigation of affected families, and in whom no complication was present; and in 15 with acute pyelonephritis or infection of the lower urinary tract during the period

Table 12.

Patients Studied

Minimal Lesion Glomerulonephritis (8)

Membranous Glomerulonephritis (22)

Proliferative Glomerulonephritis (181). This category includes: S.L.E. glomerulonephritis (28), membranoproliferative glomerulonephritis (13), post-streptococcal glomerulonephritis (10), focal sclerosing glomerulonephritis (4), acute necrotising glomerulonephritis (13)*, glomerulonephritis associated with Henoch Schönlein purpura (9), polyarteritis nodosa (12)*, Goodpasture's syndrome (2), subacute bacterial endocarditis (1), cryoglobulinaemia (1), Wegener's granulomatosis (1), systemic sclerosis (1), Alport's syndrome (3), Fabrey's disease (1), partial lipodystrophy (1), carcinomatosis (1), others (86).

* Seven patients with polyarteritis nodosa have also been included in the category of acute necrotising glomerulonephritis.

of active infection.

For control purposes urine F.D.P. concentration was measured in 63 healthy, normotensive subjects of both sexes aged 15 to 65 years. Moreover, serial determination for 14 consecutive days was performed on 23 of these. F.D.P. could be detected in minute concentrations in 38% of samples, but the highest recorded value was 0.25 μg/ml. Care was taken to avoid vaginal contamination in menstruating females.

4.3 RESULTS

The maximum concentrations of serum and urine F.D.P. recorded in the first 172 patients studied are shown in Fig. 68 where all proliferative forms of glomerulonephritis apart from S.L.E. and membranoproliferative glomerulonephritis are considered together (unspecified). The results in the remaining 39 cases do not differ from those which are discussed below. Serum F.D.P. concentrations were commonly elevated in proliferative forms of glomerulonephritis. However, pronounced daily variations were recorded in individual patients, so that an individual measurement may have been misleading. A rise in serum F.D.P. above the upper limit of normal (20μg/ml) was also seen in minimal lesion and membranous glomerulonephritis although this occurred much less frequently than in the proliferative forms.

Despite massive proteinuria (2-25G/day) and prolonged periods of testing (up to 70 days) urinary F.D.P. concentration was never greater than 2μg/ml in patients with minimal lesion or membranous glomerulonephritis. Infusion of human albumin solution which is known to increase the renal clearance of plasma proteins (Hardwicke and Squire, 1955)

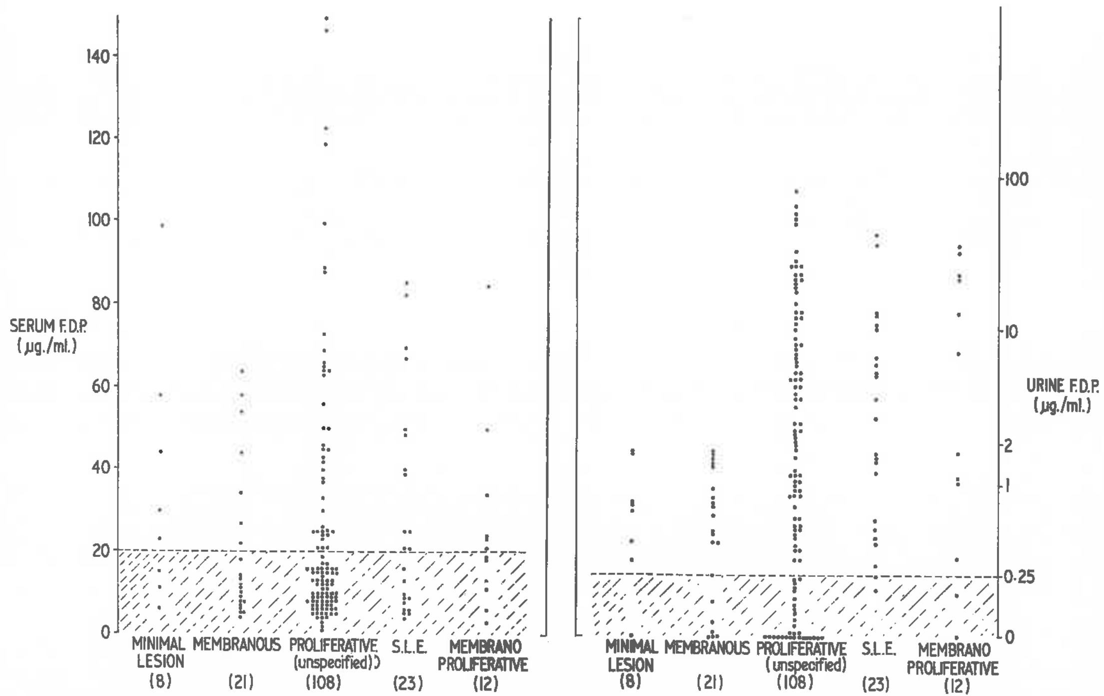


Fig. 68. Maximum serum and urinary F.D.P. concentrations recorded in the first 172 cases of glomerulonephritis studied. Normal ranges are shaded and urinary F.D.P. are plotted on a logarithmic scale.

was performed on five occasions in two patients with minimal lesion and on four occasions in two patients with membranous glomerulonephritis. Although valid clearance data cannot be obtained for fibrinogen or F.D.P., the concentration of urine F.D.P. did not rise with this infusion and remained below 2.0 μg/ml in each instance.

By contrast, wide ranges of urinary F.D.P. concentration were found in proliferative forms of glomerulonephritis. Daily measurement often showed pronounced fluctuations even in the presence of consistently normal serum F.D.P. concentrations.

Albumin infusion performed on at least 2 occasions in each of 5 patients with proliferative glomerulonephritis resulted in a 2 to 5 fold increase in urine F.D.P. concentration often to levels exceeding 50 μg/ml. Such high levels were not recorded in other circumstances.

No significant abnormality was detected in the patients with uncomplicated polycystic disease, and in those with pyelonephritis or lower urinary tract infection the values never exceeded 0.5 μg/ml.

4.3.1 ACTIVITY OF DISEASE

In each patient with glomerulonephritis, the activity of disease was assessed in the light of information available at the time of the F.D.P. study. Cases were considered to be in an active phase if any one or more of the following clinical or pathological features were present: Decreasing creatinine clearances, progressive rise in the concentration of serum creatinine, proteinuria in excess of 1G per 24 hours, red blood cell casts in the urine, focal or diffuse proliferation of endothelial or mesangial cells, or fibrin within the

glomerular capillary as detected by electron microscopy. The disease was considered inactive or resolving if none of these clinical features were present, or if histology showed only foci of glomerular sclerosis with or without capsular adhesions. In 114 patients concurrent clinical and histological data were analysed, in 83 only clinical material was considered because the renal biopsy was performed before one month of the urinary analysis, and in 12 only biopsy and incomplete clinical evidence was assessed. While objection may be raised to some of the criteria for activity in this study, in practice their use has provided consistent results. In particular the arbitrary decision to use IG per day or greater of proteinuria may in some instances have led to the inclusion in the "active" disease list of some patients whose disease was resolving at the time of study e.g. mesangial proliferative glomerulonephritis, and some cases of recovering post-streptococcal glomerulonephritis. On the other hand it allowed inclusion of the cases of minimal lesion and membranous glomerulonephritis which on the other criteria would in most instances have been excluded. In 114 cases, biopsy was performed during or within a period of one month of the study and in these the pathological and clinical evaluations of disease activity were in close agreement (see Table 13). Of the 85 whose disease was deemed active, 11 had membranous or minimal lesion glomerulonephritis, and "activity" was based on degree of proteinuria. The remaining 74 patients had a form of proliferative glomerulonephritis, and in only 3 of these was there no pathological evidence of clinical activity (Table 13). All patients with histological or ultrastructural evidence of active proliferative glomerulonephritis also had clinical

Table 13.

Assessment of "activity" in 74 patients with proliferative glomerulonephritis whose renal biopsy was performed at the time of, or within one month of, the urinary study, and whose disease was considered in an "active" phase.

Clinical Indices of Activity	No.	Pathological Indices of Activity	No.
Decrease in Creatinine Clearance	43	Active Proliferation of Endothelial or Mesangial Cells	71
Progressive Rise in Serum Creatinine	45	Electron Microscopic Identification of Fibrin	56*
Proteinuria in Excess of 1G per 24 Hours	62		
Red Blood Cell Casts in the Urine	69		
Total Number Showing One or More Clinical Features of Activity	74	Total Number Showing Pathological Features of Activity	71

* All sections were not examined by electron microscopy.

evidence of "activity". Of interest was the finding of endothelial cell proliferation in association with each instance of glomerular capillary fibrin deposition as detected by electron microscopy. Because of this close relation it was reasoned that "activity" could be satisfactorily defined on only incomplete clinical and histological data in the remaining patients.

The numbers of patients studied with "active" disease in each clinico-pathological category is shown in Table 14.

4.3.2 RELATION BETWEEN SERUM F.D.P. CONCENTRATION AND DISEASE ACTIVITY

Although serum F.D.P. concentrations were commonly raised in all forms of proliferative glomerulonephritis, this elevation was dependent for the most part on disease activity (Table 15). In general terms, the degree of elevation was related to the severity of the disease in both a clinical and pathological sense. For example the most consistently elevated readings were found in those cases where diffuse glomerular proliferation was demonstrated, and in those where necrotising features and/or crescent formation was prominent. However, no abnormal levels were recorded in 32 out of the 101 (32%) whose disease was considered inactive.

A rise in serum F.D.P. was also seen at some stage in 5 of 8 patients with minimal lesion and 8 of 22 patients with membranous glomerulonephritis, though in some individual patients it occurred much less often than in the proliferative forms. An impression was formed that these elevations occurred in association with maximal blood

Table 14.

The number of patients in each disease group considered to have "active" disease during the period of study. (Total number studied in each group is shown in brackets.)

Minimal Lesion Glomerulonephritis	8	(8)
Membranous Glomerulonephritis	22	(22)
Membranoproliferative Glomerulonephritis	7	(13)
Systemic Lupus Erythematosus	17	(28)
Proliferative Glomerulonephritis (unspecified*)	77	(140)
Total	131	(211)

* Includes post-streptococcal (10), acute necrotizing (13), polyarteritis nodosa (12), Henoch-Schönlein purpura (9), Alport's syndrome (3), Goodpasture's syndrome (1), Wegener's granulomatosis (1), S.B.E. (1), partial lipodystrophy (1), mixed cryoglobulinaemia (1) and Fabrey's disease (1), in all of whom the disease was considered "active".

Table 15.

Range of maximum serum F.D.P. concentrations found in patients with proliferative forms of glomerulonephritis.

Normal range for serum F.D.P. is 1-20 $\mu g/ml$.

Maximum Serum F.D.P. Concentration $\mu g/ml$	S.L.E. Glomerulonephritis		Membrano-Proliferative Glomerulonephritis		Unspecified Proliferative Glomerulonephritis	
	Active	Inactive	Active	Inactive	Active	Inactive
0-20	4	7	1	5	27	46
20-50	8	4	2	1	25	15
50-100	5	-	3	-	15	2
>100	-	-	1	-	10	-
Total	17	11	7	6	77	63

volume depletion, and it is in such circumstances that acute renal failure develops in these patients (Conolly et al, 1968).

4.3.3 RELATION BETWEEN URINE F.D.P. CONCENTRATION AND DISEASE ACTIVITY

Apart from four patients, all cases of active proliferative glomerulonephritis excreted F.D.P. in a concentration greater than 2 μg/ml, and in those in whom the disease was inactive, the F.D.P. concentration never exceeded 2 μg/ml (Fig. 69). Thus the urinary measurement provided a more accurate and sensitive index of disease activity than the serum F.D.P., given that sufficient daily readings were performed to account for the commonly observed periodic fluctuations. In most cases of active disease daily measurements showed pronounced fluctuations, so that cyclical elevations of urinary F.D.P. concentration were interspersed with periods of low levels. The nature of this cyclical pattern became more apparent after the study of diseases which were either self-limiting, rapidly progressive, or responded to therapy. The fluctuations persisted when the disease remained active often becoming more pronounced and closer together in rapidly progressive cases (Fig. 70b). In more benign cases, the peaks in F.D.P. concentration were less pronounced, lasted for shorter periods, and were separated by longer intervals (Fig. 71). This pattern was characteristically observed in those cases which histologically showed minor focal proliferative changes within the glomeruli and little change in renal function during long periods of observation. In post-streptococcal glomerulonephritis, moderate fluctuations were observed during the

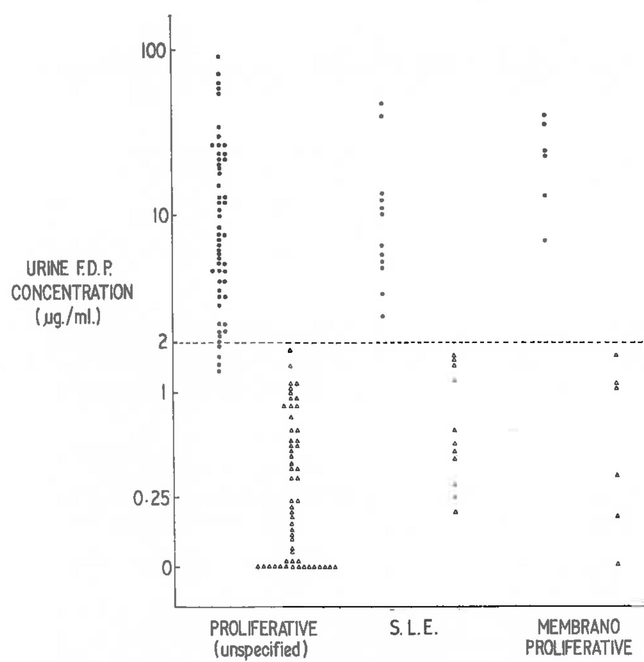


Fig. 69. Maximum urine F.D.P. concentrations recorded in proliferative forms of glomerulonephritis.

● = Active disease.

△ = Inactive disease.

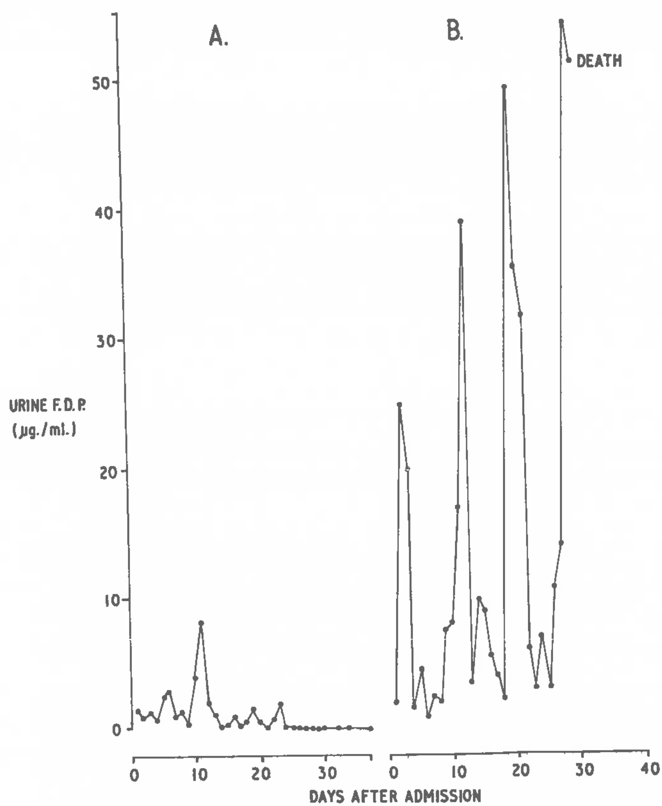


Fig. 70. Daily urinary F.D.P. concentrations in two patients with proliferative forms of glomerulonephritis. In post-streptococcal glomerulonephritis (A) abnormal F.D.P. excretion was found during the active phase and ceased thereafter. Acute necrotizing glomerulonephritis (B) was associated with pronounced periodic rises before death.

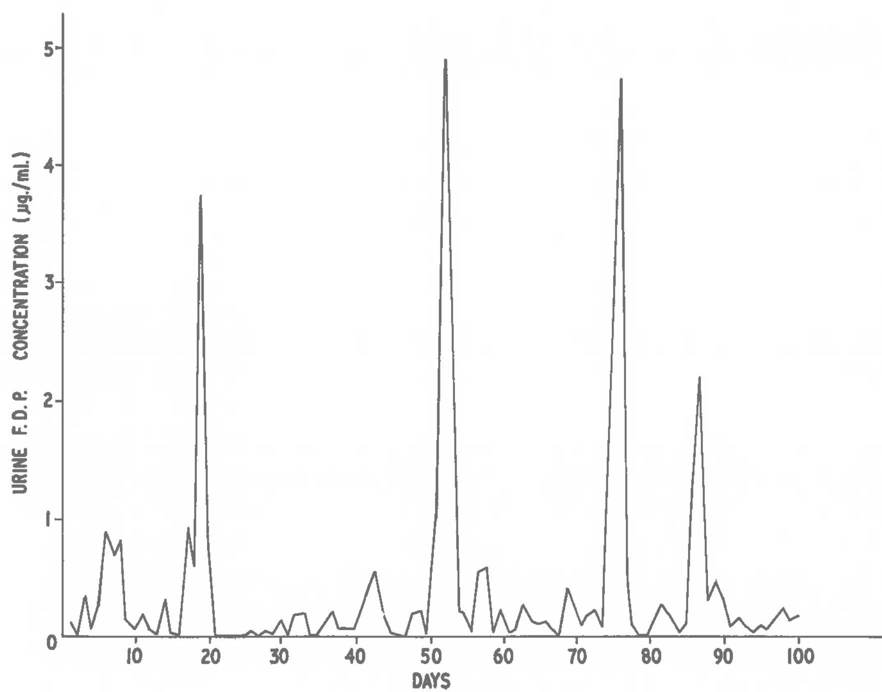


Fig. 71. Daily urinary F.D.P. concentrations recorded for 100 days in a patient discovered to have mild focal proliferative changes in most glomeruli; after presenting with recurrent limited attacks of haematuria and proteinuria.

period of disease activity, but urine F.D.P. disappeared or remained at low levels after clinical recovery (Fig. 70a). In these cases serum F.D.P. decreased and euglobulin lysis times increased during this period (Fig. 72). Thus in more active and severe diseases urine F.D.P. concentrations were higher and fluctuations, closely related together in time, were persistent. Maximum recorded urine F.D.P. concentrations in each case of proliferative glomerulonephritis are shown in Table 16. Apart from patients with Henoch-Schönlein purpura and post-streptococcal glomerulonephritis, the highest values were recorded in patients with the more severe clinical and pathological features. Indeed, 34 out of 59 (57%) patients with levels above 10 g/ml have died from renal failure or are maintained by chronic haemodialysis, and if patients with post streptococcal and Henoch-Schönlein nephritis are excluded the percentage is even higher (69%). From study of biopsy and clinical details of the remaining cases in this category, it seems that the majority will eventually suffer the same fate, as in each instance renal function is deteriorating and histological features of progression are present. By contrast, only 6 of 121 (5%) whose urine F.D.P. was below 10 $\mu g/ml$ during the period of study have died.

It may be argued therefore that serial urine F.D.P. measurements provide a sensitive index of both the activity and severity of disease. In most instances however, the severity may be assertained by carefully assessing more routine tests of renal function, and greater value of the urine measurement might lie in its documentation of the natural history of the disease under study.

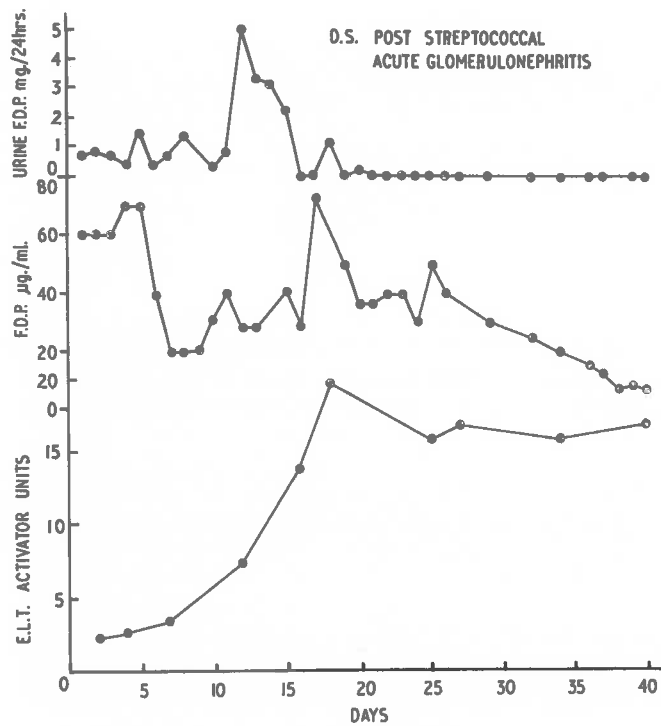


Fig. 72. Serial plasma plasminogen activator, serum and urinary F.D.P. concentrations in a 16 year old boy with acute post-streptococcal glomerulonephritis. Clinical recovery was associated with return to normal of these indices.

Table 16.

Maximum urine F.D.P. concentrations recorded in the cases with proliferative forms of glomerulonephritis. The figures in brackets represent the number of patients in each category who have either died from renal failure or have been admitted to a chronic haemodialysis program.

Maximum Urine F.D.P. µg/ml	S.L.E.	Membrano-Proliferative	Post-Streptococcal	Henoch Schönlein Purpura	Polyarteritis Nodosa	Wegener's Granulomatosis	Acute Necrotising	Good-pasture's Syndrome	Others
0-1	6	3	-	-	-	-	-	-	48
1-2	5	3 (1)	-	-	-	-	-	-	19
2-5	3 (1)	2 (1)	2	1	4	-	-	-	8
5-10	4	2 (1)	2	3	1 (1)	-	-	-	5 (1)
10-20	5 (1)	-	2	3	2 (2)	-	2 (1)	-	5 (3)
$>$ 20	5 (4)	3 (3)	4	2 (1)	5 (5)	1 (1)	4 (4)	2 (2)	14 (7)

4.3.4 THE SIGNIFICANCE OF URINARY FIBRIN EXCRETION

a. URINE F.D.P. AND NATURAL HISTORY OF PROLIFERATIVE GLOMERULONEPHRITIS

As can be seen from the previous section, urine F.D.P. concentration, if measured serially for long periods, provides a reliable index of the natural history of proliferative forms of glomerulonephritis. It is not with regard to the self-limiting and rapidly progressive diseases that its value lies however, for in these cases the natural history is self-evident. Rather, it is in those instances where disparity between clinical and pathological features exist that it has its most potential. Figures 73, 74 and 75 illustrate three such examples where relatively normal and stable renal function existed in the face of marked and progressive histological changes. In each case gross abnormalities of urine F.D.P. existed for some time before rapid deterioration of renal function ensued.

In the less dramatic but equally perplexing cases of focal proliferative glomerulonephritis, where histology and renal function may be only slightly abnormal, further study for longer periods than shown in Fig. 71 may reveal the true nature of the condition.

Equally important are the cases of resolving acute glomerulonephritis and mesangial proliferative glomerulonephritis where proteinuria of moderate degree and cellular proliferation are residues of the acute disease. In these cases urinary F.D.P. are consistently low — usually below 3 $\mu g/ml$ — and fail to show the periodic fluctuations characteristic of the more active, progressive cases.

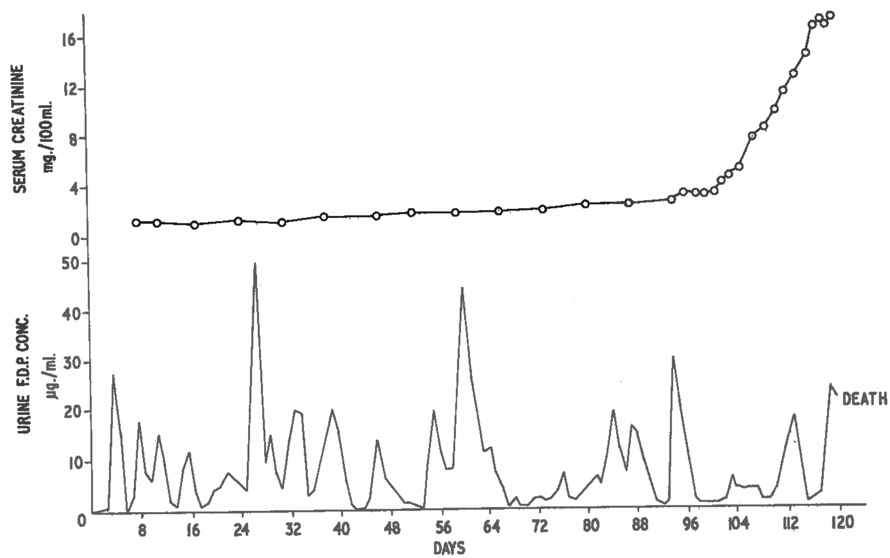


Fig. 73. Daily urinary F.D.P. and serial serum creatinine concentrations in a 23 year-old man with progressive proliferative glomerulonephritis. The persistent gross abnormality of urinary F.D.P. preceded rapid deterioration of renal function by 3 months.

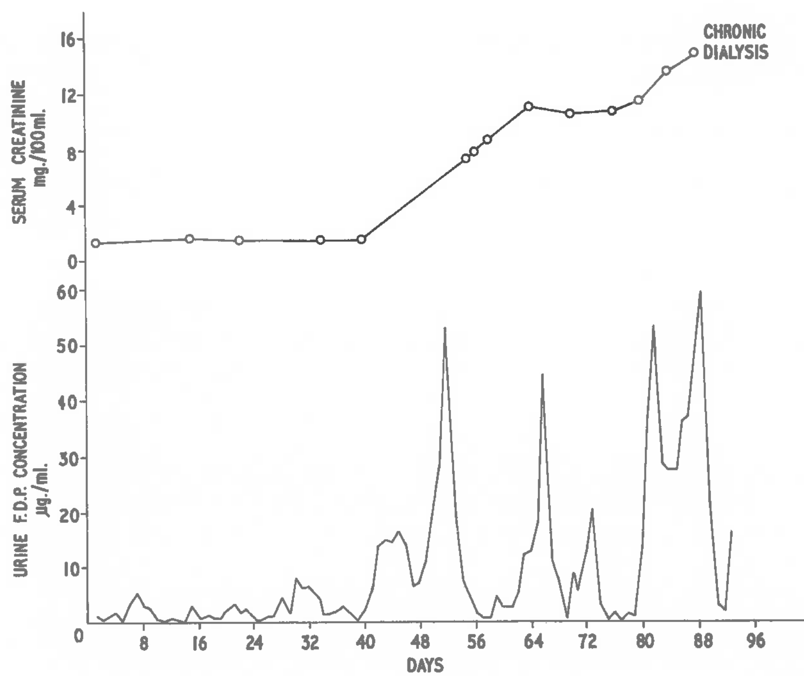


Fig. 74. Urinary F.D.P. and serum creatinine concentrations in a 23 year-old man with known proliferative glomerulonephritis of seven years duration. Sudden deterioration of renal function occurred during the study and was associated with worsening of the already abnormal urinary F.D.P. excretion pattern.

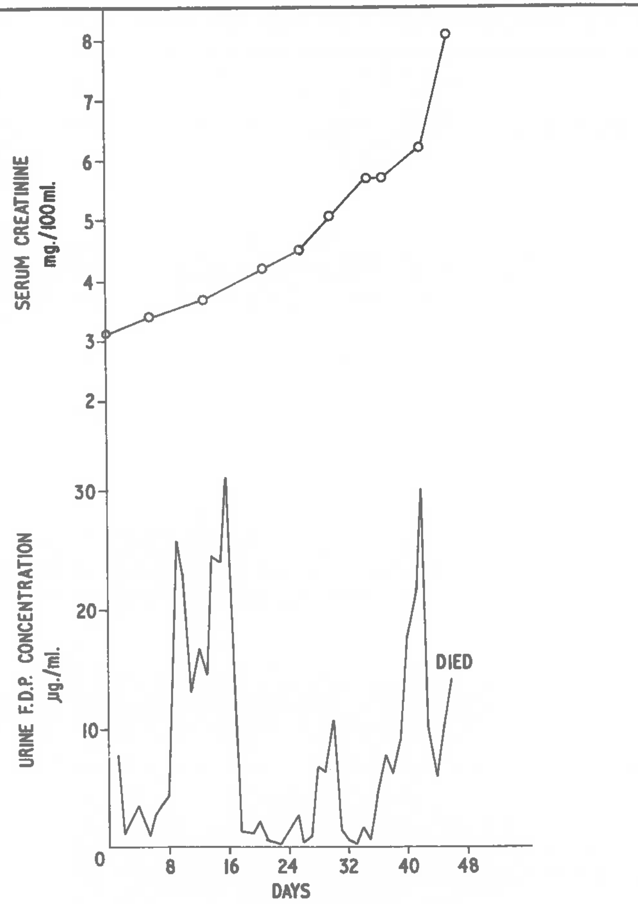


Fig. 75. Daily urinary F.D.P. and serial serum creatinine concentrations in a 68 year-old man with membrano-proliferative glomerulonephritis. Persistent abnormality of urinary F.D.P. was associated with rapid deterioration of renal function prior to death.

b. DIAGNOSIS

In terms of differential diagnosis, urinary F.D.P. concentration measured serially is of value in the nephrotic syndrome, for in the presence of gross proteinuria, there is a clear distinction between proliferative forms and those with minimal lesion and membranous glomerulonephritis. All 8 patients with minimal lesion and 8 of the 22 patients with membranous glomerulonephritis had nephrotic syndrome during the period of study, and 25 patients with proliferative glomerulonephritis suffered the same consequences of massive proteinuria. At no time was the urine F.D.P. concentration found to be greater than 2.0μg/ml in minimal lesion and membranous glomerulonephritis, despite long periods of testing, albumin infusion, and in one case increased oedema, proteinuria and blood pressure during the third trimester of pregnancy which is usually associated with higher urinary F.D.P. levels. Maximum values ranging between 4.6 and 64.5μg/ml were found in cases of proliferative glomerulonephritis with nephrotic syndrome.

c. RELATION BETWEEN URINE F.D.P. EXCRETION AND THE PRESENCE OF FIBRIN WITHIN THE GLOMERULUS

ELECTRON MICROSCOPIC STUDY

In 51 patients with proliferative glomerulonephritis in whom the renal biopsy was taken during or within one month of the urinary study, the degree of intraglomerular fibrin as detected by electron microscopy was correlated with the maximum recorded urinary F.D.P. concentration during that period. Material such as that described in Section 1.6 was taken as representing fibrin or its degradation products,

and the extent of its presence within the glomerular capillaries was graded as:

0 = no fibrin detected.

+ = rarefied layer between the basement membrane and endothelium containing odd strands of fibrin (Fig. 76).

++ = dark, dense deposits between the basement membrane and endothelium (Fig. 77).

+++ = fibrin projecting into the capillary lumen and between swollen and proliferating endothelial cells (Fig. 78).

++++ = glomerular capillary thrombosis (Fig. 79).

The results are shown in Table 17. While not suitable for statistical analysis they indicate a close relationship between the F.D.P. concentration and the extent of the intraglomerular fibrin deposition. It is perhaps of interest that fibrin was not detected ultrastructurally in cases of minimal lesion and in only odd glomerular capillaries in 2 cases of membranous glomerulonephritis.

IMMUNOFLUORESCENCE STUDY

In a similar study 47 consecutive renal biopsies were stained with fluorescein conjugated human anti-fibrinogen serum and the deposition of fibrin within the kidney compared with the maximum recorded urine F.D.P. concentration during the period of study. The resolution of the microscope used for immunofluorescence was such that the degree of fibrin deposition could not be accurately determined. Thus intra-renal fibrin was graded as:

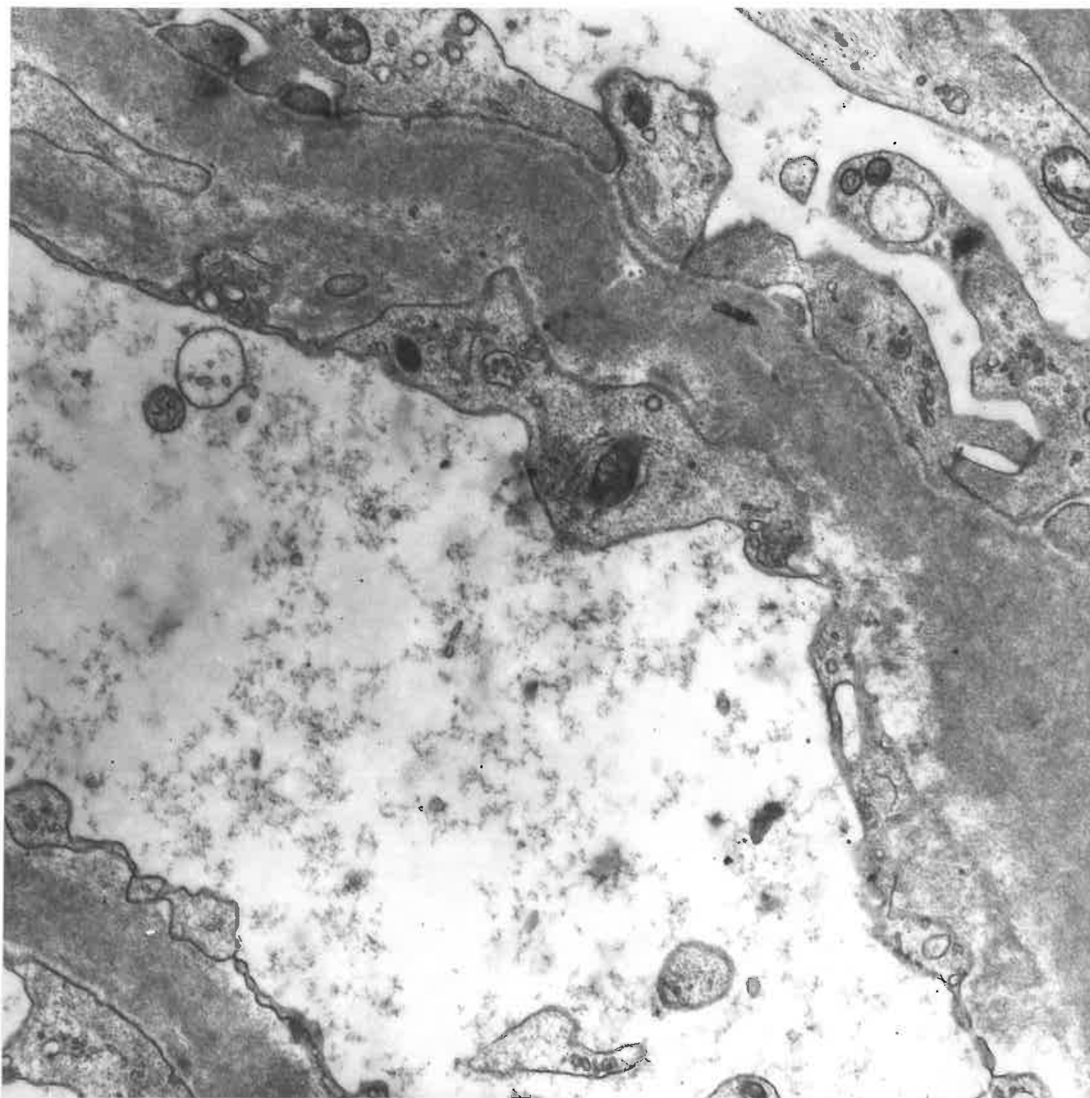


Fig. 76. Electron microscopic grading of glomerular capillary fibrin. Where there was a rarefied layer between the basement membrane and endothelium containing odd strands of fibrin, glomerular capillary fibrin was graded as +. (x 20,000)

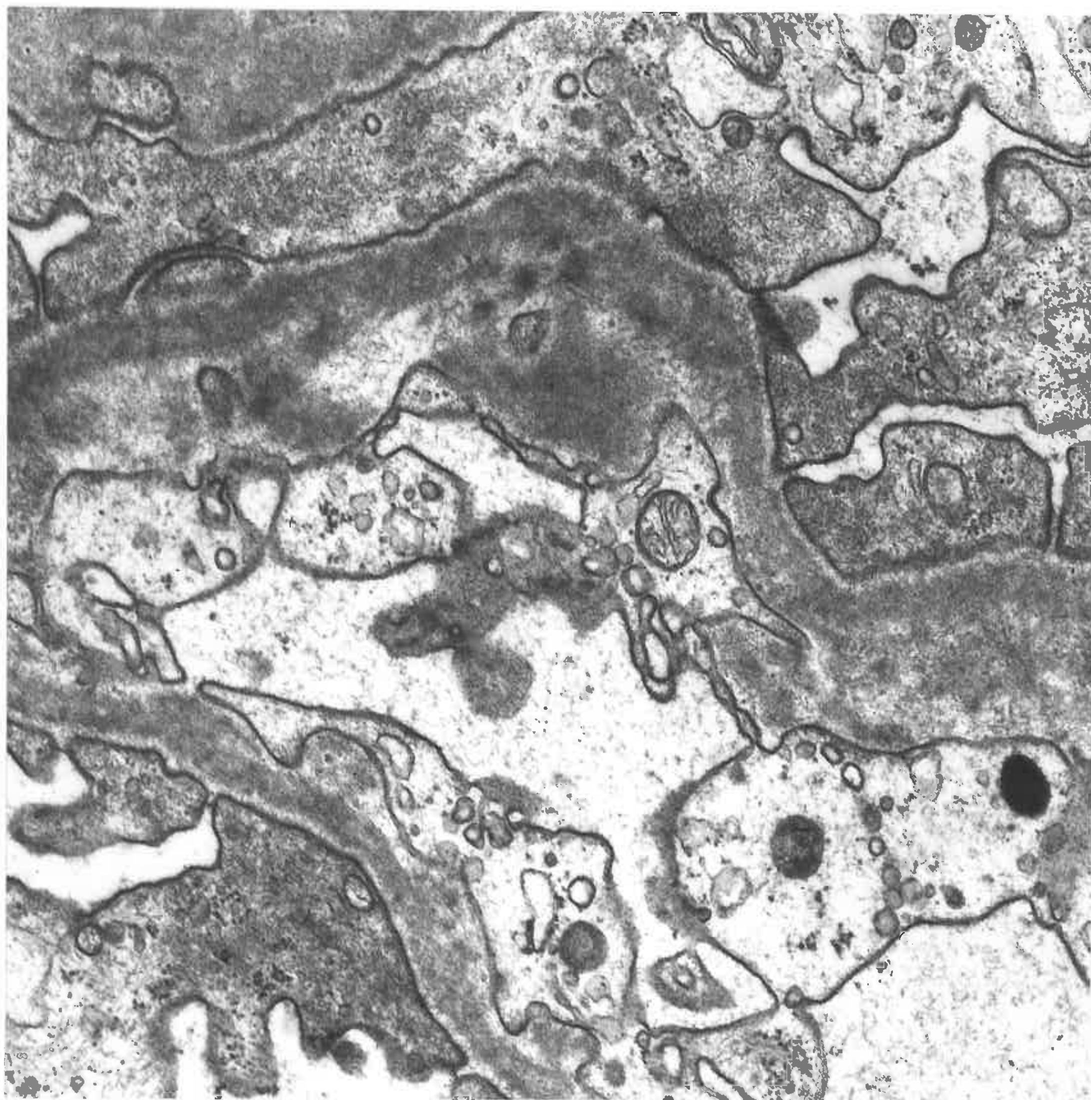


Fig. 77. Electron microscopic grading of glomerular capillary fibrin. The presence of dark, dense deposits between the basement membrane and endothelium was graded as ++. (x 34,000)

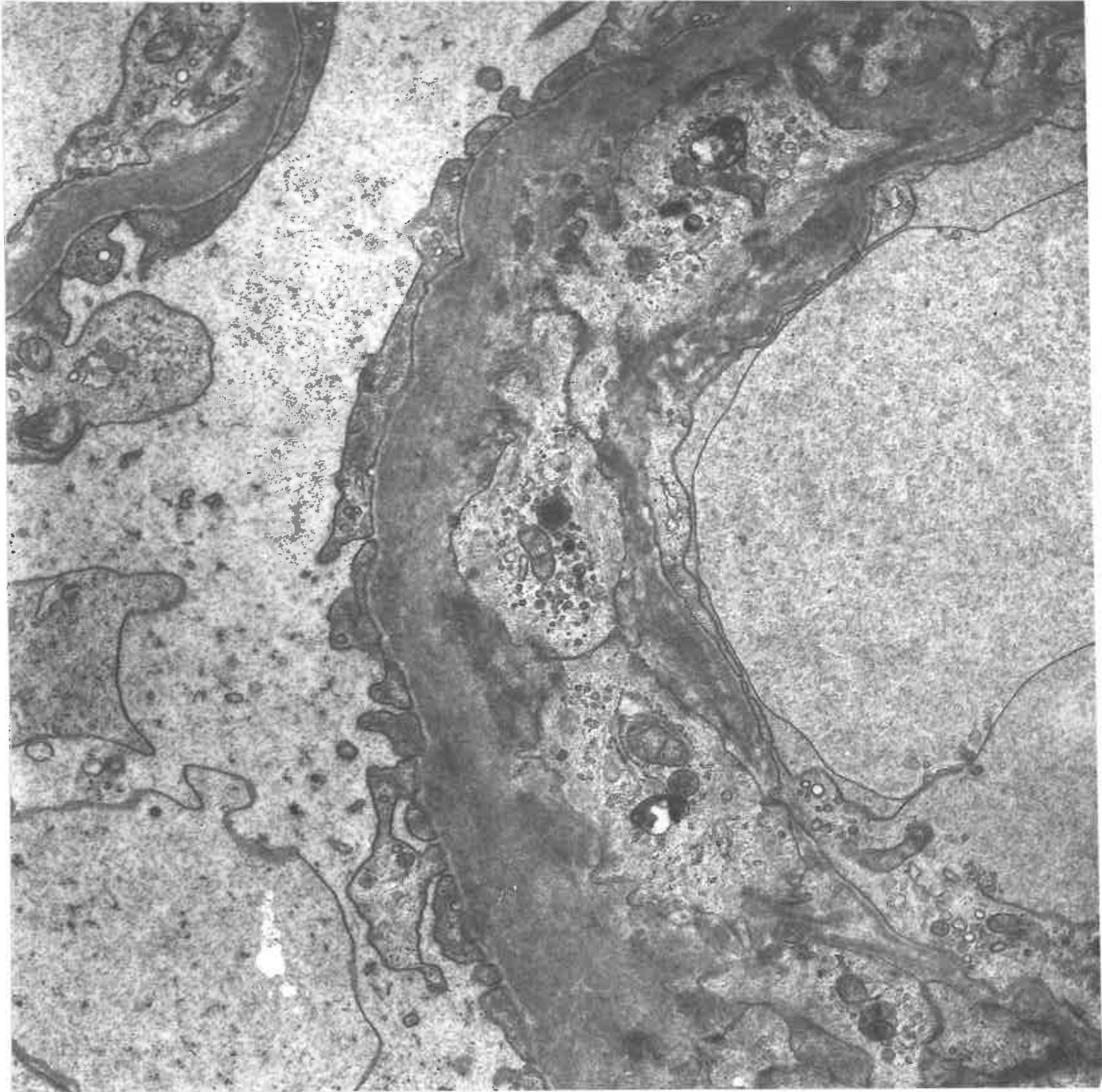


Fig. 78. Electron microscopic grading of glomerular capillary fibrin. When fibrin was seen projecting into the capillary lumen and between swollen and proliferating endothelial cells it was graded as +++. (x 12,000)

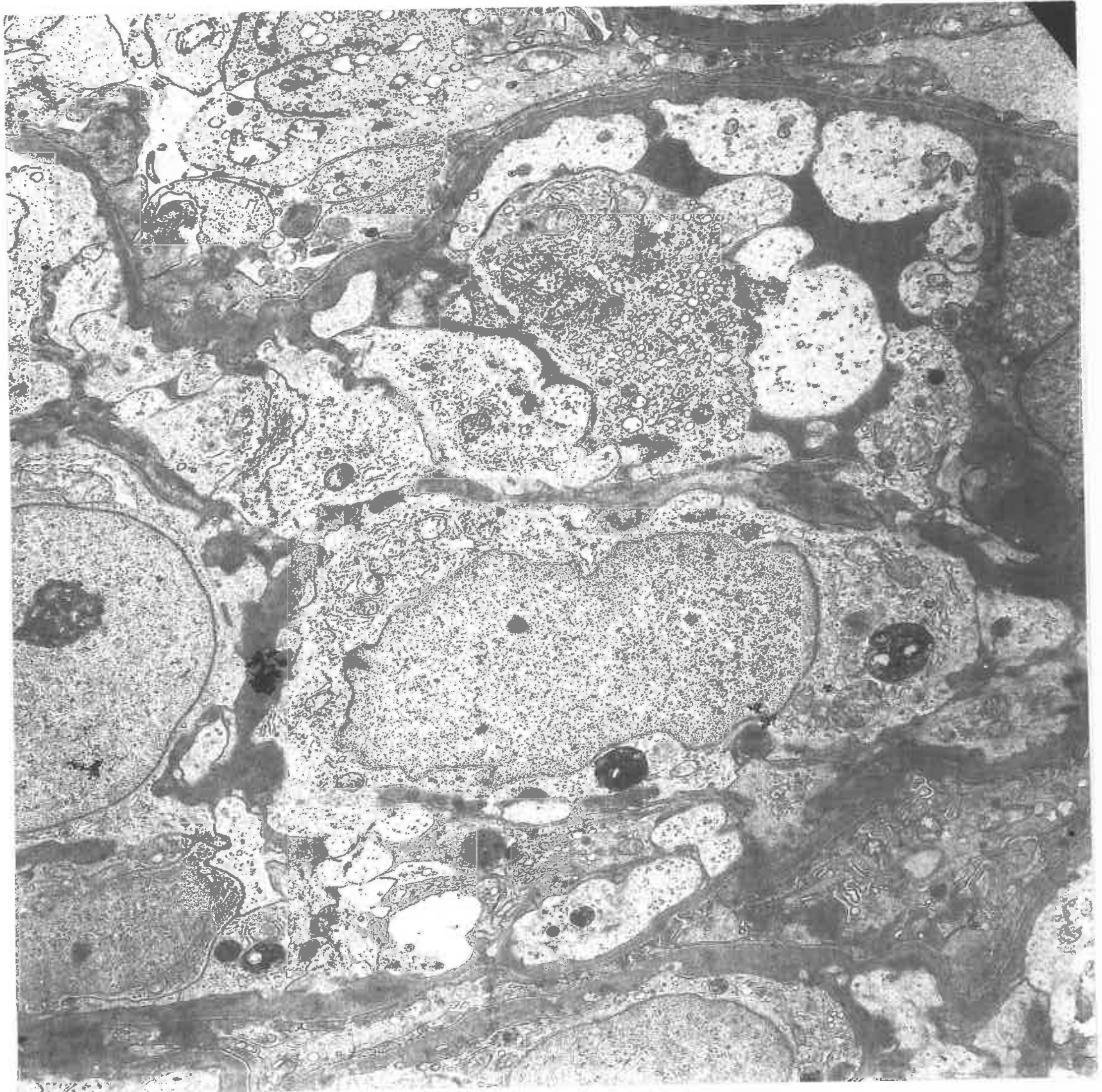


Fig. 79. Electron microscopic grading of glomerular capillary fibrin. If there was complete occlusion of the glomerular capillary lumen by fibrin and proliferating endothelial cells, it was graded as ++++.

Table 17.

Relation between maximum urine F.D.P. concentration and the extent of intraglomerular fibrin deposition in proliferative glomerulonephritis.

Glomerular fibrin electron microscopic grading	Maximum Urine F.D.P. Concentration ($\mu g/ml$)					
	<1	1-2	2-5	5-10	10-20	>20
0	6	-	1	-	-	-
+	1	7	11	2	2	1
++	-	3	3	3	1	2
+++	-	-	-	1	1	4
++++	-	-	-	-	-	2

0 = no fibrin present.

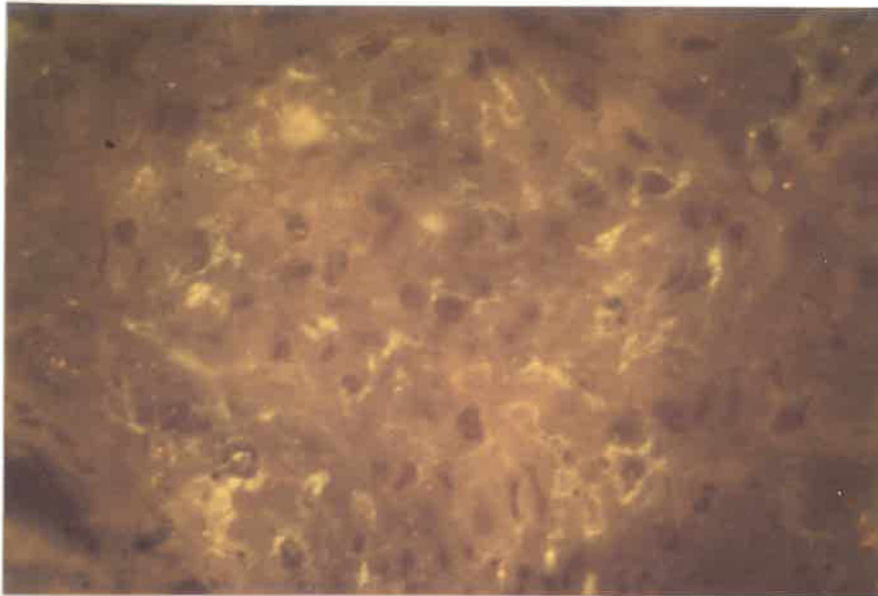
+ = capillary wall alone or interstitium alone (Fig. 80).

++ = capillary wall and mesangium or interstitium alone (Fig. 81).

+++ = fibrin occluding the glomerular capillary lumen (Fig. 82).

++++ = intraglomerular capillary fibrin together with fibrin within crescents and/or interstitium, and/or tubular cells (Fig. 83).

The results are shown in Table 18 where it can be seen that the cases in which fibrin is more widely dispersed throughout the kidney, the urine F.D.P. excretion is higher. Of interest was the observation that in cases where there was only mesangial deposition of fibrin, urinary excretion did not exceed 2 μg/ml. This pattern was observed in patients with resolving proliferative glomerulonephritis where the mesangial cells seemed to be able to cope with removal of fibrin. On the other hand, in rapidly progressive and acute necrotizing glomerulonephritis, little or no mesangial immunofluorescence was observed, whereas fibrin was commonly seen occluding the capillary lumen, within crescents, interstitium and tubular cells, and the urinary fibrin excretion was uniformly elevated. Thus, in these cases the mesangium seemed to play little part in the removal of fibrin and other mechanisms notably urinary excretion played a greater part. It was also notable that the highest circulating levels of F.D.P. were found in these cases and it is possible that the abnormal coagulation within the kidney contributes to this abnormality. This theory was put to the test in one patient with acute necrotizing glomerulonephritis in whom blood samples were obtained from catheters introduced into the renal artery,



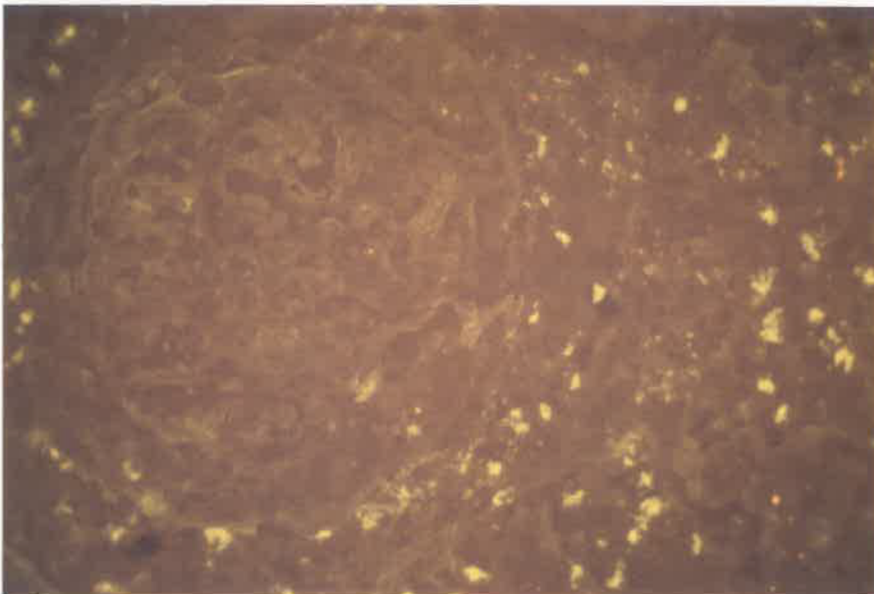
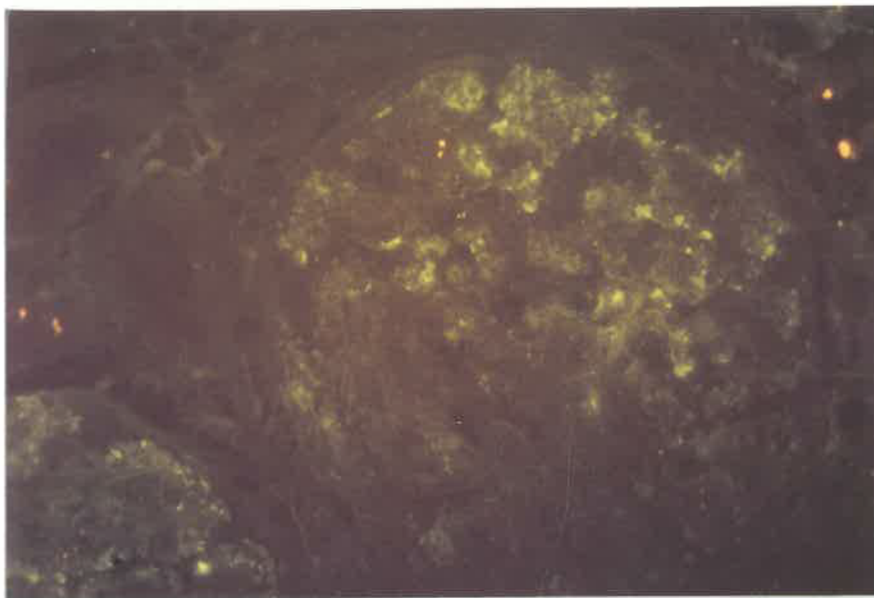


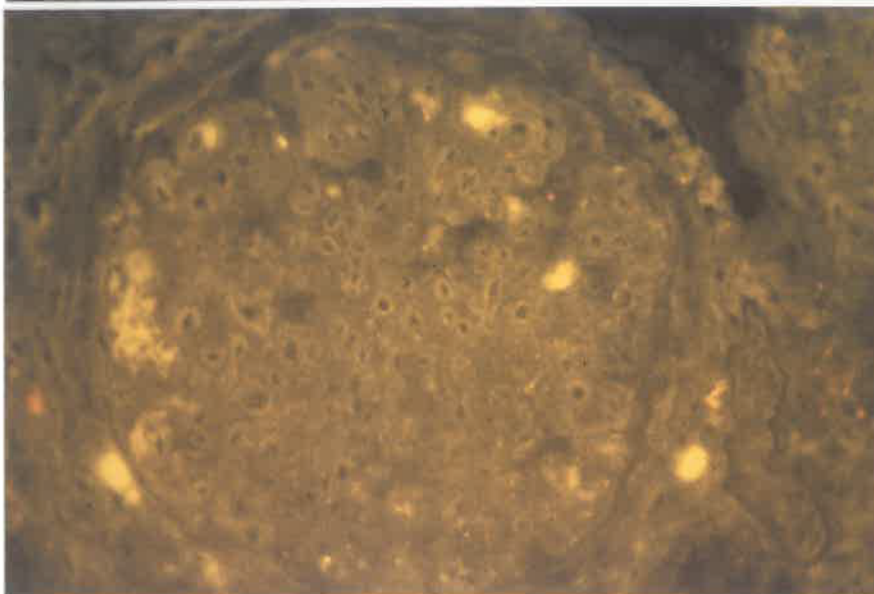
Fig. 80. Immunofluorescence grading of intrarenal fibrin (Grade +):

A. Fibrin deposition confined to the glomerular capillary wall;

B. Fibrin deposition confined to the interstitium.



A



B

Fig. 81. Immunofluorescence grading of intrarenal fibrin (Grade ++).

A. Fibrin present in glomerular capillary wall and mesangium;

B. Fibrin present in mesangium alone.

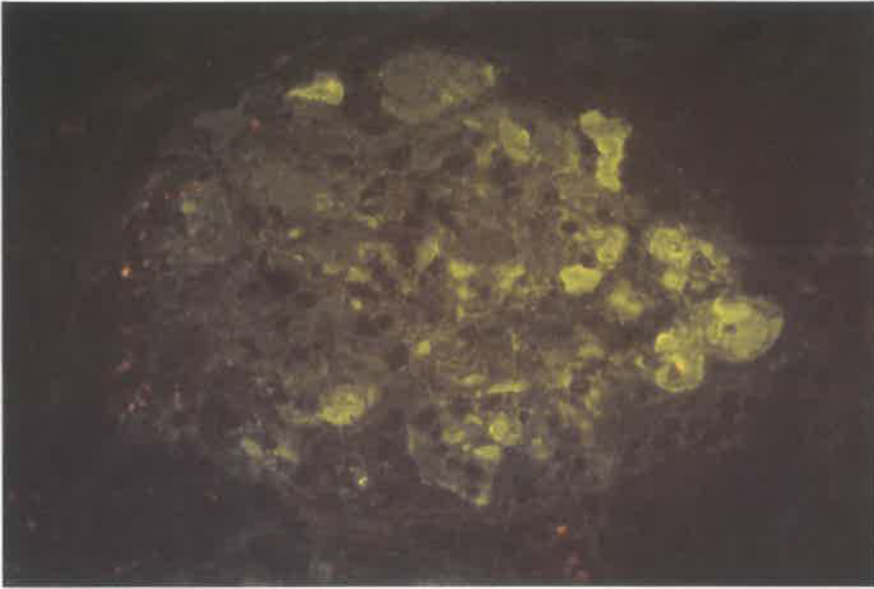
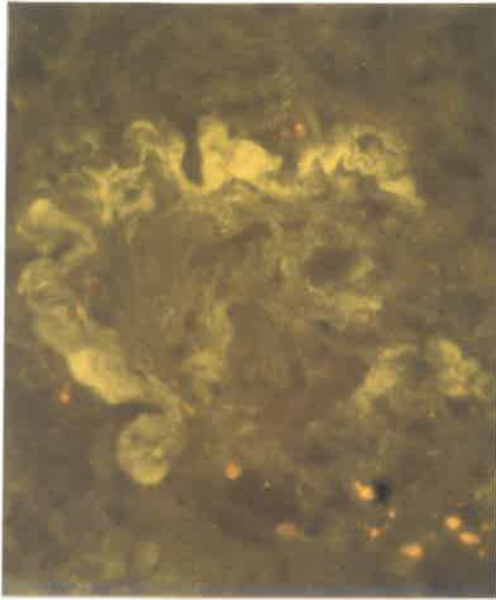
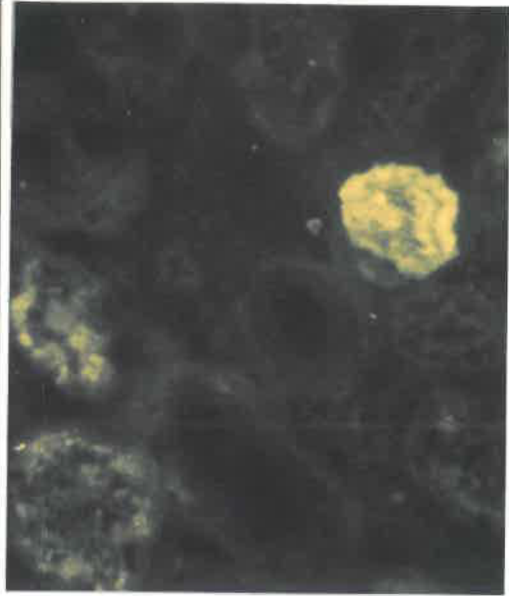


Fig. 82. Immunofluorescence grading of intrarenal fibrin (Grade +++). Fibrin occluding the glomerular capillary lumen.



A



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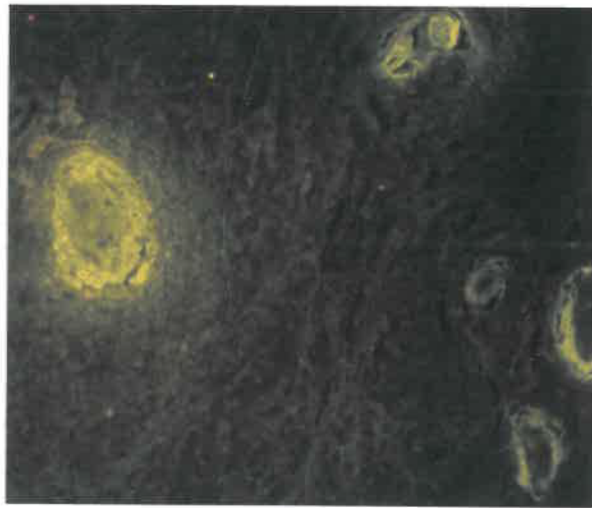

C

Fig. 83. Immunofluorescence grading of intrarenal fibrin (Grade ++++):

A. Intraglomerular capillary fibrin together with fibrin within a crescent;

B. Intraglomerular capillary fibrin together with fibrin within tubular cells;

C. Intraglomerular capillary fibrin together with fibrin within interstitium (in this case arteries).

Table 18.

Relation between maximum urine F.D.P. concentration and the disposition of intrarenal fibrin as determined by immunofluorescence of 47 consecutive renal biopsies.

Intrarenal Fibrin Immunofluorescent Grading	Maximum Urine F.D.P. Concentration (μg/ml)					
	< 1	1-2	2-5	5-10	10-20	>20
0	6	5	-	-	-	-
+	3	1	5	-	-	-
++	1	-	3	3	1	-
+++	-	-	-	3	3	2
++++	-	-	-	3	3	5

renal vein and the inferior vena cava. The results (Table 19) indicated that fibrinogen, F.D.P. and S.F.M.C. were removed by the kidney and that in this case the kidney did not contribute to the abnormally high serum F.D.P. level. Rather, it was felt that this case represented one of disseminated intravascular coagulation, and this impression was confirmed at autopsy where infarct necrosis was found in many organs.

4.3.5 PROTEINURIA AND URINARY F.D.P. EXCRETION

Critical to the concept of urinary F.D.P. concentration as a measure of disease activity, progression and natural history is its relation with the degree and selectivity of proteinuria. No benefit would accrue from the study of fibrin excretion if it was merely a reflection of the renal loss of protein or of changes in glomerular permeability to protein.

In 143 patients, the urine F.D.P. concentration, total protein excretion and selectivity of plasma proteins were measured on the same 24 hour specimen. No relation was found between F.D.P. concentration or 24 hour F.D.P. excretion and the degree of proteinuria in proliferative glomerulonephritis when the population of these cases were considered as a whole ($r = 0.06$, $p > 0.1$) or as individual disease groups. A single example of this lack of correlation is shown in Fig. 84. In minimal lesion and membranous glomerulonephritis, the F.D.P. concentration and excretion were closely related to the degree of proteinuria ($r = 0.79$, $p < 0.001$ for membranous, $r = 0.70$, $p < 0.001$

Table 19.

Coagulation studies performed on blood drawn simultaneously from renal artery, renal vein and lower inferior vena cava in a 16 year-old girl with acute necrotizing glomerulonephritis

	Renal Artery	Renal Vein	Inferior Vena Cava (below renal vein)
Plasma Fibrinogen (mg/100ml)	688	636	686
Plasma S.F.M.C. (O.D. units)	0.77	0.55	0.74
Serum S.F.M.C. (O.D. units)	0.51	0.41	0.51
Serum F.D.P. (μg/ml)	148	91	148
Plasma Plasminogen Activator Content (units)	2.20	2.16	2.39
Plasma Plasminogen (casein units/ml)	1.89	1.93	2.04

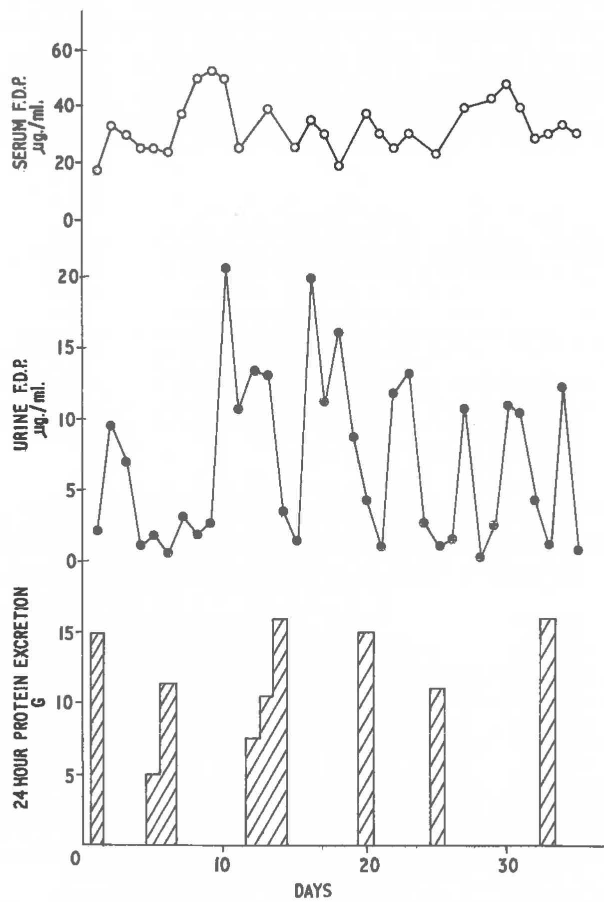

Fig. 84. Daily urinary F.D.P. concentration and urinary protein excretion in a 35 year-old man with membrano-proliferative glomerulonephritis and nephrotic syndrome. There is no apparent relationship between urinary F.D.P. and protein excretion or between serum and urinary F.D.P. concentrations1

for minimal lesion). The results for all patients are summarized in Fig. 85.

In no group of cases, nor in the population as a whole was any statistically significant relation found between the urinary F.D.P. concentration or excretion and the selectivity of proteinuria ($r = 0.07$, $p > 0.1$), (Fig. 86). These findings are open to question however, as the validity of comparing the excretion of a substance with the clearance of another is not known. Preferably, clearance data for fibrinogen or individual degradation products would have been desirable, but at the present time means of acquiring this are not available.

4.3.6 UROKINASE AND URINARY F.D.P. EXCRETION

The presence in the urine of plasminogen filtered through the glomerular basement membrane with other plasma proteins in glomerulo-nephritic patients raises the possibility that filtered fibrinogen or degradation products may be further degraded in the urinary tract by plasmin rendered active by urokinase or filtered plasma activators. Thus urine F.D.P. concentration may be influenced by the urine concentration of plasminogen activator.

Urokinase and F.D.P. were measured on the same 24 hour urine specimen for between 14 and 29 days in 13 patients — unspecified proliferative (5), Henoch-Schönlein (2), minimal lesion (2), membranous (2), S.L.E. (1) and membrano-proliferative (1). No significant

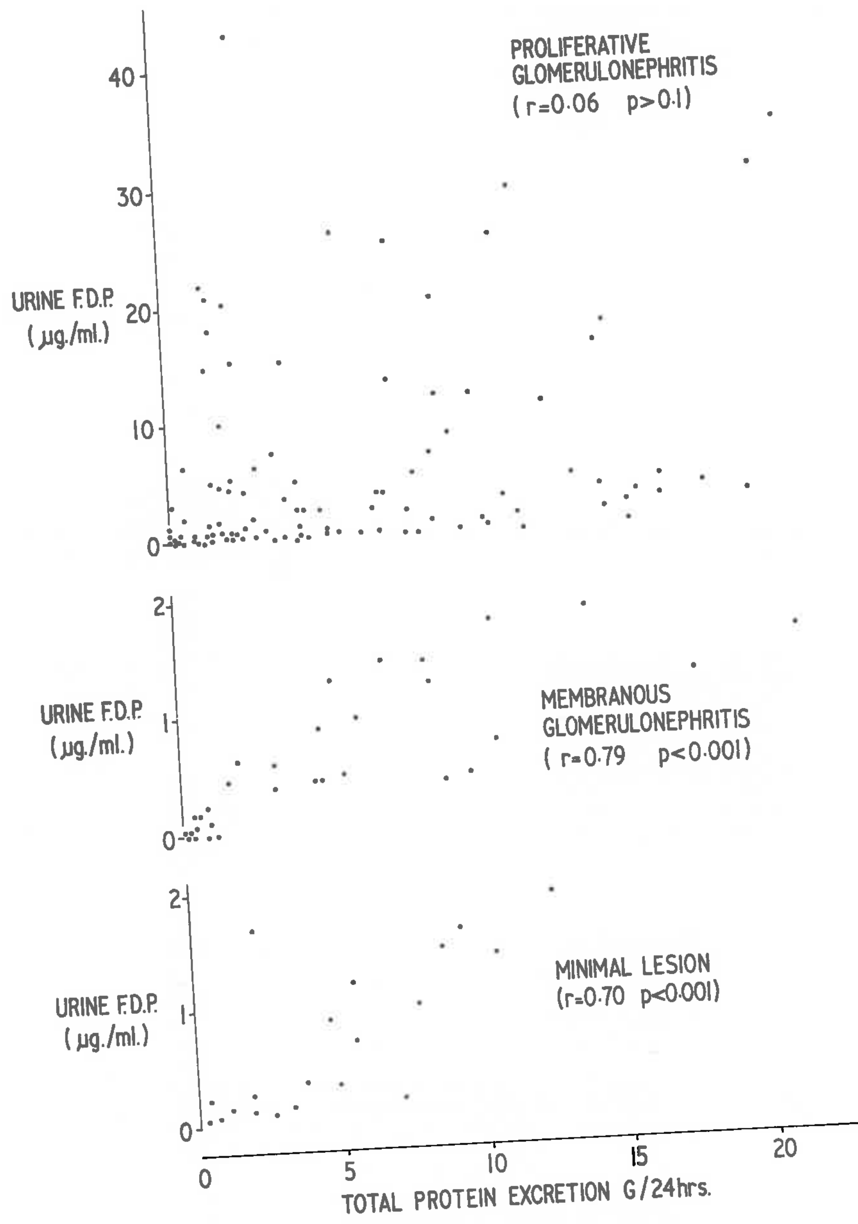

Fig. 85. Relation between total daily protein excretion and urinary F.D.P. concentration in the different categories of glomerulonephritis. All proliferative forms are considered together.

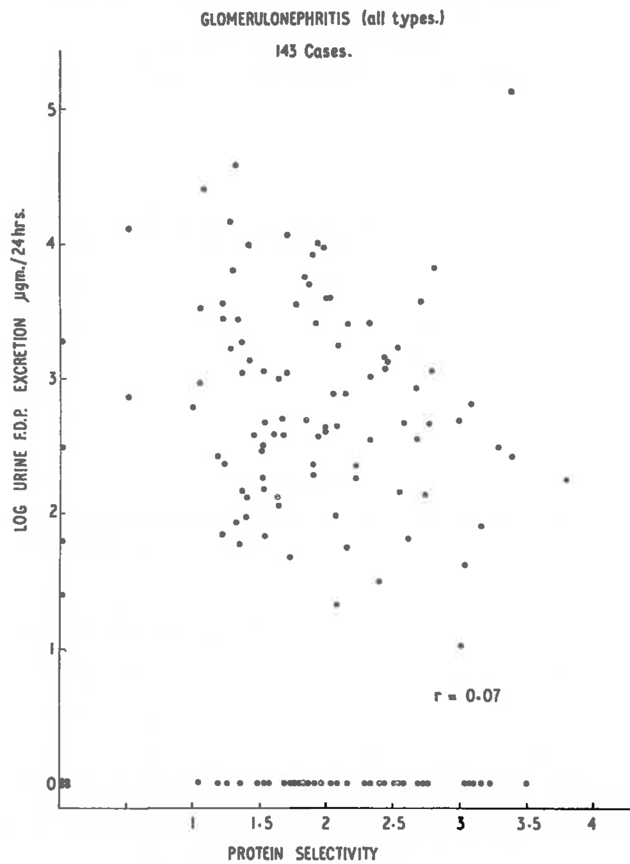

Fig. 86. Urinary F.D.P. excretion plotted in relation to protein selectivity in 143 cases of the different categories of glomerulonephritis. No relationship was found although the validity of comparing an excretion rate with a clearance is questioned.

correlation was found between the values obtained when considered as a single population ($r = 0.05$, $p > 0.1$) as separate diseases, or in individual patients ($p > 0.1$ in each instance). An example is shown in Fig. 87. It seems unlikely therefore that urinary F.D.P. excretion is greatly modified during the passage of urine to the exterior. Moreover, fluctuations in urokinase excretion could not account for the large periodic increase in urine F.D.P. concentration seen in many cases of active proliferative glomerulonephritis.

4.3.7 COLUMN CHROMATOGRAPHIC ANALYSIS OF URINARY F.D.P. IN GLOMERULONEPHRITIS

CHARACTERIZATION OF FIBRIN DEGRADATION PRODUCTS

Previous workers have demonstrated that the proteolysis of fibrinogen or fibrin results in degradation products whose size depends on the length of time lysis is continued. Fibrinogen has a molecular weight of approximately 300-340,000, and after short periods of lysis fragments X of molecular weight 240-270,000 is formed. Further proteolysis of of fragment X results in the formation of two further fragments, Y and D of molecular weights 150,000 and 90,000 respectively (second stage products). Cleavage of fragment Y produces a further fragment D and fragment E of molecular weight 50,000. (Third stage products.) Fragments D and E are resistant to any further lysis by plasmin.

The individual fragments have been characterized by a combination of procedures including column chromatography, immune-electrophoresis and ultracentrifugation, and collected in sufficient quantities by

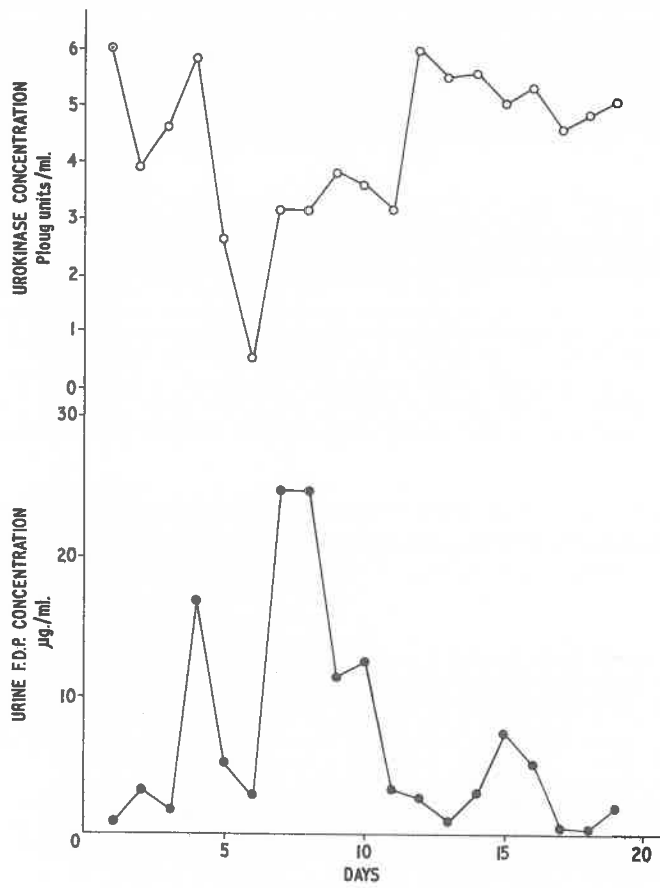

Fig. 87. This figure illustrates the lack of correlation between urinary F.D.P. and urokinase excretion in a 38 year-old male with Henoch Schonlein purpura.

Pevikon block electrophoresis to enable further purification for antiserum production in animals (Marder, Shulman and Carroll, 1969; Marder, James and Sherry, 1969).

Prior to examination of urinary fibrin derivatives by column chromatography, standardisation of the column containing "Biogel - A - 1.5M" (8% Agarose) with respect to fibrin degradation products was performed in the following manner. A series of samples of fibrinogen (200 mg in 5 ml saline) were eluted and fractions analysed by the T.R.C.H.I.I. for their reactivity in this assay, and by protein analysis. The elution pattern for fibrinogen was thus established and used as a baseline for comparison with lysed samples. In each lysis experiment, 200 mg fibrinogen in 5 ml saline was lysed at room temperature with 5,000 Ploug units of urokinase. Lysis was stopped at 5, 10, 30, 45, 60 minutes and 24 hours by the addition of excess quantities of trasylol. Each sample was then subjected to column chromatography and immuno-electrophoresis and eluate fractions were examined for protein and F.D.P. concentrations.

In separate chromatographic runs the presence of the blue dextran marker dye, urokinase and trasylol was not found to influence the T.R.C.H.I.I. to an extent that interference with haemagglutination inhibition end-point recording occurred.

The results are summarised in Fig. 88. Under the conditions which held, little change in the elution pattern occurred until 10 minutes, when fragment X appeared characterised by a differing elution peak and immuno-electrophoretic pattern. At 45 minutes, lysis had

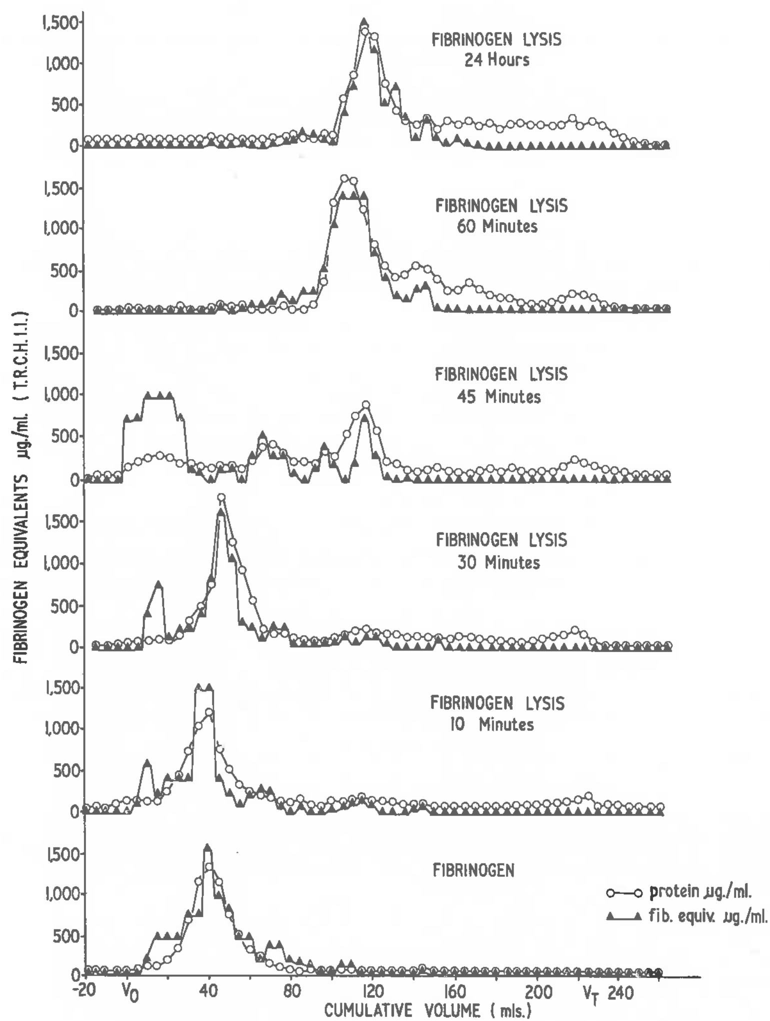

Fig. 88. The elution profiles of fibrinogen and its degradation products formed <u>in vitro</u> by lysing fibrinogen with urokinase for specific periods. The eluates were studied for protein concentration and their reactivity in the T.R.C.H.I.I.

progressed so that the intermediate products Y and D were apparent on chromatography, and a 3 arc pattern was seen on immuno-electrophoresis. By 60 minutes, lysis was virtually complete and only minor further changes in the chromatographic pattern had occurred at 24 hours, although immuno-electrophoresis demonstrated further shift of D and E to the cathode and anode respectively. While it was initially thought that fragments D and E could be separated on 8% agarose, immuno-electrophoresis of this peak revealed 2 distinct bands, fragment D migrating towards the cathode and E towards the anode. Serial changes on immune-electrophoresis are demonstrated in Fig. 89.

The elution positions (Kd) of each fragment were ascertained and are shown in Table 20. It is recognised that the interpretation of this data is subject to question, especially with regard to the validity of using whole anti-fibrinogen serum when detecting individual fragments by the T.R.C.H.I.I. The reaction in the test system of purified fragments D and E kindly supplied by Dr. V.J. Marder showed that the data was valid in a qualitative but not quantitative sense, for only 50% of the total estimated content of D and 10% of E was detected. Various available sources of anti-fibrinogen sera were also tested but the brand used in all the reported studies (Behringwercke) was found the most sensitive for these lower molecular weight fragments.

Finally the proteins, thyroglobulin, albumin and ferritin were chromatographed, for after analysis of available published data (Marder et al, 1969; Dudek et al, 1970), it was considered that thyroglobulin should elute between fibrinogen and X, ferritin between X and Y, and

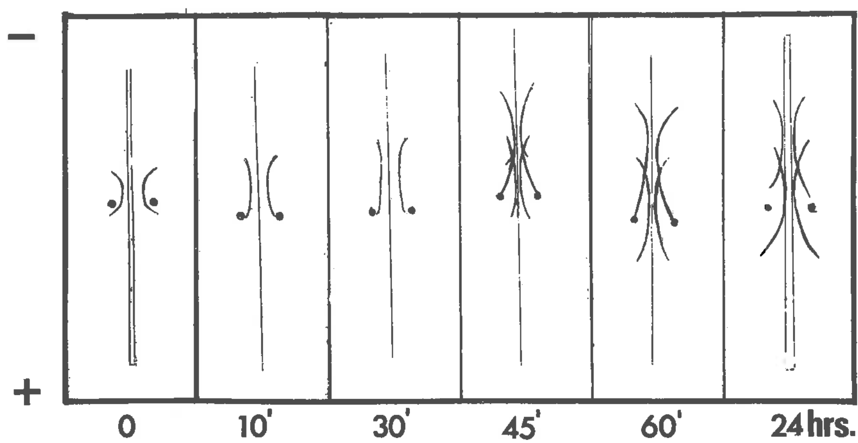

Fig. 89. Immuno-electrophoresis of timed urokinase digests of human fibrinogen under standard conditions. Cathode at top, anode at bottom of each slide. Origin indicated by the dot. The figure has been re-touched to improve presentation.

Table 20.

The elution positions of the major peaks observed during the progressive lysis of fibrinogen

	Mean KD Value	Range	No. of Recordings
Fibrinogen	0.125	0.115-0.140	5
Fragment X	0.230	0.200-0.272	5
Fragment Y	0.375	0.350-0.415	4
Fragments D & E	0.505	0.490-0.520	8
?	0.638	0.610-0.655	8

albumin between D and E. The calibration of the column is shown in Fig. 90 in which personally obtained results are compared with published figures. The molecular weights of X, Y, D and E calculated from the Stoke's radius are in agreement with those found by Marder et al (1969) and Dudek et al (1970). A further unknown peak with K_D of 0.638 and Stokes radius of 24 was calculated to have a molecular weight of 28,000. It was not possible to separate this peak from D and E by immune-electrophoresis.

CHROMATOGRAPHIC ANALYSIS OF URINARY F.D.P.

From twelve patients with active proliferative glomerulonephritis, three patients with membranous and two patients with minimal lesion glomerulonephritis concentrated urine specimens were analysed by column chromatography in 8% agarose. Individual fractions were concentrated 6-10 times by rapid freezing in dry ice so that protein and poly-peptides were present in sufficient concentrations to be detected by the T.R.C.H.I.I. Profiles were compared with those obtained in the fibrinogen lysis experiments.

In minimal lesion and membranous glomerulonephritis small amounts of fibrinogen, fragments X, Y and D were found while in all cases of active proliferative glomerulonephritis, large amounts of fragments D and E were found in addition. Analysis of the data revealed that more than 90% of the urinary F.D.P. in proliferative glomerulonephritis was present as fragments D and E. The elution profiles of 2 patients from each group are shown in Fig. 91. Of interest was the observation that the elution pattern of purified human fibrinogen placed in normal

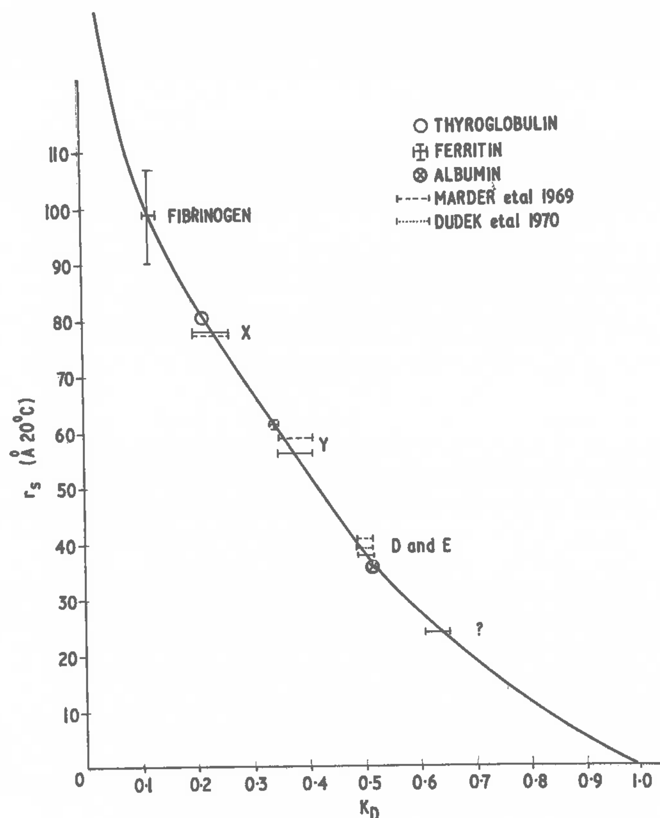

Fig. 90. Calibration of the 8% agarose column used in the majority of the elution studies. The molecular weights of fragments X, Y, D and E derived from this data are in general agreement with those found by other authors. Personal results are indicated thus ├──┤

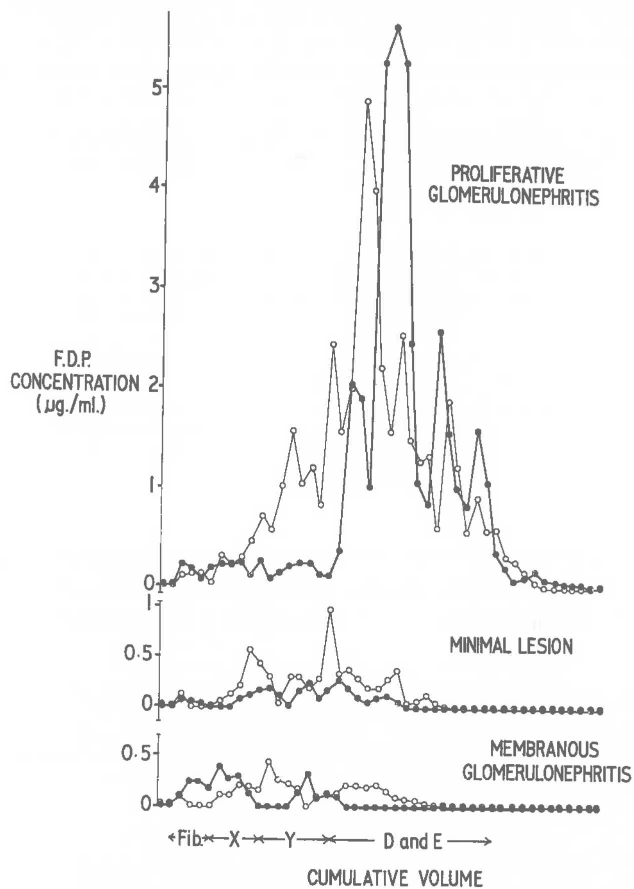

Fig. 91. Results of the T.R.C.H.I.I. for fibrinogen and derivatives in eluates obtained from column chromatography of concentrated urine samples. The elution positions of fragments from fibrinogenolysis are shown for comparison.

urine and incubated for 12 hours at 37°C was similar to that seen in minimal lesion and membranous glomerulonephritis and markedly different from that of maximally lysed fibrinogen and active proliferative glomerulonephritis (Fig. 92). Immuno-electrophoresis was not sufficiently sensitive for precipitin arcs to be detected even on concentrated urine specimens.

4.4 DISCUSSION

The primary aim of this investigation was to determine the forms of glomerulonephritis in which abnormal coagulation played a role. For practical purposes study was limited in most cases to serial examination of serum and urinary F.D.P. concentrations. However, it soon became apparent that the urine estimation provided some advantages in terms of sensitivity in reflecting intrarenal events, and in terms of convenience to the patient, for continuous study could be accomplished for long periods even on outpatients. Previous workers have shown that serum F.D.P. concentration is correlated with the degree of activity of proliferative glomerulonephritis (Steihm and Trygstad, 1969), a finding which is supported by the data reported here. However, raised concentrations are found in non renal conditions, particularly states of disseminated intravascular coagulation (Merskey et al, 1967), deep venous thrombosis and pulmonary embolism (Ruckley et al, 1970), and the serum determination is relatively insensitive in moderate and/or localized renal involvement. For example, consistently normal serum

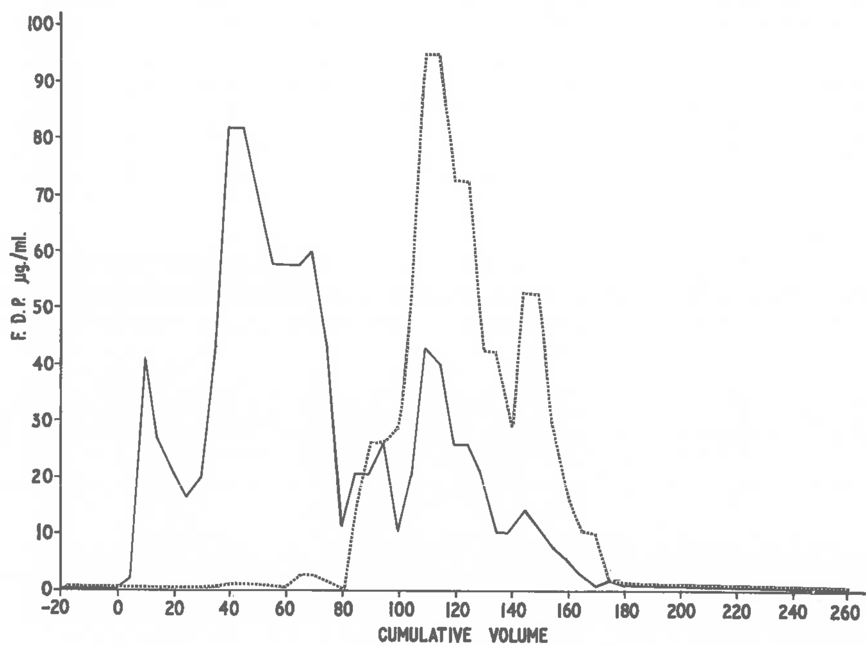

Fig. 92. Comparison of T.R.C.H.I.I. results on eluates obtained from samples taken after complete lysis of fibrinogen in buffered saline (--------) and normal urine into which was placed 200 mg. purified human fibrinogen and incubated at 37°C for 12 hours (————).

F.D.P. levels were found in 32% of cases considered to have active proliferative glomerulonephritis, whereas urinary F.D.P. concentration was above 2 μg/ml in all of these.

Healthy normotensive subjects excrete negligible amounts of F.D.P. and this is also the case in the patients with uncomplicated polycystic disease. The relatively low values found in patients with pyelonephritis and lower urinary tract infection is surprising as all had features associated with inflammation of the urinary tract. However, in view of the abnormal urinary F.D.P. concentrations found in all types of glomerulonephritis, the presence of glomerular damage seems necessary for significant urinary F.D.P. excretion to occur. Such damage is an inconstant feature of uncomplicated polycystic or infective renal disease.

In minimal lesion and membranous glomerulonephritis, in which only meagre histological or ultrastructural evidence of intraglomerular fibrin deposition is found, urinary F.D.P. concentration did not exceed 2 μg/ml despite heavy proteinuria. Nevertheless, the extent of F.D.P. excretion is closely related to the degree of proteinuria. Moreover, the relatively small quantities of fibrin/fibrinogen derivatives excreted are in the higher molecular weight range (fibrinogen, fragments X, Y and D). This suggests that in these cases, the F.D.P. in the urine arises from fibrinogen, filtered through an abnormally permeable basement membrane, which undergoes partial proteolysis in the renal tract.

Urine F.D.P. concentrations, often greatly in excess of 2 μg/ml were frequently found in cases of proliferative glomerulonephritis no

matter what pathogenetic mechanism was involved, nor what clinical category of disease was present. Moreover, the extent of the elevation is closely related to the severity of the disease in terms of both renal functional and pathological changes. In these cases a close relationship also exists between the extent of F.D.P. excretion and the extent of intraglomerular fibrin deposition detected by ultrastructural study of renal biopsies and the disposition of fibrin shown by immunofluorescence. Furthermore, the data indicates that this increased concentration is related to the "activity" of the disease. Fractionation studies show that a larger proportion of the excreted F.D.P. is of low molecular weight type (fragments D and E). While there are several possible explanations for this finding, the most attractive is that it results from local breakdown of the intraglomerular fibrin. In support of this hypothesis, excretion of F.D.P. in these cases was found to be independent of the degree of proteinuria and its selectivity and also of the urinary proteolytic activity as measured by urokinase excretion. Thus it seems probable that F.D.P. in the urine in active proliferative forms of glomerulonephritis results from 2 sources:

1. The complete degradation of fibrin formed within the glomerular capillaries (fibrinolysis) giving preferentially D and E products, and

2. Limited proteolysis of filtered fibrinogen as in minimal lesion and membranous glomerulonephritis.

In this study, useful clinical information was obtained from the daily estimation of urinary F.D.P. This was especially the case in patients with the nephrotic syndrome in whom the concentration of F.D.P. was $2\mu g/ml$ or more. In these circumstances the diagnosis of a proliferative form of glomerulonephritis was almost certain. Daily measurement in all types of proliferative glomerulonephritis including S.L.E. and membranoproliferative forms also provided information, hitherto unavailable, relevant to the activity, natural history and prognosis. In post-streptococcal glomerulonephritis, for example, after a limited period of activity there was abrupt cessation of F.D.P. excretion at the time of clinical recovery. In mesangial proliferative glomerulonephritis where fluorescence is confined to the mesangial cells, F.D.P. excretion was limited and did not show the periodic fluctuations seen in cases where glomerular capillary wall fluorescence was detected. Where clinical evidence of activity persisted, large fluctuations of F.D.P. concentration occurred which increased in magnitude in progressive disease and were similar to the cyclical peaks noticed in renal homograft rejection (see Section 6).

This cyclical pattern may provide a clue to the nature of the inflammatory stimulus in proliferative glomerulonephritis. It may represent periodic attempts by systemic or intrarenal fibrinolytic mechanisms to degrade intraglomerular fibrin although the absence of parallel changes between excreted urokinase and F.D.P. are against this hypothesis. More likely, it may reflect a fluctuating inflammatory activity which is of presumed immunological origin.

Finally and perhaps most significant of all, in proliferative glomerulonephritis, the daily estimation of urine F.D.P. may provide a tool, which at minimal inconvenience to the patient, facilitates the continual review of specific therapy.

Section 5.

TREATMENT OF GLOMERULONEPHRITIS BASED ON THE URINARY EXCRETION OF FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS (F.D.P.)

As abnormal F.D.P. excretion is seen in the more severe and active cases of proliferative glomerulonephritis, and a continued high level of excretion is associated with a poor prognosis, drugs which cause its reduction may have a beneficial effect on the course of these diseases. Early in 1970, serial assays in a 17 year-old male with persistent oedema, proteinuria and haematuria 6 weeks after a classical attack of Henoch-Schönlein purpura revealed a marked reduction of F.D.P. concentration associated with indomethacin therapy. Vermylen et al (1970) in Belgium reported a similar observation in 8 patients with chronic proliferative glomerulonephritis. The purpose of this study was to analyse the effect of indomethacin and other anti-inflammatory drugs such as aspirin and prednisolone on the urinary F.D.P. excretion in patients with various forms of glomerulonephritis.

5.1 PATIENTS AND METHODS

PATIENTS: In each of the 35 patients studied, the diagnosis was made on the basis of clinical evaluation and light and electron microscopic examination of renal biopsies. At the time of study 31 patients had an "active" form of proliferative glomerulonephritis, 2 suffered from minimal lesion and 2 from membranous glomerulonephritis. Some clinical details are given in Table 21.

TREATMENT: A total of 47 treatment schedules were carried out in the 35 patients. Twenty-six patients received indomethacin alone in a dosage of 75-150 mg/day and of these 13 are still taking the drug. This therapy was only temporary in 7 and was discontinued in the remaining 6 because of gastro-intestinal side-effects (2), admission to a chronic haemodialysis program (1), a fatal outcome (2), and to observe the effects of

Table 21.

Group B. Prednisolone Alone

Case No.	Age	Sex	Diagnosis	Biopsy Features	Clinical Features	Duration of Therapy (weeks)	F.D.P. Response
3	16	F	S.L.E.	Diffuse proliferation; crescents	Progressive disease (see Fig. 94)	7 (see Group C)	-
27	36	F	S.L.E.	Diffuse proliferation; some necrosis	Progressive disease, 6 months	24 (continuing)	+
28	15	F	S.L.E.	Focal proliferative; necrotizing	Progressive disease, 2 years	40 (continuing)	+
29	25	F	S.L.E.	Diffuse proliferation; crescents	Progressive disease, 6 months	32 (continuing)	+
30	63	M	Acute oliguric G.N.	Necrosis; proliferation; crescents	Rapidly progressive disease, 1 month	12 (discontinued)	+
17	59	M	Proliferative	Diffuse proliferation; crescents	Progressive disease, ?polyarteritis	8 (discontinued)	-
31	38	M	H.S.P.	Diffuse proliferation; crescents	Progressive disease; nephrotic, 1 month	32 (continuing)	-
32	16	F	H.S.P.	Diffuse proliferation; crescents; sclerosis	Rapidly progressive disease	4 (continuing)	-
21	48	M	Proliferative	Diffuse proliferation	Recurrent nephrosis, 8 years	30 (discontinued)	+
22	25	M	Proliferative	Diffuse proliferation; sclerosis	Recurrent nephrosis, 5 years	32 (continuing)	+
23	12	M	Minimal Lesion	—	Recurrent nephrosis, 6 years	22 (discontinued)	+
24	70	F	Minimal Lesion	—	Recurrent nephrosis, 8 years	12 (discontinued)	+

Table 21.

Group A. Indomethacin Alone (Continued)

Case No.	Age	Sex	Diagnosis	Biopsy Features	Clinical Features	Duration of Therapy (weeks)	F.D.P. Response
15	22	M	Proliferative	Diffuse proliferation	Recurrent nephrosis, 3 years	46 (continuing)	+
16	64	M	Proliferative	Diffuse proliferation; crescents	Progressive disease; Nephrotic	26 (continuing)	+
17	59	M	Proliferative	Diffuse proliferation; crescents	Progressive disease; ?Polyarteritis	10 (discontinued)*	+
18	52	M	Proliferative	Diffuse proliferation; obliteration	Proteinuria, hypertension, 6 months	28 (continuing)	+
19	16	M	Proliferative	Focal proliferation; Interstitial inflammation	Alport's syndrome	8 (continuing)	-
20	19	M	Proliferative	Diffuse proliferation; crescents	Fabrey's disease	18 (continuing)	-
21	48	M	Proliferative	Diffuse proliferation	Recurrent nephrosis, 8 years	3 (discontinued)*	-
22	25	M	Proliferative	Diffuse proliferation; obliteration	Recurrent nephrosis, 5 years	3 (discontinued)*	-
23	12	M	Minimal Lesion	—	Recurrent nephrosis, 6 years	2 (discontinued)	-
24	70	F	Minimal Lesion	—	Recurrent nephrosis, 8 years	3 (discontinued)	-
25	19	F	Membranous	—	Proteinuria, 4 years	16 (continuing)	-
26	42	M	Membranous	—	Proteinuria, 7 years	2 (discontinued)	-

Table 21.

Clinical, pathological and therapeutic details of the 35 patients and 47 treatment schedules.

Group A. Indomethacin Alone

Case No.	Age	Sex	Diagnosis	Biopsy Features	Clinical Features	Duration of Therapy (weeks)	F.D.P. Response
1	18	M	H.S.P.	Diffuse proliferation; Small crescents	Progressive disease, 3 months	26 (discontinued)	+
2	14	M	H.S.P.	Focal proliferation	Recurrent haematuria	4 (discontinued)	+
3	16	F	S.L.E.	Diffuse proliferation; Crescents	Progressive disease, 4 months	2 (discontinued)	+
4	63	M	P.S.G.N.	Diffuse proliferation; Exudative	Progressive disease, 4 months	60 (continuing)	+
5	42	M	Membrano-proliferative	Some glomerular obliteration	Progressive disease, 4 years	66 (continuing)	+
6	35	M	Membrano-proliferative	Crescents common	Unresolved after 5 months	75 (continuing)	+
7	46	M	Membrano-proliferative	Glomerular obliteration lobulation	Proteinuria, hypertension	18 (continuing)	+
8	31	F	Membrano-proliferative	Mesangial swelling and lobulation	Proteinuria, haematuria, 8 years	4 (discontinued)	-
9	68	M	Membrano-proliferative	Marked glomerular obliteration	Progressing renal failure; Nephrotic	2 (died)	-
10	23	M	Proliferative	Diffuse proliferation; Crescents; obliteration	Progressing renal failure; Nephrotic	2 (dialysis)	-
11	23	M	Proliferative	Diffuse proliferation; crescents; obliteration	Progressing renal failure	2 (dialysis)	-
12	21	M	Proliferative	Diffuse proliferation; Exudative	Persistent proteinuria; haematuria	52 (continuing)	+
13	19	F	Proliferative	Focal proliferation	Recurrent haematuria	10 (discontinued)*	+
14	27	M	Proliferative	Diffuse proliferation	Progressive disease, 3 months	32 (continuing)	+

Table 21.

Group C. Prednisolone and Indomethacin

Case No.	Age	Sex	Diagnosis	Biopsy Features	Clinical Features	Duration of Therapy (weeks)	F.D.P. Response
3	16	F	S.L.E.	Diffuse proliferation; crescents	Progressive disease (see Fig. 94)	52 (continuing)	+
29	25	F	S.L.E.	Diffuse proliferation; crescents	Progressive disease; crescents	14 (continuing)	+
31	38	M	H.S.P.	Diffuse proliferation; crescents	Progressive disease; nephrotic, 1 month	16 (discontinued)*	+
32	16	F	H.S.P.	Diffuse proliferation; crescents; sclerosis	Rapidly progressive disease	4 (continuing)	+
17	59	M	Proliferative	Diffuse proliferation; crescents	Progressive disease; ?polyarteritis	5 (discontinued)	+

Group D. Aspirin Alone

Case No.	Age	Sex	Diagnosis	Biopsy Features	Clinical Features	Duration of Therapy (weeks)	F.D.P. Response
33	32	M	Proliferative	Focal proliferation	Recurrent haematuria	8 (discontinued)	+
34	45	F	Proliferative	Focal proliferation	Proteinuria; recurrent haematuria	4 (discontinued)	+
35	70	M	S.L.E.	Diffuse proliferation	Progressive disease; nephrotic	64 (continuing)	+
20	19	M	Proliferative	Diffuse proliferation; crescents	Fabrey's disease	8 (discontinued)	-

+ = Significant fall in urine F.D.P. concentration after treatment
- = No change in urine F.D.P.
* = Drug discontinued because of side effects
H.S.P. = Henoch Schönlein Purpura
P.S.G.N. = Post-streptococcal glomerulonephritis

drug withdrawal (1). These details are represented in Group A of Table 21. Twelve patients received prednisolone alone for between 4 and 40 weeks (Group B, Table 21) in doses varying between 15 and 90 mg/day. Indomethacin 75 mg/day was added to the prednisolone therapy in 5 of these when it was found that urinary F.D.P. excretion was not being controlled by the dose of prednisolone currently employed (Group C, Table 21). Aspirin alone (1200-1800 mg/day) was given to four patients (Group D, Table 21) in three for periods between 4 and 8 weeks and the fourth for 64 weeks.

RENAL FUNCTION: Renal function was assessed periodically during the course of therapy by measuring blood urea concentration, creatinine clearance and 24 hour urinary protein excretion.

F.D.P.: Urine F.D.P. concentration was measured on consecutive daily early morning urine specimens in all patients for periods no shorter than 14 days prior to drug administration, and throughout the period of study. In 20 such patients, serum F.D.P. were measured daily before and after starting therapy.

CHROMATOGRAPHY: Concentrated urine specimens from patients with "active" proliferative glomerulonephritis collected during therapy with indomethacin (6), prednisolone (2) and aspirin (1) were subjected to column chromatography in 8% agarose. Individual eluates were analysed by the T.R.C.H.I.I. and compared with those analysed from the same patients before treatment.

CONTROL STUDIES: In 17 healthy subjects, daily studies of fibrinolysis were performed before, during and after the administration of

indomethacin 75 mg/day in 7, aspirin 1200 mg/day in 7, and prednisolone 30 mg/day in 3. Each subject was studied for 4-5 days prior to taking the drug, for 4-10 consecutive days during therapy and for 4-7 days after the drug was stopped. The following indices were measured: serum and urinary F.D.P., plasma plasminogen activator, urinary plasminogen activator, and the percentage release of PF_4 from platelet rich plasma following the addition of 5 M A.D.P. Results for each subject were expressed as a percentage of the mean values obtained during the pre-drug period.

5.2 RESULTS

5.2.1 F.D.P.

In 14 of the 26 treated with indomethacin alone, 4 of the 10 with prednisolone alone, and 3 of the 4 treated with aspirin alone, there was a sudden fall of urine F.D.P. concentration which occurred within 2 to 3 days of commencing therapy (Fig. 93). This reduction was drug dependent as shown in Fig. 93 and did not depend on the excretion of the drug into the urine as indomethacin, aspirin or prednisolone placed in the urine *in vitro* caused no alteration in F.D.P. concentration. In all patients who showed such a response to indomethacin and aspirin the urinary F.D.P. concentration was maintained consistently below 1.0 g/ml while therapy was continued. In 6 patients treated with prednisolone alone recurrence of increased F.D.P. excretion coincided with reduction in dosage. In these cases, increasing the dose of prednisolone or introducing indomethacin caused prompt reduction of

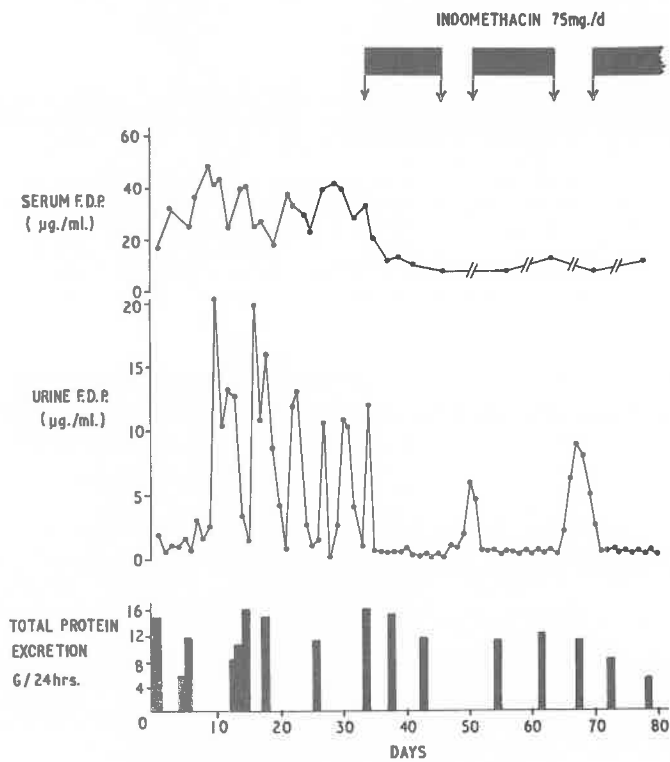

Fig. 93. Daily serum and urinary F.D.P. concentrations, and random 24 hour urinary protein excretions before, during and after three separate courses of indomethacin in Case 6, a 35 year-old man with membrano-proliferative glomerulonephritis.

urinary F.D.P. concentration (Group C, Table 21) which was maintained during the period of treatment (Fig. 94). In four instances (Cases 21-24) where there was no response to indomethacin therapy urine F.D.P. was significantly reduced in concentration after 10-21 days of prednisolone at a time coinciding with diuresis, loss of oedema and reduction of proteinuria. Cases 23 and 24 had minimal lesion and in Cases 21 and 22, while definite diffuse proliferative features were identified histologically, the proteinuria was of a highly selective nature. This gradual reduction in urinary F.D.P. was thought to reflect changes in glomerular permeability and was different from the sudden reduction described above.

Twelve of the 26 patients in Group A, 4 in Group B and 1 in Group D, showed no change in urine F.D.P. concentration with therapy. In 4 patients on prednisolone (Cases 3, 17, 31 and 32) this may have resulted from the employment of a dose (15-40 mg/day) too low to effect a response, as the initial dose used in those who did respond was above 50 mg/day in each case. Of the 12 patients in whom reduction did occur with indomethacin or aspirin, three developed terminal renal failure soon after therapy was commenced (Cases 9, 10 and 11). One had membranoproliferative glomerulonephritis (Case 8) and the others had forms of glomerulonephritis different in both clinical and pathological expression from those who did respond (Cases 19-26).

Serum F.D.P. was measured serially in eleven patients in whom reduction of urinary F.D.P. was associated with treatment. There was significant reduction in serum F.D.P. concentration in 5 (Fig. 93) while

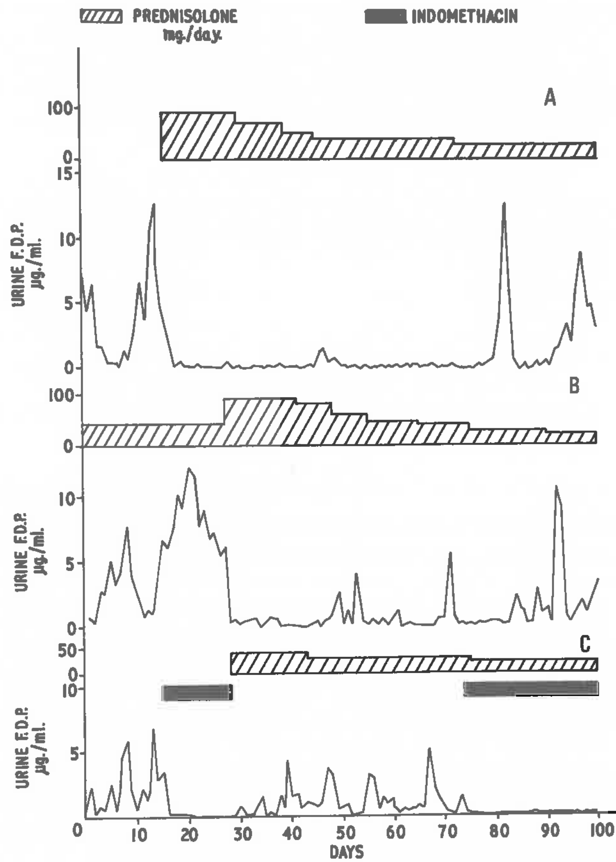

Fig. 94. Urine F.D.P. concentrations in relation to treatment in cases 27 (A), 29 (B) and 3 (C), each of whom had diffuse proliferative glomerulonephriti associated with S.L.E.

no change occurred in the remainder who maintained normal serum concentrations throughout.

5.2.2 CHROMATOGRAPHY

In "active" proliferative glomerulonephritis prior to treatment the excreted fibrin derivatives consisted predominantly of the plasmin-resistant fragments D and E. The rapid fall in urine F.D.P. following therapy was due to disappearance of these fragments, so that the elution profile was similar to that of minimal lesion and membranous glomerulonephritis, and consisted of fibrinogen, fragments X, Y and small quantities of D (Fig. 95).

5.2.3 RENAL FUNCTION

Prior to treatment creatinine clearance had been deteriorating over varying intervals in 63% of the patients studied (70% in F.D.P. responders, 50% in non-responders) and was stable in the remainder.

The mean changes in proteinuria, blood urea concentration and creatinine clearance after 2 weeks and 6 months are shown in Table 22, where F.D.P. responders and non-responders are separated. There was a significant reduction in proteinuria in patients where rapid F.D.P. reduction was observed. The fall in protein excretion occurred within the first few days of therapy, was more pronounced after 6 months, and was not associated with a change in protein selectivity. In the 6 patients followed for 12 months or longer, proteinuria remained at low levels. The blood urea concentration often rose soon after

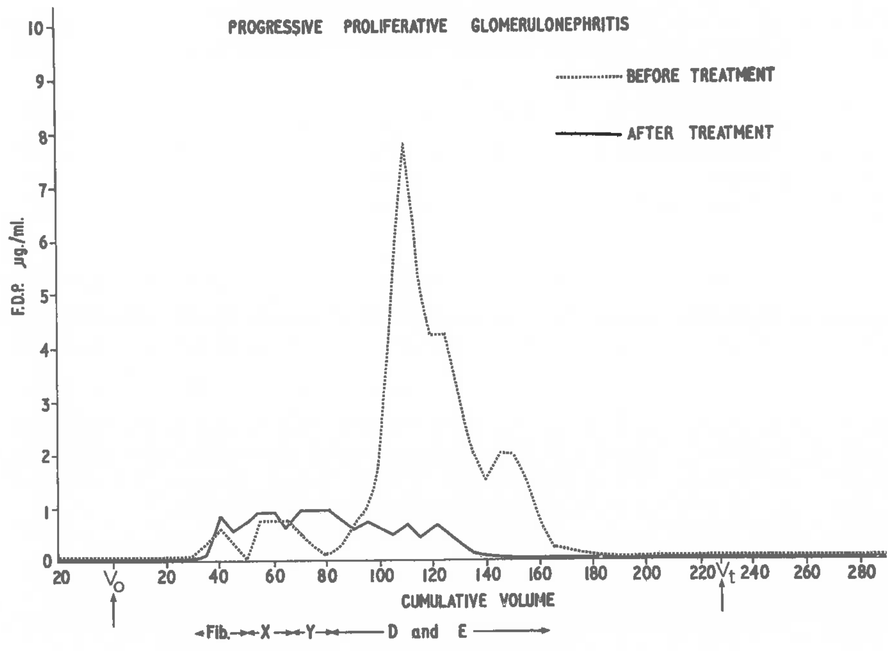

Fig. 95. Chromatography of urine before and after therapy with indomethacin in Case 17.

Table 22.

Mean and standard deviation of protein excretion, blood urea and creatinine clearance before and after treatment at 2 weeks and 6 months. Patients receiving prednisolone are not included and the short term data of those receiving drugs as outpatients were not recorded.

Renal Function in Patients Treated with Indomethacin and Aspirin

Short Term (2 weeks) 21 Patients

	Proteinuria (G/24hrs)		Blood Urea (mg/100ml)		Creatinine Clearance (ml/min)	
	Before	After	Before	After	Before	After
All (21 Patients)	6.77 ± 5.52	4.65 ± 6.35	80.4 ± 45.7	103.4 ± 64.9	43.5 ± 26.6	42.4 ± 27.1
	$p < 0.3 > 0.20$		$p < 0.2 > 0.1$		$p > 0.9$	
F.D.P. Responders 13 Patients	5.87 ± 4.30	2.38 ± 3.75	83.9 ± 46.3	91.0 ± 43.2	48.9 ± 25.3	47.2 ± 24.5
	$p = 0.05$		$p < 0.7 > 0.6$		$p > 0.9$	
F.D.P. Non Responders 8 Patients	8.23 ± 6.60	8.34 ± 8.12	74.8 ± 47.1	123.6 ± 89.9	36.3 ± 27.9	31.8 ± 29.3
	$p > 0.9$		$p < 0.3 > 0.2$		$p < 0.9 > 0.8$	

Long Term (6 months) 15 Patients

	Proteinuria (G/24hrs)		Blood Urea (mg/100ml)		Creatinine Clearance (ml/min)	
	Before	After	Before	After	Before	After
All (15 Patients)	6.23 ± 5.17	1.65 ± 3.79	68.4 ± 44.8	60.1 ± 40.8	57.9 ± 31.0	72.9 ± 42.0
	$p < 0.2 > 0.1$		$p < 0.7 > 0.6$		$p < 0.3 > 0.2$	
F.D.P. Responders 13 Patients	6.00 ± 4.74	0.72 ± 1.15	67.6 ± 43.8	56.9 ± 36.3	59.9 ± 30.3	77.9 ± 41.2
	$p < 0.01 > 0.001$		$p < 0.6 > 0.5$		$p < 0.3 > 0.2$	

commencing treatment, but by 6 months had returned to values at or below pre-treatment levels in those showing F.D.P. response. Creatinine clearance remained stable or slightly improved. Insufficient numbers of F.D.P. non-responders have been studied for 6 months to make a valid comparison between these groups.

5.2.4 CONTROL STUDIES

Normal serum F.D.P. concentrations and negligible urinary F.D.P. concentrations were found throughout the period of study in all healthy subjects and there was no change during drug administration. Serial changes in plasma activator concentration and urinary activator excretion rate for all subjects receiving indomethacin are presented in Fig. 96 and are representative of the changes occurring in those treated with aspirin and prednisolone. The alterations occurred in parallel but those of urinary activator were more marked and consisted of an increase in the first day of therapy followed by a decrease in the next 2 days. Thereafter, the levels regained control values where they remained until cessation of treatment, when a reverse of the pattern observed in the first 3 days of therapy was seen. During therapy with indomethacin and aspirin, PF_4 was not released from platelet rich plasma after stimulation with $5\mu M$, A.D.P., *in vitro*. This concentration of A.D.P. was known to provoke PF_4 release in all subjects prior to treatment. However, similar or increased amounts were released in those taking prednisolone.

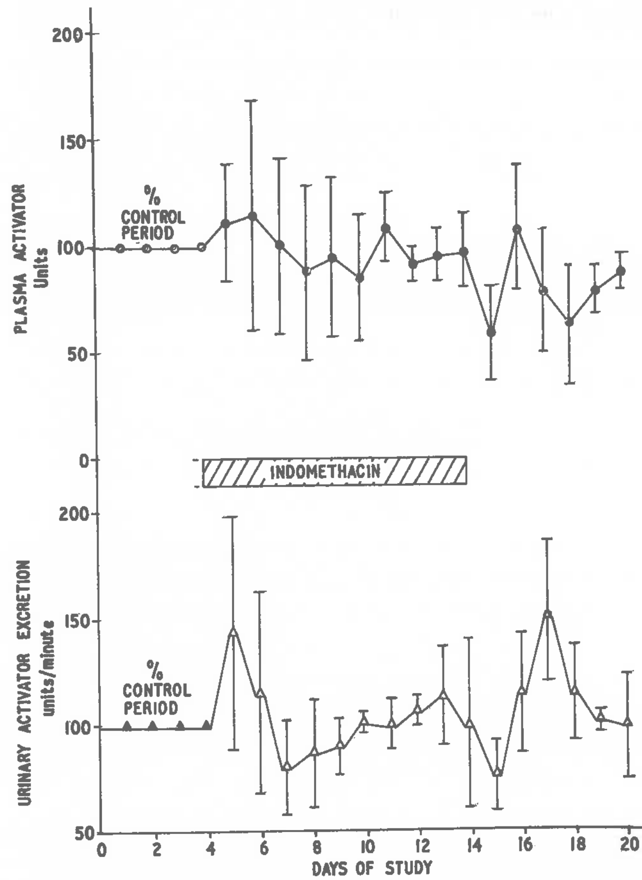

Fig. 96. Means and standard deviations of the percentage changes in plasma activator content and urinary activator excretion rate in 7 healthy subjects during and after therapy with indomethacin.

5.3 DISCUSSION

Urinary F.D.P. excretion in glomerulonephritis is dependent on glomerular damage and may result from local inflammation and/or changes in the glomerular capillary basement membrane permeability to plasma proteins. In proliferative glomerulonephritis F.D.P. are excreted in amounts proportional to the degree of intraglomerular fibrin deposition and the "activity" and severity of disease rather than the degree of proteinuria. In minimal lesion and membranous glomerulonephritis, F.D.P. excretion is proportional to the degree of proteinuria and is thought to result from increased glomerular permeability to circulating fibrinogen. Reduction in F.D.P. excretion after treatment may thus represent a decrease in either glomerular inflammation or permeability.

Analysis of the changes after indomethacin, aspirin and prednisolone suggests that each drug is capable of lowering urinary F.D.P. excretion within two to three days in a large proportion of patients (63% in this study) with proliferative forms of glomerulonephritis. Moreover, this low excretion was maintained in all patients who continued therapy with indomethacin (75 mg/day) or aspirin 1200 mg/day). Reduction in the dose of prednisolone, however, was associated with a return of significant F.D.P. excretion which in turn could be suppressed by increasing the dose or by introducing indomethacin. Furthermore, in three patients who showed no F.D.P. decrease after 30-40 mg/day of prednisolone, addition of indomethacin caused prompt reduction. In terms of F.D.P. response to prednisolone therefore, there seems to be a clear dose-response relationship which varies from one

individual to another and which was not encountered with the small doses of indomethacin and aspirin employed. This was seen most strikingly in patients with glomerulonephritis associated with S.L.E. and confirms the long-held clinical impression that there is an optimal dose for each patient below which signs of inflammation reappear. It may also explain why high dose steroid therapy has been found more beneficial than low doses in S.L.E. glomerulonephritis (Pollak et al, 1961), and why no apparent benefit accrued to patients with proliferative glomerulonephritis in the M.R.C. controlled trial of prednisolone in which the average dose was 20-30 mg/day (Black et al, 1970).

Fractionation studies indicate that this acute reduction in urinary F.D.P. is due to the disappearance after therapy of the smaller molecular weight fragments D and E. Similar qualitative data were obtained by Vermylen et al (1970) who found that F.D.P. were detected in pre-treatment urine samples by both the staphylococcal clumping test (S.C.T.) and the T.R.C.H.I.I. whereas only the S.C.T. detected F.D.P. in post-treatment specimens. The S.C.T. is insensitive to the lower molecular weight fragments D and E (Allington, 1967; Lipinski et al, 1967; Hawiger et al, 1970; Thomas et al, 1970; Leavelle et al, 1971; Marder et al, 1971). The high concentrations of D and E before treatment are thought to be due to lysis and subsequent excretion of the intraglomerular fibrin deposits, but there are several possible explanations for their absence after therapy. On the basis of the findings in healthy subjects, it seems unlikely that indomethacin, aspirin or prednisolone (contrary to the findings

of Isaacson, 1970) cause long-term depression of fibrinolysis, although these studies did indicate an acute biphasic effect on plasma and urinary activator content. It is also unlikely that these drugs influence the glomerular permeability to D and E in proliferative glomerulonephritis, as fractionation of urine after treatment revealed the presence of fibrinogen and the higher molecular weight products in a proportion similar to those observed in untreated minimal lesion and membranous glomerulonephritis. Correction of the permeability defect by prednisolone therapy in two cases of minimal lesion and two cases of proliferative glomerulonephritis with highly selective proteinuria caused gradual reduction in urinary F.D.P. over the course of three weeks; a pattern not observed in the other cases of proliferative glomerulonephritis. The investigations of Michielsen et al (1969) indicate that histological evidence of glomerular inflammation is reduced in patients taking indomethacin. After limited personal experience with post-treatment biopsies, this finding is supported. Moreover, electron microscopy reveals a lack of glomerular capillary fibrin (Figs. 97 and 98). Available evidence, therefore, points to inhibition of intraglomerular fibrin formation as the basis of the sudden reduction of urinary F.D.P. excretion.

Indomethacin inhibits chemotactic neutrophil migration (Phelps and McCarty, 1967), the haemolysis of red cells following various insults (Brown and Mackey, 1968), the phagocytosis of polystyrene latex particles by human leukocytes (Kvarstein and Stormoken, 1971), the histamine induced contraction of smooth muscle (Northover, 1971)

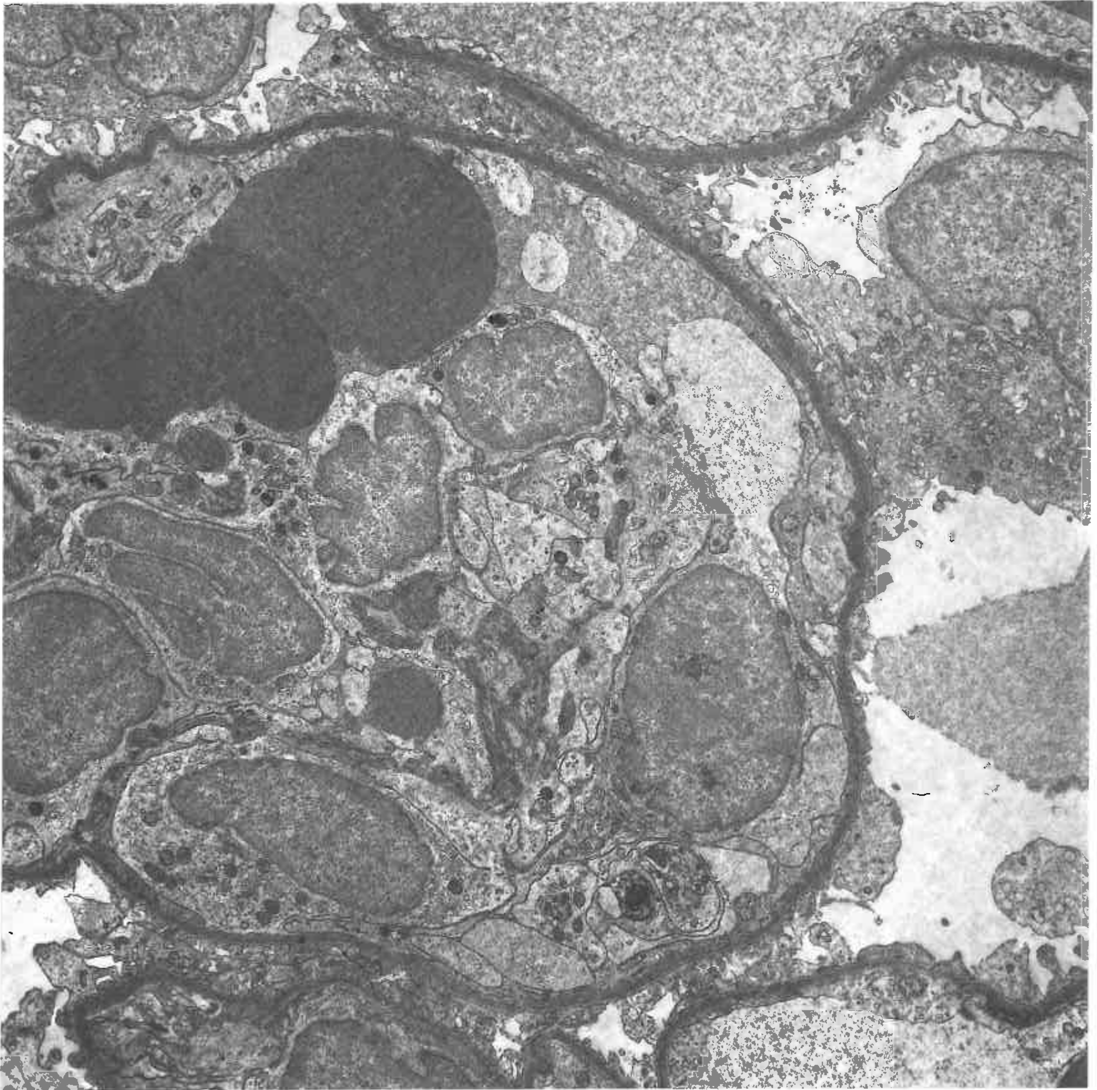

Fig. 97. Electron micrograph of a glomerulus from Case 4, a 63 year-old man who had persistent haematuria, proteinuria, oedema and progressive azotaemia for 4 months after an episode of acute post-streptococcal glomerulonephritis. The entire capillary is occluded by proliferating endothelial cells, fibrin and entrapped erythrocytes. (Case 4; x 3,000)

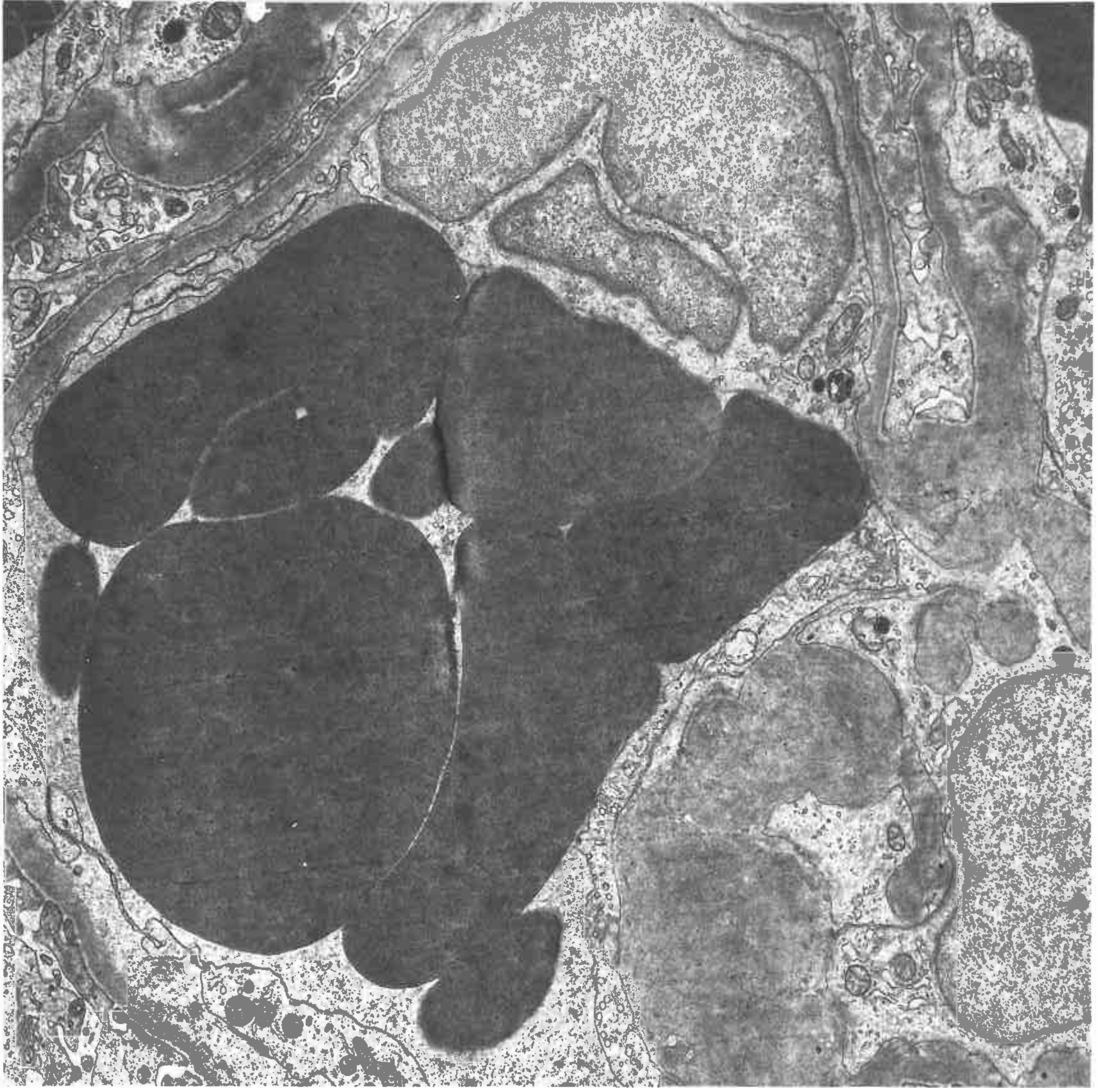

Fig. 98. Glomerular capillary from the same patient as Fig. 97, four weeks after commencing indomethacin. The endothelial cell proliferation and fibrin deposition is reduced. The reason for the intracapillary erythrocyte accumulation which was seen in each glomerulus examined is not clear. Fibrin was still present, however, in mesangial regions. (Case 4; x 5,000)

and causes salt and water retention. A common mechanism at the cellular level such as the inhibition of prostaglandin synthesis (Vane, 1971) may account for these diverse actions and for those of aspirin, and for the inhibition of calcium uptake into the cell which may alter intracellular enzyme function (Northover, 1971). It is, however, the effect on platelet aggregation which may be of vital importance to the reduction of urinary F.D.P. excretion (Vermylen et al, 1970). Adrenaline, A.D.P. and collagen fail to aggregate *in vitro* platelets of subjects taking indomethacin or aspirin (O'Brien et al, 1970; Zucker et al, 1970) possibly because of inhibited prostaglandin production within the platelets (Smith and Willis, 1971). The platelet release reaction, during which the procoagulant platelet factors 3 and 4, histamine and serotonin are liberated is thus inhibited and the tendency for platelet aggregates to form a nidus for fibrin formation within glomeruli prevented. Subsequent lysis and excretion does not, therefore, occur. The finding of absent PF_4 release during therapy supports this hypothesis.

However, it is difficult to reconcile the actions of indomethacin and aspirin with those of prednisolone which is not known to interfere with platelet function *in vitro*, and it may be that the anti-inflammatory actions of these drugs are based on as yet unknown mechanisms.

When reduction in urinary F.D.P. concentration was sustained for 6 months or longer there was a significant diminution of proteinuria and maintenance of stable and sometimes improved renal function. The reduction in proteinuria occurred within the first three days in the

majority of cases, but was often associated with an acute rise in blood urea concentration although the creatinine clearance remained stable. However, if therapy was continued these features resolved and by 6 months blood urea concentrations were at or below pre-treatment values. Despite the initial rise in blood urea no significant alteration in creatinine clearance occurred.

While these observations are of interest to nephrologists treating patients with glomerulonephritis, they should be interpreted with considerable caution as many treatments for kidney disease have failed to stand the test of controlled study. Many of the patients described were indeed selected for study on the basis of abnormal urinary F.D.P. excretion and a comparably large group of untreated patients have not been observed. Moreover, the length of study is too short for any positive effect on the natural history to be noticed. On the other hand, persistance of abnormally high urinary F.D.P. excretion is associated with a poor prognosis in proliferative glomerulonephritis and homotransplantation (see Section 6), and measures taken to lower it may be beneficial. Although recourse to controlled clinical trial is necessary for full evaluation of indomethacin and aspirin, some substance may be given by these studies, to the reported beneficial effects claimed for indomethacin (Michielsen et al, 1969) and anti-coagulants (Kincaid-Smith, et al, 1970) on proliferative glomerulonephritis. They may, furthermore, point to the disease entities where particular attention should be focussed e.g. Henoch Schönlein purpura,

systemic lupus erythematosus, and progressive unspecified proliferative glomerulonephritis. In these conditions F.D.P. reduction occurred in the majority of cases in this study. In addition 3 of the 5 patients with membranoproliferative glomerulonephritis showed a similar response. Moreover, as over one third of the total population of patients did not show a response to any drug, a controlled trial may be weighted heavily by this factor and the possible beneficial effects recorded in those responding disguised. A more practical approach may be to conduct trials on known responders.

Of equal importance may be the finding that the anti-inflammatory drugs used had no effect on the F.D.P. excretion in a significant proportion of patients with glomerulonephritis. This was perhaps not surprising in minimal lesion and membranous glomerulonephritis, but raises the possibility that the coagulation mechanism may be activated by different means in individual cases of proliferative glomerulonephritis. In such cases anti-coagulation may produce the desired response. Unfortunately there are no means presently available whereby these groups may be identified apart from trial and error.

Finally, and most unequivocally, it has been demonstrated that urine F.D.P. concentration can be lowered by several therapeutic agents, some of which have only minor adverse effects. A strong case is thus made out for the continued evaluation of drugs in glomerulonephritis using serial measurements of urinary F.D.P. by the T.R.C.H.I.I.

Section 6.

FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS FOLLOWING RENAL HOMOTRANSPLANTATION

6.1 INTRODUCTION

Although functional and pathological changes in human renal allografts have an immunological basis, the mechanisms of tissue injury are incompletely understood. Early experience with the pathology of both human and animal grafts led to the recognition of the predominance of mononuclear cell infiltrations of interstitial tissue and much early work was concentrated on furthering the understanding of the host's cellular response to the graft and its suppression. The successful containment of the cellular response in the human and experimental situation by what may now be regarded as conventional immuno-suppressive measures, provided the impetus for large-scale clinical renal transplantation. At the same time, the striking vascular lesions, whose significance had been obscured by the attention paid to cellular mechanisms of injury, assumed greater prominence. Detailed studies of canine grafts by Altman (1963), Porter et al (1964), Murray et al (1964) and Horowitz et al (1965) and human grafts (Porter et al (1963), Dempster et al (1964), Hamburger et al (1965) and Kincaid-Smith (1967), in which gross obliterative vascular lesions were described, further showed that the ultimate fate of the graft may depend on these lesions induced by humoral rather than cellular means. Moreover, the detection of pre-existing humoral antibodies against donor cells in cases of hyper-acute rejection (Kissmeyer-Nielsen et al, 1966) clearly demonstrated the importance of humoral immunity in graft rejection. Unfortunately, circulating antibodies active against

donor cells are rarely found after transplantation and the relative contributions of cellular and humoral immunity to the ultimate rejection of the graft can only be implied by evaluation of such imprecise parameters as histological changes, tissue typing, mixed lymphocyte culture and the clinical response to various therapeutic agents, the actions of which are still not completely understood.

As seen in clinical practice, rejection has been arbitrarily divided into three fairly distinct categories which may however merge in individual cases.

1. Hyperacute rejection which occurs within hours of anastomosis and results in primary graft failure. The morphological consequence of this reaction is massive destruction of graft vasculature (Kissmeyer-Nielsen et al, 1966; Williams et al, 1968; Starzl et al, 1968; Myburgh et al, 1969) in which fibrin and platelet thrombi are deposited in amounts sufficient to cause physical obstruction to blood flow and cortical necrosis. Although infiltration with polymorphs is often a feature, round cells are not prominent.

2. Acute rejection episodes are usually seen within the first 6-12 weeks after transplantation and are characterised by acute deterioration in a graft previously functioning satisfactorily. Histological examination reveals acute vascular injury which may be present in the vessels from the anastomosis to the renal glomerulus and intertubular venules and capillaries. It is most prominent, however, in the cortical arteries and arterioles and consists of fibrinoid necrosis within the media, endothelial proliferation and accumulation of foamy lipophages within the intima, occasional luminal thrombosis and

infiltration with polymorphonuclear leukocytes. The acute glomerular lesions may be of similar quality and severity. Acute tubular necrosis and interstitial oedema are frequent, but round cell infiltration is variable. The response to treatment with conventional immunosuppressive agents is usually satisfactory, but is not uniformly good.

3. Chronic rejection is the slow process of destruction occurring in most grafts which eventually leads to organ failure. It is variable in its time of clinical expression after grafting, but may be found in biopsy specimens long before deterioration in renal function occurs. Many of the histological features are thought to develop as a result of repeated small episodes of acute vascular injury. Thus organisation of mural and intraluminal thrombi eventually leads to markedly hyperplastic arteries and arterioles with diffuse or focal thickening of the intima by very cellular fibroblastic tissue; medial atrophy and fragmentation of the internal elastic lamina. Glomerular changes are similarly sclerotic in nature, and consist of diffuse thickening of the glomerular basement membrane which seems to result from organisation of material deposited in the subendothelial regions of the glomerular capillary, and from expansion and hypertrophy of mesangial areas. Polymorph infiltration is not a prominent feature and round cell infiltration is variable throughout the interstitium. Tubular changes are in keeping with the degree of glomerular obliteration.

There is considerable evidence now available which suggests that the pathogenesis of vascular lesions associated with graft rejection is based on abnormal coagulation and platelet function. Recently, a

careful analysis of the role of vascular injury in early graft failure by Busch et al (1971) has strengthened the hypothesis presented by Kincaid-Smith (1970) that thrombosis and residual lesions following organisation of thrombi are the major changes in vessels and glomeruli that lead to graft failure. Furthermore, it has been claimed that these lesions may be considerably modified by anticoagulant and anti-thrombotic therapy (Kincaid-Smith, 1970). If this claim is confirmed by data obtained by controlled clinical trial this postulated mechanism of pathogenesis will be further substantiated.

For some time it has been recognised that the earliest signs of rejection are accompanied by a diminished renal blood flow (Kountz et al, 1963; Lewis et al, 1968). This is reflected by changes in urinary urea and osmolar excretion, elevation of blood urea and fall in creatinine clearance. In some cases significant proteinuria may develop. These tests provide the most consistently reliable indices of rejection, but all are subject to wide variation with diet, degree of hydration and in many instances may be non-specifically altered by such insults as infection and drug administration. The diagnosis of rejection can be based on functional changes, or on the histological changes seen on renal biopsy. However, biopsy cannot be regarded as a routine investigative tool, and is often performed only when functional changes have been observed. Moreover, histological changes do not necessarily correlate with function (Ogden, 1967). Many other tests for detecting rejection episodes which involve enzyme, radiological, radio-isotope and clearance techniques are non-specific, time-consuming and unsuitable

for daily use, and not readily available. Investigative tools yielding more precise and specific information and which cause no inconvenience to the patient are urgently required.

Using the techniques of immuno-diffusion and immuno-electrophoresis, fibrin degradation products have been detected in the urine of patients undergoing transplant rejection (Braun and Merrill, 1968; Antoine et al, 1969). However, the low sensitivity of the methods used and the inability to adapt them to quantitate the material measured severely limits their practical application. The T.R.C.H.I.I., on the other hand, fulfils these requirements and was used in the present investigation.

6.2 PATIENTS AND METHODS

6.2.1 PATIENTS

Fifty-one patients have been studied. Three received a kidney from living donors and forty-eight from cadaver sources. Immunosuppressive therapy consisted of prednisolone and azathioprine in conventional doses. Acute rejection crises were treated by increasing the dose of prednisolone, and with actinomycin C in a dose of 200 mg immediately and repeated after 4 days. In addition, 8 patients received heterologous anti-lymphocyte serum (A.L.S.), and five had anticoagulant therapy during the period of investigation. Thirty-three patients were studied daily from the time of transplantation until discharge. These, along with eighteen patients who had received grafts between 3 months and 8 years before this investigation began were followed as outpatients

at 1-4 weekly intervals. From this group twelve were selected for continuous daily estimations on early-morning urine samples delivered to the laboratory by post.

In the first 16 patients, serum samples were obtained daily during the period of hospitalization which varied from 16-51 days.

6.2.2 METHODS

Urine and serum samples were prepared and analysed as previously described using the T.R.C.H.I.I. Unlike the studies on glomerulonephritis where the patient's condition and therapy were personally known, in this study all assays were performed without knowledge of the patients' condition. Clinical rejection was diagnosed and treated independently by members of the Nuffield Transplantation Unit's clinical staff. This unit is based in the Western General Hospital, 3 miles distant from the Medical Renal Unit at the Royal Infirmary in Edinburgh. The diagnosis was based on clinical examination and observation of trends in the serum creatinine, creatinine clearance, blood urea, total urinary proteins, urine sodium content, and urine volume.

6.3 RESULTS

6.3.1 SERUM F.D.P.

Serum F.D.P. concentrations were commonly elevated in the post-operative periods but became normal in all patients as the grafts began to function. In the 16 patients in whom serial measurements were made, 6 episodes of clinical rejection were diagnosed. In 3

of these (50%) a rise in serum F.D.P. concentration above normal occurred, the maximum recorded figure being 54.4 μg/ml. In the other 3 and the remaining 10 patients in whom no rejection episode was diagnosed during the period of study, serum F.D.P. concentrations remained normal once graft function was established.

6.3.2 URINARY F.D.P.

a. INPATIENT SERIES: All patients receiving cadaver organs experienced a variable period of post-operative oliguria. The urine produced during the immediate post-operative period contained large amounts of F.D.P., probably due to contamination with blood. Substantial quantities of F.D.P. remained once haematuria had cleared however, while the oliguria persisted, but the onset of spontaneous renal function was always accompanied by a significant elevation of urinary F.D.P. excretion, over and above these values. The previous investigations (Section 3) of urinary F.D.P. excretion in acute ischaemic renal failure suggest that this post-operative elevation, and the elevation seen as the homograft begins to function is largely due to peri-operative ischaemia.

After this early post-operative response, all episodes of acute clinical rejection were associated with a significant increase in F.D.P. excretion which occasionally clearly preceded clinical detection by more than 24 hours. Figs. 99 and 100 illustrate these 2 sorts of F.D.P. rise associated with peri-operative ischaemia and rejection. Following successful reversal of rejection, F.D.P. excretion returned

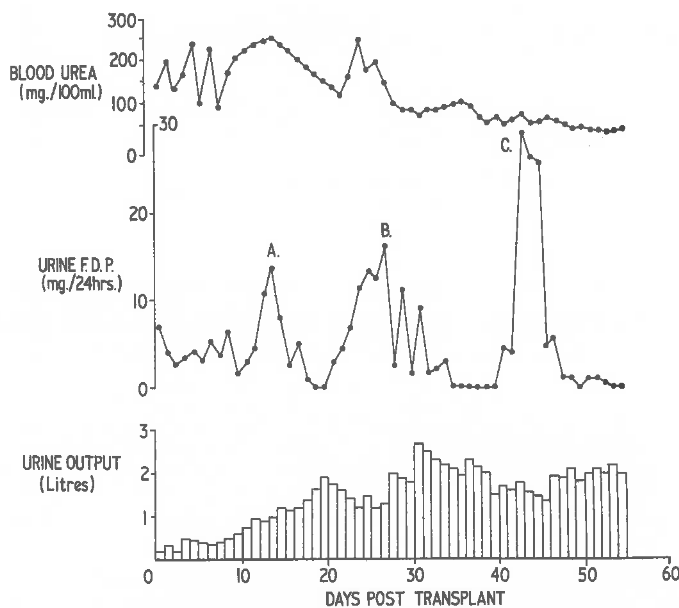

Fig. 99. Urine volume, urine F.D.P. and blood urea changes in a patient studied for 55 days after cadaver renal transplantation. Urinary F.D.P. elevation A represents that associated with recovering renal tubular necrosis, B an episode of clinically diagnosed acute rejection, and C a response which followed 12 days of A.L.S. administration.

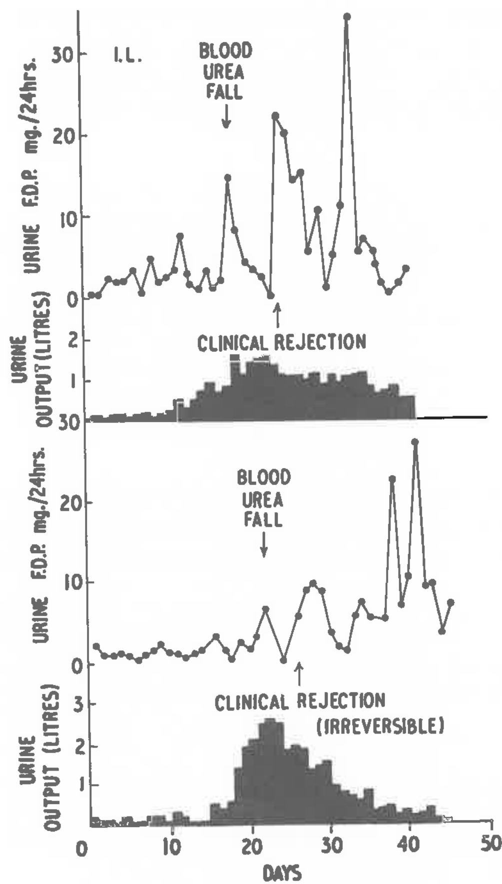

Fig. 100. Elevated urine F.D.P. concentrations were observed in these 2 cases associated with acute tubular necrosis, and with acute rejection. Both episodes of rejection responded poorly to therapy, and F.D.P. concentration remained elevated.

to low levels, but high values were maintained if renal function continued to deteriorate. Apart from the rise in urinary F.D.P. excretion associated with clinically-identified rejection, elevations were seen in 2 other circumstances. The first of these occurred for no apparent reason, was unrelated to infection or change in therapy and was usually short-lived and apparently self-limiting (Fig. 101). The only interpretation seemed that these elevations represented acute, spontaneously reversible episodes of rejection which did not reach clinical significance. Such changes were seen in 27 out of the 33 (82%) patients studied in this acute phase. The second was seen in 8 patients and occurred 10-14 days after the commencement of an intravenous course of A.L.S. Examples are illustrated in Fig. 99 (peak C) and Fig. 102, and are representative of the changes which occurred in the 6 other similarly treated patients. On each occasion the F.D.P. excretion reached considerable proportions, but lasted for only 10-15 days and was followed by a period of low F.D.P. excretion. The elevation coincided in each case with the detection of circulating immune complexes related to the equine serum (James et al, 1971).

In this series of inpatients, five were treated with anticoagulant therapy as an adjunct to conventional immuno-suppression. Heparin in 2 cases and warfarin in one case were associated with a marked fall in urine F.D.P. excretion but no significant change in the pattern of F.D.P. excretion was observed in the remaining 2 patients who were treated with both drugs. The responses to heparin and warfarin are shown in Figs. 103 and 104 and lack of response with warfarin in

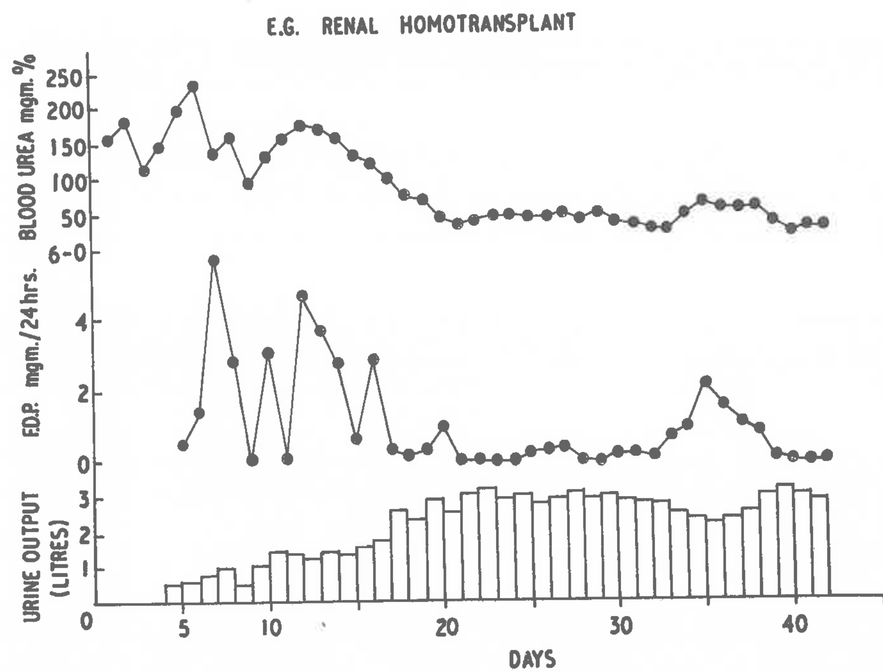

Fig. 101. Urinary F.D.P. excretion in a patient with uniform improvement in renal function after acute tubular necrosis. An unexplained but minor elevation of F.D.P. occurred between the 34th-39th post-operative days.

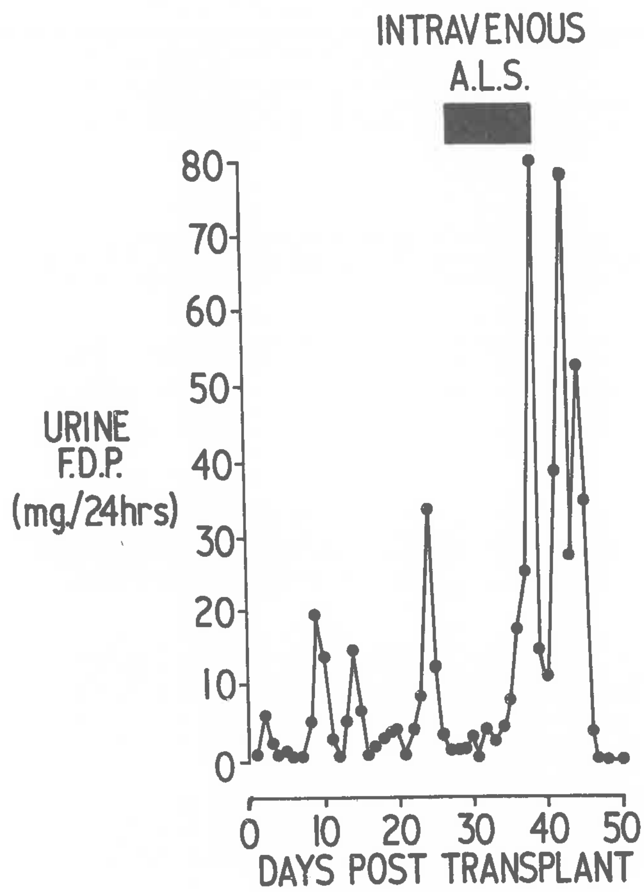

Fig. 102. A further example of the increased F.D.P. excretion which occurred 10-12 days after an intravenous course of A.L.S.

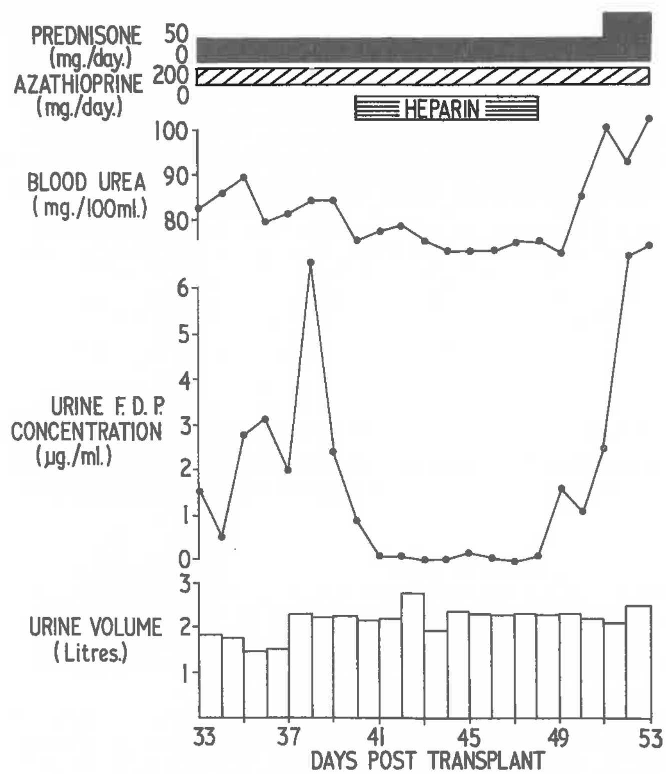

Fig. 103. The changes in urine volume, blood urea, and urine F.D.P. content before, during and after a 12-day course of intravenous heparin in a recipient of a cadaver renal allograft.

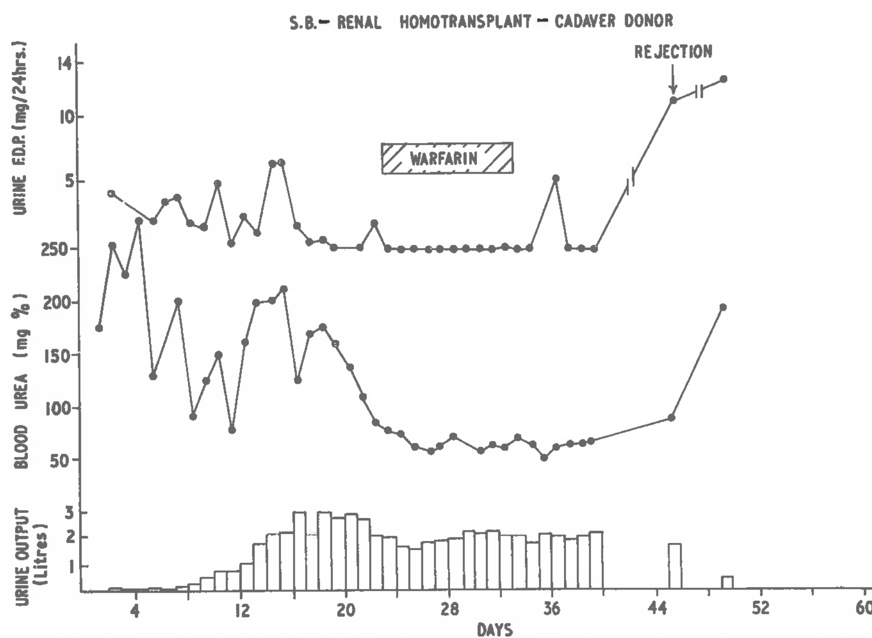

Fig. 104. Changes in urinary F.D.P. excretion, blood urea concentration and urinary output before, during and after warfarin treatment in a patient with a cadaver graft.

Fig. 107. In those who responded, withdrawal of anticoagulants was associated with an apparent rebound increase in F.D.P. excretion.

b. OUTPATIENT SERIES: In the outpatient group all episodes of clinical rejection were associated with a markedly abnormal elevation of urinary F.D.P. However, in patients seen at regular intervals, it became evident that even in those with apparently stable and often excellent graft function, significant F.D.P. excretion was periodically recorded. The frequency, magnitude and significance of these changes in patients seen so infrequently was difficult to interpret. Accordinly, 12 patients were asked to forward daily early morning urine specimens by post. Data from 4 of these patients (2 cadaver and 2 live donor grafts) with excellent stable renal function, and with one exception whose therapy did not change throughout the period of study are summarised in Fig. 105. Minor, periodic but abnormal elevations were recorded in all such patients. Figures 106 and 107 illustrate the grossly abnormal excretion of F.D.P. in 2 patients whose renal function deteriorated during the study. In the patient represented in Fig. 106 rejection was diagnosed on the basis of a rise in serum creatinine concentration from 1.0 to 2.0 mg/100 ml and a highly satisfactory functional response to treatment was observed. The increased dose of prednisolone and azathioprine apparently caused a significant reduction in urine F.D.P. excretion which was maintained for a long period thereafter. On the other hand, the patient represented in Fig. 107 underwent progressive irreversible deterioration of graft function despite intensive therapy, including warfarin, and chronic haemodialysis had to be re-instituted.

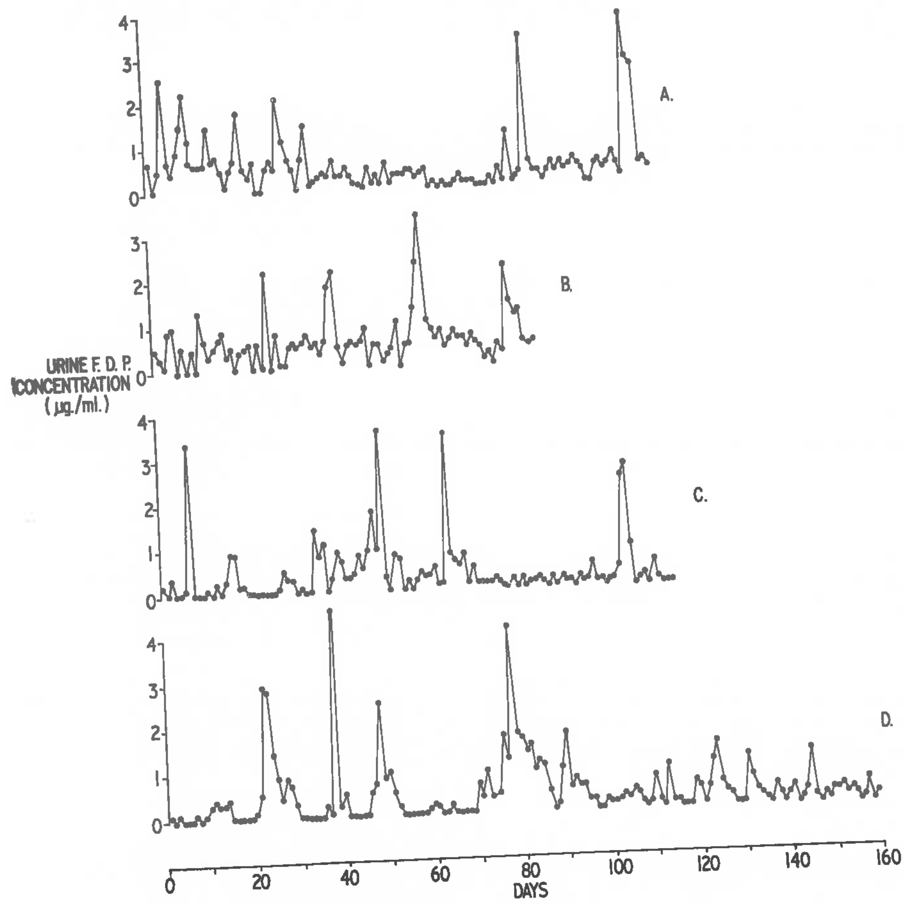

Fig. 105. Daily urine F.D.P. concentration in three outpatients (A, B and C) with stable graft function and therapy. In patient D, the azathioprine dose was reduced on day 68 and prednisolone was required to control the subsequent clinical rejection which was evident on day 80.

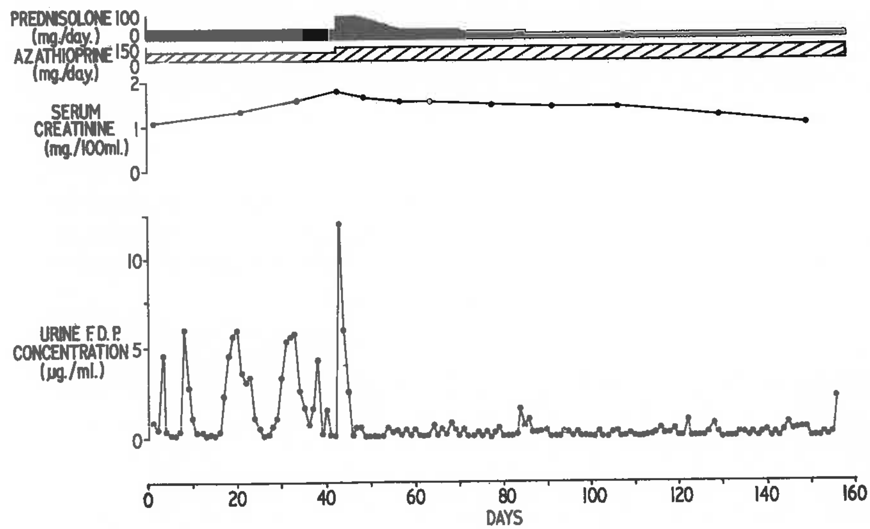

Fig. 106. Urinary F.D.P. and serum creatinine changes in an outpatient studied 11 months after receiving a cadaver renal allograft. Clinical rejection was diagnosed on day 43 of the study. The responses to increased immunosuppression are demonstrated.

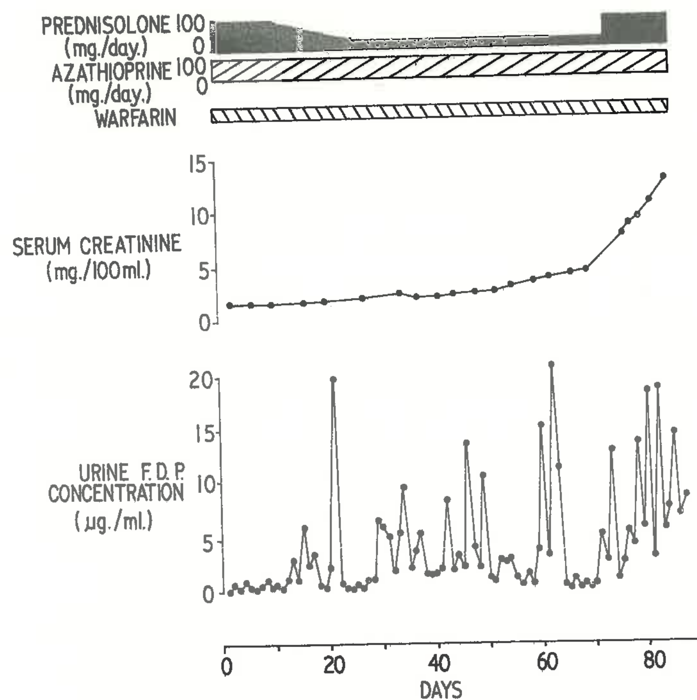

Fig. 107. Urinary F.D.P. and serum creatinine changes in a patient who had received a cadaver renal allograft 20 months previously. Despite intensive immunosuppressive therapy and oral anticoagulation irreversible rejection supervened.

The changes in urine F.D.P. content during a period of over 200 days following transplantation in a 35 year-old female recipient of a cadaver graft are illustrated in Fig. 108. The post-operative period was uneventful and graft function remained excellent despite the appearance of abnormalities similar to those shown in Fig. 105 on day 80.

6.4 DISCUSSION

It is considered that the early diagnosis of rejection is an important factor in determining the longevity of renal allograft function (Chisholm et al, 1969). However, techniques currently employed to make this diagnosis are relatively insensitive and in some instances non-specific. Considerable evidence exists that platelet aggregation and coagulation activation occurs in the foreign renal tissue soon after it is implanted, and continues throughout the life of the graft despite measures taken to prevent it. This evidence is based on the electron microscopic appearance of platelets and deposited fibrin within arterioles and glomerular capillaries, and the detection of fibrin by immunofluorescent staining, in all phases of the rejection process (McKenzie and Whitingham, 1968; Rosenau et al, 1969; Kincaid-Smith, 1970; Busch et al, 1971). There is also ample evidence for similar events occurring in experimentally transplanted animals (Rosenberg et al, 1969). However, the recording of abnormalities of coagulation, fibrinolysis and platelets in the systemic blood or by

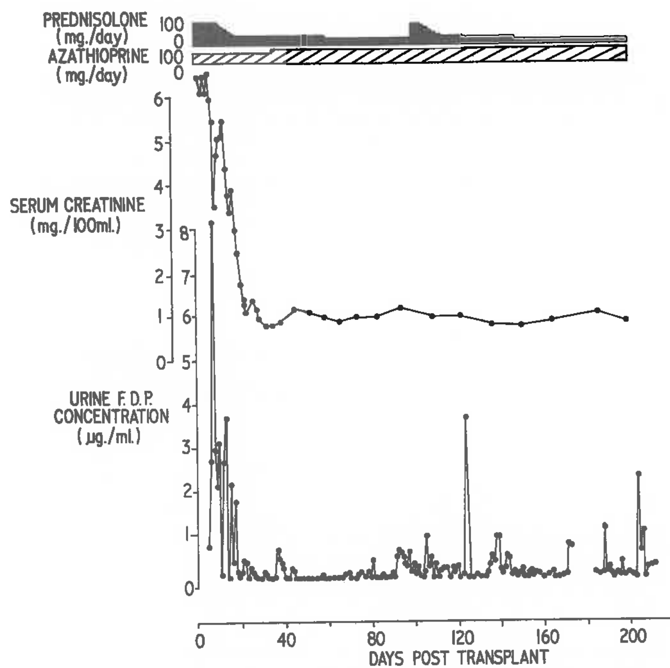

Fig. 108. Daily urinary F.D.P. and serial serum creatinine values in a patient followed as an inpatient and outpatient for over 200 days after cadaver renal transplantation.

gradient changes across the kidney from renal artery to renal vein has not been uniform. Starzl et al (1970) found that kidneys implanted into a host with known anti-donor antibodies, sequestered host platelets white cells and clotting factors. On the other hand, Colman et al (1969) could find little evidence of consumption coagulopathy in patients with hyperacute and acute forms of rejection and concluded that the intravascular coagulation occurring was a localized reaction confined to the graft. Wardle et al (1971) have found that a rise in serum F.D.P. concentrations when accompanied by a fall in the platelet count is associated with rejection. In the present study, serum F.D.P. concentrations were elevated in 3 of 6 clinically identified rejection episodes, and because of its lack of sensitivity when compared with the urine assay the serum measurement was not thereafter performed routinely. Thus, from this limited evidence, it is believed that if intravascular coagulation is localized, or of minimal intensity the presently used techniques are not sensitive enough to detect changes in the serum.

As with glomerulonephritis, the availability of urine makes the kidney a unique organ in which localized and minimal fibrinolytic activity may readily be studied. In the renal allograft situation, this was first recognized by Braun and Merrill (1968) and later confirmed by Antoine et al (1969). Both these studied were confined to immuno-assays which while highly specific, are considerably less sensitive than the T.R.C.H.I.I. In addition, only rough quantitative estimates were made, and the tests were performed on urine samples

obtained at intervals separated by at least three weeks. Thus while of great interest, the data from these earlier investigations could be regarded as less precise than desirable.

The early elevation of urine F.D.P. excretion seen before the graft function is sufficient to maintain dialysis — free existence is due to ischaemic renal failure and peri-operative trauma. Further evidence in support of this view was the absence of an early peak in all living-donor renal transplants investigated. Thus, the assay of urinary F.D.P. in the post-operative oliguric and early diuretic phases is probably of little value in the diagnosis of acute rejection. However, these results are of some interest for Salaman (1970) has suggested that no significant uptake of I^{125}-labelled human fibrinogen occurs in cadaver renal transplants during the phase of acute tubular necrosis, unless rejection is underway. This apparent discrepency with our data may reflect differences in principal and degrees of sensitivity between the two techniques.

One of the most unequivocal conclusions which emerged from this investigation was that all episodes of clinical rejection which resulted in an adjustment of immuno-suppressive therapy, were associated with a marked elevation of urinary F.D.P. excretion. However, in the in-patient series, where a careful daily assessment of the clinical and biochemical status of the patient was possible, it was found that similar elevations occurred which did not appear to be associated with evidence of rejection. While it may be claimed that those elevations accompanying acute rejection frequently began at least 24 hours before

the clinical diagnosis, and therefore could be of diagnostic assistance the presence of significant peaks occurring independently of acute rejection episodes renders its diagnostic value tenuous in this situation.

In considering the problem of early diagnosis of renal allograft rejection, Carpenter and Austen (1968) have emphasized that part of the complexity in the interpretation of currently available techniques results from the number of unknown factors. Specifically they have referred to the question of whether rejection is, in fact, a continuous process, or episodic in nature, so that mild rejection episodes may reverse spontaneously. In the present study, the sudden apparently self-limiting elevations of urinary F.D.P. excretion occurring without biochemical or clinical evidence of rejection would lend support to an "episodic hypothesis". Presumably periodic intensive immunological activity is matched on most occasions by intrinsic homeostatic mechanisms. However, some patients develop what can best be described as a continuous fluctuation of urine F.D.P., which if of sufficient magnitude and duration seems eventually to lead to clinical rejection. Busch et al (1971) have observed areas of fibrinoid necrosis at various stages of healing in wedge biopsies and nephrectomy specimens from patients with rejection and it is possible that the variable onset of these lesions is reflected in the urinary events.

From the clinical point of view, the results of the outpatient study may prove in time to have significant practical consequences. It was clear that in some patients, substantial cyclical elevations

in urinary F.D.P. were present for many weeks before rejection was diagnosed. During this time, the patients were apparently well, at home, and seen regularly at follow-up clinics and no adjustments to immuno-suppressive therapy were made as rejection was not suspected (Fig. 106). In other patients, these regular, rhythmic and substantial elevations in urinary F.D.P. excretion did not occur. Instead there were occasional, sometimes prolonged periods of minimal but abnormal activity, interspersed with infrequent short-lived, but more pronounced elevations (Fig. 105). It is thought that the changes seen in these patients may represent a feature of low grade chronic rejection. Within the limits of the numbers studied, and the duration of investigations there appeared to be no significant difference in the overall patterns of the F.D.P. excretion in patients whose kidneys were derived from either cadaver or living sources.

Inasmuch as most patients were treated with several drugs at the same time, and the period of study in some was limited to the length of hospital admission, responses of F.D.P. excretion to any single therapeutic manouever were difficult to evaluate. However, three observations were distinct and unequivocal. The first was seen in patients treated for rejection with increased doses of prednisolone. If this therapy was successful in reversing rejection there was a marked diminution in F.D.P. excretion following the dose change, which was maintained for long periods thereafter (Fig. 106). The second consisted of a similar reduction following the introduction of anticoagulant therapy in the form of heparin or warfarin (Figs. 103 and 104).

These responses to changes in therapy were paralleled by a functional response manifested by improved renal function. Unfortunately not all patients treated in this way showed the same reduction in F.D.P. excretion. In these cases relentless deterioration in graft function occurred (Fig. 107). The third concerns treatment with A.L.S.

Some workers have claimed that prolonged intramuscular administration of A.L.S. produces no significant renal damage (Starzl et al, 1967; Traeger et al, 1969; Fries et al, 1970) while others have stressed that certain anti-lymphocyte antibody preparations may contain sufficient levels of anti-glomerular antibody to cause renal lesions (Guttman et al, 1967; Diethelm et al, 1969; McMorr et al, 1970). Recent studies in mice have emphasised the potential dangers of immune complex serum sickness which may accompany A.L.S. therapy, and it seems from the study of dogs (Iwasaki et al, 1967) that intravenous administration of A.L.S. increases these dangers. Thus, the observed elevated F.D.P. excretion from renal allografts during the periods in which 8 patients were receiving A.L.S. was not unexpected, and suggests that this therapy was associated with acute, but self-limiting renal damage. The onset of the post-A.L.S. elevation did not coincide with the initiation of A.L.S. therapy, but occurred 10-14 days after, and was associated in time with the detection of circulating immune complexes related to the equine serum (James et al, 1971). Unfortunately electron microscopic evidence of immune-complexes in the glomerular basement membrane was not obtained.

Certain conclusions can be drawn from this study. If the excretion of F.D.P. by transplanted kidneys is similar to that of kidneys with glomerulonephritis, then it can be assumed that fibrin deposition plays a role in most rejection episodes. Using the sensitive and semi-quantitative T.R.C.H.I.I. these episodes are always detected. Moreover, by continuous observation, abnormalities are observed which have been interpreted as subclinical episodes of rejection. For the clinician, therefore, this assay may provide a method for gauging the activity of occult rejection and determining the natural history of graft survival. At the same time, finer individual titrations of immuno-suppressive support may be possible. Whether attempts to maintain a near zero urinary F.D.P. excretion will be possible, and whether this in turn will prolong graft survival remains a matter of conjecture. Perhaps what may be of equal importance in terms of patient morbidity may be the means by which it is done; the introduction of anti-coagulants, increases in immuno-suppression, or the use of new less toxic preparations. For the transplant immunologist these investigations may also be of interest as they could provide a basis for experimental and clinical models to study the nephrotoxicity of A.L.S., assessment of new forms of immuno-suppression, and by revealing episodes of occult rejection provide a more rational approach to the study of the role of tissue typing in renal transplantation.

Section 7.

CONCLUSION

The work presented in this thesis represents part of an extensive study into the role of coagulation in renal diseases. Although many subjects have been dealt with in detail, the study is by no means complete, as can be seen from the paucity of data on such subjects as malignant hypertension, pre-eclamptic or pregnancy toxaemia and diabetes mellitus. In each of these conditions study of renal biopsy and autopsy material displays the presence of fibrin within the kidney. This may be overt and massive as in malignant hypertension where the fibrinoid necrosis of arterioles, occasional luminal thrombosis and variable glomerular changes are similar to the abnormalities observed in the haemolytic uraemic syndrome. Electron and immunofluorescence microscopy confirm the presence of fibrin in these lesions. In pre-eclampsia, the fibrin is often apparent only after electron and immunofluorescence microscopy, and lies beneath and between swollen glomerular capillary endothelial cells. In these conditions evidence of intrarenal fibrin deposition usually disappears after successful treatment of the hypertension on the one hand or delivery of the infant on the other. Glomerular capillary fibrin is also occasionally observed under the electron microscope in renal biopsies from patients with diabetes mellitus.

Studies of coagulation and fibrinolysis in such patients have been meagre in comparison with the work presented in the preceding sections, but in general confirm the abnormalities demonstrated on renal biopsies. They are presented in brief, general terms.

In malignant hypertension serum F.D.P. concentrations are elevated especially in the early stages of hospitalization when blood pressure is not controlled. Concentrations remain elevated if the blood pressure is not successfully treated, and if progressive renal failure ensues, but revert to normal when treatment causes a fall in blood pressure without further renal functional impairment. In the 27 patients studied for between one and 5 weeks, 23 showed progressive impairment of renal function resulting in death or admission to a chronic haemodialysis programme, and in 4 the sequence of events was successfully, but temporarily, halted. The changes in urinary F.D.P. excretion, although following the same general pattern as the serum changes were less clearly defined. Urine from 19 patients periodically contained excessive concentrations in the order of 20-35 μg/ml. However, 8 patients, at no stage of testing, excreted F.D.P. in a concentration greater than 10 μg/ml. There was no relationship between the level of urinary F.D.P. and blood urea concentration or mean blood pressure, and in particular 2 of the 4 patients who were successfully treated continued to excrete large concentrations of F.D.P. Part of this confusion may have resulted from the fact that 25 of these patients were referred to a renal unit for management of renal failure. Clearly more work is necessary in this area with long term study of patients with essential hypertension being mandatory.

Patients with pre-eclampsia (see Section 3) were studied for periods varying between 21 and one days before delivery. Serum F.D.P. concentrations were elevated above 20 μg/ml in 5 out of 11 of these

patients, while abnormalities in F.D.P. excretion were observed in all. Moreover, F.D.P. excretion demonstrated the same cyclical increases as seen in proliferative glomerulonephritis and transplant rejection, and was most marked in those most severely affected, but disappeared at varying times after delivery in all patients. While two women who showed no evidence of pre-eclampsia did not excrete F.D.P. in a concentration greater than 0.5 μg/ml during the last month of pregnancy, a much larger controlled study is needed to confirm the observations recorded here.

Abnormal urinary F.D.P. excretion has been demonstrated in 3 of 12 diabetic patients studied for periods longer than one month.

Based on these observations and those reported in the previous sections it is possible to define the diseases in which intrarenal coagulation does and does not usually occur. These are shown in Table 23.

Table 23.

INTRARENAL COAGULATION USUALLY PRESENT	INTRARENAL COAGULATION USUALLY ABSENT
Acute ischaemic renal failure	Minimal lesion glomerulonephritis
Haemolytic uraemic syndrome	Membranous glomerulonephritis
Proliferative glomerulonephritis (all types)	
Renal homotransplant rejection	
Malignant hypertension	
Pregnancy hypertension	
Diabetes Mellitus	

The use of serial tests of coagulation and fibrinolysis has enabled a much clearer definition of the role intrarenal coagulation plays in the natural history of these diseases. In particular serum and urinary F.D.P. measurements have demonstrated several distinct patterns of intrarenal coagulation which seem to be independent of the cause of the renal damage. Thus there are diseases in which there are:

1. Single or short lived episodes of intrarenal coagulation;
2. Recurring episodes which cease on removal of the cause;
3. Recurring episodes which unless interrupted cause progressive destruction of the kidney;
4. No signs of intrarenal coagulation.

The first category includes acute ischaemic renal failure, some cases of the haemolytic uraemic syndrome, post-streptococcal and other self-limiting forms of glomerulonephritis. While these diseases may often cause severe renal dysfunction, there is a strong tendency for complete renal recovery when the damage caused by disease has spontaneously or naturally resolved. It is unlikely that specific therapy is necessary in these cases.

In the second category there are possibly many diseases but the one most commonly seen is pre-eclamptic toxaemia. From the small amount of data available it would seem that the stimulus to intrarenal coagulation is recurrent, but ceases a short time after delivery. Once again, it is unlikely that specific therapy other than removal of the cause will prove beneficial.

The third category of diseases includes a large number of cases of proliferative glomerulonephritis, most cases of the haemolytic uraemic syndrome, renal homotransplant rejection, malignant hypertension and possibly diabetes mellitus. The studies reported here indicate a cyclical form of injury which is of variable intensity and frequency and in most cases the causative factor of which is unknown. If left unchecked, the continuation of this process is likely to be associated with progressive destruction of renal function. It is to this category that most therapeutic attention should be paid for by interrupting the cyclic pattern of injury renal function may be maintained. Maintenance of near zero urinary F.D.P. excretion is possible in approximately two thirds of patients, with a variety of commonly used drugs. It is not known as yet whether this will prolong renal function. What may be of greatest importance, however, is the way in which this maintenance is achieved, as some of the drugs used have serious adverse effects.

Diseases such as minimal lesion and membranous glomerulonephritis belong to the fourth category.

This broad classification of glomerular diseases may be of some practical value, for it is often possible to determine the category a patient is likely to fall into by a series of simple tests. Furthermore, if diseases falling into categories one and two are defined, unnecessary treatment may be avoided.

While our studies have indicated a role for abnormal coagulation in many renal diseases, its significance or importance as a pathogenetic

factor has not been determined. The question whether the abnormal coagulation is a primary or secondary event is also unresolved. However, the presence of intrarenal fibrin in so many diseases, of such diverse origins, leads to the conclusion that its deposition lysis and removal is part of the normal response of the kidney to injury.

Perhaps the answer to its significance will become apparent when adequate controlled trials of anticoagulant and antiplatelet therapy have been carried out. Clues of equal relevance may exist in the diseases minimal lesion and membranous glomerulonephritis where the reasons for the lack of inflammatory reaction and fibrin deposition remain a complete mystery.

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