EXPERIMENTAL GLOMERULONEPHRITIS AND NATURE OF NEPHROTOXIN

(Short Title)

Wei-wei Ng

EXPERIMENTAL STREPTOCOCCAL GLOMERULONEPHRITIS IN RABBITS AND FURTHER STUDIES ON THE NATURE OF NEPHROTOXIN

(Abstract)

The effect of the extracellular, dialysable nephrotoxin produced by a group A type 12 streptococcus on the rabbit was studied critically for the similarities to human acute glomerulonephritis. Nephrotoxin-treated rabbits have been found to have renal changes upon light and electron microscopic observations. In the latter case, numerous "humps" and cellular proliferation and edema were evident in the glomeruli. No consistent glomerular deposition of autologous gamma globulin could be found by using immunofluorescence microscopy. No alterations in serum complement activity were noted. Chemical characterisation of purified nephrotoxin was performed by end-group analysis, ultraviolet, fluorometric and infrared spectroscopy and quantitative assays, and following acid hydrolysis by thin-layer, paper and gas-liquid chromatography. Nephrotoxin is found in a semi-purified extract consisting chiefly of polypeptides but itself upon further purification appears to be a substance containing a prominent substituted aromatic or heterocyclic residue.

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by

Wei-wei Ng

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Department of Microbiology & Immunology
McGill University, Montreal

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I. INTRODUCTION

An animal model of poststreptococcal acute glomerulonephritis was launched by the experiments of Reed and Matheson (1954*a*) in which localised skin infection with nephritogenic group A type 12 streptococci or intravenous injections of the streptococcal culture filtrates in rabbits could induce renal disease. The active substance, referred to as nephrotoxin, was shown to be dialysable, heat-stable, and chiefly polypeptide in nature.

In the continuing study of the nephrotoxin induced acute glomerulonephritis in rabbits, two major areas of investigation were explored.

Firstly, if the animal model is to reflect the disease as it occurs in man so that a detailed study of pathogenesis can be made, it must fulfill as many criteria of the human disease as possible. In the field of the study of acute glomerulonephritis, this fact has become especially important in recent years since immunohistological and ultrastructural studies of human biopsy material have revealed possible structural basis for functional anomaly, and animal experiments conducted by Germuth, Dixon, and many others indicate that renal disease induced by known immunological means give rise to similar findings. Therefore the *in vivo* effects of nephrotoxin must be critically examined and compared to those seen in human acute glomerulonephritis. Different aspects of the changes in rabbits which received nephrotoxin were followed, including evidence of hypertension, urinary abnormalities, alteration in serum complement levels, immunofluorescent staining for the presence of host gamma globulin deposits in the kidneys, as well as

light and electron microscopic examinations of kidney sections for signs of acute or progressive lesions.

Secondly, the nature of purified nephrotoxin should be ascertained so that comparison of it can be made with inactive, corresponding substances that are produced by non-nephritogenic strains of streptococci, and that the nephrotoxin can be discussed with regard to other biologically active substances which have been chemically characterised. Previous purification of nephrotoxin preparations had indicated that activity resided in a homogeneous fast migrating band obtained by electrophoretic separation of a semi-purified nephrotoxin preparation (Cheuk, 1960). The yield of nephrotoxin purified in this manner was too low. Therefore, in the work which was undertaken, attempts were made to devise further purification of the semi-purified nephrotoxin on a preparative scale. The nature of the further purified nephrotoxin was investigated and compared with the corresponding non-toxic components obtained from non-nephritogenic type 6 streptococcal culture filtrates and from the growth medium, Todd-Hewitt broth, alone. Ultraviolet, infra-red, and fluorescence spectrophotometry, as well as thin-layer and gas-liquid chromatographic methods, among others, were used for examination of the purified substances.

II. REVIEW OF THE LITERATURE

The enigma of acute glomerulonephritis as it occurs in man has provided impetus for much research with accessible data from man and a quest for the understanding of its pathogenesis through varied animal experiments. Earliest descriptions of the clinical and pathological manifestations of what is primarily a kidney disease were given by Bright (1827) and Langerhans (1879). Since then general consensus have been reached concerning the clinical and pathological symptoms of the disease.

A. Clinical and Pathological Manifestations of Poststreptococcal Acute Glomerulonephritis

Clinically in man, acute glomerulonephritis (AGN) presents a picture of edema, hypertension, and urinary abnormalities. The kidney damage is usually of a generalised nature, involving the glomeruli, and to a much lesser extent, the tubules in the kidney. The degree of hypertension appears to be correlated with the severity of renal lesions (Earle, 1965). Urinary abnormalities usually include hematuria, proteinuria, and the presence of red blood cells and granular and leukocyte casts in the urine (Heptinstall, 1966). In severe cases, oliguria or anuria may occur. Since the advent of renal biopsies, it has also been found that minimal or no urinary abnormalities may accompany typical nephritic lesions (Cohen and Levitt, 1963; Berman and Vogelsang, 1963; Kandall *et al.*, 1969). Other concomitant laboratory findings may include

an increase in blood urea nitrogen and a decrease in the glomerular filtration rate (Bradley *et al.*, 1950; Earle *et al.*, 1951). Serum complement is decreased in almost all cases (Fischel and Gajdusek, 1952; Lange *et al.*, 1960; Gotoff *et al.*, 1969). In the survey of 246 cases of AGN by Lange *et al.* (1960), complement levels were found to be lowered in 245 cases. Klemperer *et al.* (1965) showed that a decrease in $beta_{1c}$ might be the limiting factor for the decreased total hemolytic complement activity in AGN.

Grossly, the kidneys appear pale and enlarged, usually with a 50% increase in size, and often with petechial hemorrhages. Upon hemisection, the cortex appears thickened (Heptinstall, 1966). Light microscopic examination of the kidneys reveals diffuse involvement of the glomeruli (Kark, 1967). Enlargement of the glomeruli is due to the proliferation of endothelial and epithelial (McGregor, 1929; Bell, 1936; Ellis, 1942), and mesangial cells (Jones, 1953), cellular edema, and the exudation of polymorphonuclear leukocytes. The glomerular basement membrane (GBM) may appear thickened and split in some cases. Tubular changes are not pronounced, with occasional hyaline droplets accompanying proteinuric cases. Much of the interstitial changes such as edema, polymorphonuclear and mononuclear cell infiltration may be associated with accompanying systemic inflammatory processes. (Heptinstall, 1966).

The ultrastructural observations of the kidney in acute glomerulonephritis by electron microscopy confirm the light microscopic findings, but in addition reveal more detailed structural changes. Extensive

mesangial involvement may be seen, with an increase in the number of its characteristic cells and matrix fibrils (Jones, 1953; Bohle and Herfarth, 1958). Polymorphonuclear infiltration and mononuclear proliferation and enlargement are observed. Moreover, deposits of varying electron density, termed "humps", are found along or close to glomerular basement membranes, particularly subepithelially. (Kimmelstiel *et al.*, 1962; Movat *et al.*, 1962; Trump and Benditt, 1962; Andres *et al.*, 1964; Strunk *et al.*, 1964; Neustein and Davis, 1965; Herdson *et al.*, 1966; Ort *et al.*, 1969.)

Immunofluorescent staining of kidney sections from cases of AGN with fluorescein labelled antisera to 7S gamma globulin and $beta_{1c}$ localised granular deposits in the glomeruli. (Mellors and Ortega, 1956; Freedman *et al.*, 1960; Freedman and Markowitz, 1962; Lachman *et al.*, 1962; Seegal *et al.*, 1965; Koffler and Paronetto, 1965; Andres *et al.*, 1966; Michael *et al.*, 1964 and 1966.)

B. Streptococcal Etiology of Acute Glomerulonephritis

The relationship between group A streptococci and acute glomerulonephritis was first noted by Ophüls (1917). Since that time many epidemics of AGN associated with group A streptococci have been reported, among some of which had involved from 200 to several thousand cases. (Fleming, 1949; Stetson *et al.*, 1955; Kodama *et al.*, 1958; Symonds, 1960; Simon *et al.*, 1965; Poon-King *et al.*, 1967.) Among the group A streptococci, the type 12 organisms are the most frequently encountered

in AGN when the preceding infection had been of the upper respiratory tract. (Reed, 1953; Rammelkamp and Weaver, 1953; Wertheim *et al.*, 1953; Wilmers *et al.*, 1954; Stetson *et al.*, 1955; Pleydell and Hall-Turner, 1958; George *et al.*, 1958.) Other upper respiratory nephritogenic types encountered were 4, 25 (Rammelkamp and Weaver, 1953), and 1 (Goldsmith *et al.*, 1958), with limited evidence for 3 and 6 (Wilmers *et al.*, 1954; Rammelkamp, 1957).

Those types of nephritogenic group A streptococci which have been associated with prior skin infections include type 49 (Kleinman, 1954; Šrámek *et al.*, 1964; Anthony *et al.*, 1967; Poon-King *et al.*, 1967; Fish *et al.*, 1970; Kaplan *et al.*, 1970*a*), type 2 (Dillon *et al.*, 1968), type 52 (Top *et al.*, 1967; Potter *et al.*, 1968; Roy *et al.*, 1969), type 55 (Potter *et al.*, 1968; Lasch *et al.*, 1971), type 56 (Johnson *et al.*, 1968), type 57 (Ferrieri *et al.*, 1970; Potter *et al.*, 1971), and type 60 (Derrick *et al.*, 1970).

Some differences in response have been noted between streptococcal infections of the skin and of the upper respiratory tract. Traditionally, a high anti-streptolysin O (ASLO) response has been found to give an indication of recent streptococcal infection in 92-94% of the upper respiratory cases (Lyttle *et al.*, 1938; Bernstein and Stillerman, 1960). However, skin infections with streptococci appear to give a poor ASLO response (Anthony *et al.*, 1967; Dillon and Reeves, 1969; Kaplan *et al.*, 1970*b*). Furthermore, the antigenic make-up of the skin strains appear to be different from the respiratory strains in that M-typing of the former was found to be difficult. T-typing with aggluti-

nation tests using anti-T sera was very often the only means of classifying these skin strains (Johnson *et al.*, 1968; Potter *et al.*, 1968). The difficulty encountered in M-typing of these strains was attributed to the scarcity of surface M protein when it was discovered that additional neopeptone added to the usual growth media enhanced the M protein production and enabled the M-typing (Maxted *et al.*, 1967). The geographical distribution of the occurrence of skin and respiratory streptococcal infections also varies. This is understandable since pyoderma is more prevalent in the warmer zones where insect vectors may be another reservoir of infection (Bassett, 1970). In spite of the above differences between the physiology of the skin and respiratory strains, no difference has been found in the nature of the nephritis or the prognosis in each case (Wannamaker, 1970*b*).

Aside from streptococcus-associated AGN, cases have also been observed following infection with other microorganisms: *Diplococcus pneumoniae* (Blackman and Rake, 1932; Seegal, 1935; Hill *et al.*, 1965), viruses (Herbut, 1944; Cohen, 1955; Bates *et al.*, 1957; Yuceoglu *et al.*, 1966; Minokowitz *et al.*, 1968), and the parasite *Plasmodium malariae* (Gilles and Hendrickse, 1963; Edington and Mainwaring, 1966; Kibukamusoke *et al.*, 1967). However, the evidence for the involvement of viruses has been only circumstantial (Jensen, 1967), and it has been difficult to prove that prior streptococcal infection had not taken place. Similarly, in glomerulonephritis following quartan malaria, the glomerular lesions are often focal and segmental, and more membranous as compared to the diffuse lesions of streptococcal etiology (Edington and Mainwaring,

1966; Kibukamusoke and Hutt, 1967). In other cases where the lesions are not distinct from the poststreptococcal variety, it has been difficult to rule out possible streptococcal etiology (Wing *et al.*, 1971).

In summary, the streptococcal etiology of acute glomerulonephritis is well established and it is unlikely that any other microorganism even approaches the capacity of group A streptococci in inducing the epidemic type of AGN. Although the overall frequency of AGN following streptococcal infections is relatively low, high rates can follow infections with certain nephritogenic types in contrast to other sequelae of streptococcal infections such as rheumatic fever, in which a constant rate of susceptibility follows infections with all types (Rammelkamp and Weaver, 1953).

C. Pathogenesis of Acute Glomerulonephritis

The kidneys seen in acute glomerulonephritis appear to be sterile (Bell and Hartzell, 1922), as are the blood and urine (Longcope *et al.*, 1928). A process other than direct infection of the kidneys is therefore involved. Throughout the years workers in the field have delved into nearly every possibility. But as yet, there is no single theory which designates a specific role for streptococci in the pathogenesis of AGN which has been unequivocally proven and accepted. In efforts to understand the disease process, massive amounts of data have been gathered from attempts to develop experimental AGN in animals, in addition to exhaustive examinations of all aspects of human cases.

1. Animal Models of Acute Glomerulonephritis Induced by Unidentified Streptococcal Products

One of the best means to study a disease thoroughly is to reproduce it in animals if this is possible.. Efforts have been made to induce acute glomerulonephritis in mice, rats, rabbits, dogs, cats, sheep, monkeys, and baboons. Animal experiments involving the intravenous or subcutaneous injections of streptococci repeatedly gave negative results (LeCount and Jackson, 1914; Ophüls, 1917; Faber and Murray, 1917; Bloomfield, 1919; Cary, 1924; Bell *et al.*, 1925; Duval and Hibbard, 1926; Reith *et al.*, 1930; Helmholz, 1932). The renal lesions were either absent or not severe at all. Lukens and Longcope (1931) instead claimed to have induced focal and diffuse glomerulitis in their rabbits when they injected heat killed streptococcal vaccine.

Reed and Matheson (1954*a*, 1954*b*) first induced nephritis in rabbits by subcutaneous infection with a nephritogenic type 12 streptococcus and by the intravenous injection of its culture filtrate. The nephrotoxic activity was found to reside in a dialysable, ammonium sulfate precipitated fraction of the culture filtrate. This fraction was referred to as "nephrotoxin" and was shown to be active in rabbits (Matheson and Reed, 1959), monkeys (Reed and Matheson, 1960), and cats and dogs (Reed and Matheson, unpublished data).

Another method used in inducing experimental AGN is the implantation of intraperitoneal diffusion chambers containing viable streptococci. This method provides a source of streptococcal products produced by live streptococci which continue to metabolise *in situ*. Kelly and Winn (1958)

first reported the use of diffusion chambers in mice. Seven days following 24, 48, and 72 hour periods of intraperitoneal implantation of type 12 streptococcus, he obtained tubular necrosis and minimal glomerular damage. Hinkle *et al.* (1960) also obtained tubular involvement with very mild glomerulitis in their mice. In addition, they could not correlate the severity of lesions with the known nephritogenicity of the type 12 strain used as Kelly and Winn had found. The experiment conducted in rats by Markowitz *et al.* (1960) yielded similar results. The glomerular changes were "spotty" and did not approach the intensity nor the diffuseness of those seen in man. Tan *et al.* (1961) who also used mice obtained renal tubular lesions and abnormal urine sediment. He later attributed the lesions to the essential presence of streptolysin S (SLS) (Tan and Kaplan, 1962). Eisen *et al.* (1964) extended the usual 48 hours of exposure of the animals to the intraperitoneal chambers to as long as 42 days but still only obtained tubular damage without glomerular lesions, with no consistent proteinuria.

In an extended series of experiments with the diffusion chambers in rats, Lindberg *et al.* (1964, 1967) obtained significant proteinuria in rats after leaving the chambers *in situ* for 60 days. Kidney glomerular localisation of the M protein of a type 12 streptococcus, IgG, and later $beta_{1c}$ (Vosti *et al.*, 1970) were demonstrated by immunofluorescence methods. However, even after up to 6 months of exposure to the chambers, virtually no kidney damage was observed on light and electron microscopy (Vosti *et al.*, 1970). Elution of the antibodies from the kidneys indicated them to be IgG and reactive with type 12 streptococcal M

protein (Lindberg and Vosti, 1969).

Holm *et al.* (1967) claimed to have produced "clinical and histopathological evidence of the production of glomerulonephritis in rabbits by injecting an autolysate preparation of type 12 streptococci". After their single intravenous injection, almost immediate hypertension was obtained. The blood pressures climbed to 90 to 130 mm Hg from an initial reading of less than 30 mm Hg, and remained sustained for as long as 2 months. They measured the blood pressure by using a modification of Grant and Rothschild's (1934) method, as Reed and Matheson had done, by working with the peripheral ear artery. Their 20 rabbits were sacrificed at different time intervals. Most of the rabbits had proteinuria while about half had microscopic hematuria. Histopathological lesions were seen in the kidneys of most of the 12 rabbits which were sacrificed 1 to 60 days following injection, as opposed to the normal kidneys found in the 2 control rabbits.

In an effort to simulate natural respiratory infections in man, Miyamoto *et al.* (1960) inoculated type 6 and type 12 streptococcal strains into the paranasal sinuses of rabbits. Out of 33 rabbits many died in the early stages due to septicemia, and only 3 of the remaining showed histopathological lesions in the kidneys. The most severe case was one which had been inoculated with a type 12 organism, whereas the other two which had been inoculated with type 6 streptococci exhibited only glomerulitis. The urines of many rabbits were found to contain albumin and occasionally red blood cells. Blood pressures were again measured using a method similar to Reed and Matheson's. Although some

rabbits in both the type 6 and type 12 groups exhibited increased blood pressure, no significant differences could be found.

Another Japanese worker (Torii, 1960) successfully induced acute diffuse glomerulonephritis in rabbits by first sensitising them with type 12 vaccine and then triggering the disease by injecting the group specific polysaccharide. The clinical and histological findings were again much milder than those seen in man. In an attempt to increase the severity of the renal lesions produced in this manner, Maki *et al.* (1968) used a sonicated preparation of a nephritogenic type 25 organism as the trigger. In 5 rabbits thus treated, proteinuria and hematuria were obtained. The kidneys showed generalised diffuse proliferative glomerulonephritis apparently similar to poststreptococcal AGN. Rats treated in the same fashion exhibited much less severe lesions.

Meanwhile, Becker and Murphy (1968) attempted to reproduce AGN using the largest population of rabbits to date in one experiment. They infected 106 animals by various routes with the nephritogenic strains of streptococci isolated from man, each rabbit being serially infected with different strains. Of these, 22 were found to have proteinuria, frequently with hematuria, and occasionally azotemia. Fifteen of the 22 were sacrificed at different time intervals for kidney histological studies. Of these, 10 showed renal changes typical of "acute or recurrent acute glomerulonephritis in man". Similar histological findings were obtained in 3 other animals whose urined were not examined. Unfortunately, electron microscopic studies were not done on these kidneys.

Taranta *et al.* (1969) infected the tonsils and skin of 12

baboons, each with different types of group A beta hemolytic streptococci, including types 12, 52, 1, 2, 3, 6, and 49, over a period of 9 months. Only one animal produced persistent proteinuria in conjunction with increased blood urea nitrogen. Upon biopsy of this baboon and 10 others, no histological evidence of AGN were noted.

Therefore, animal experiments in which the whole streptococcal cells, whole cell sonicates, or the entirety of their extracellular products were used to induce AGN have so far met with variable success. The extent of AGN contraction has been low. With few exceptions, the kidney lesions found were not as severe as those seen in human cases of poststreptococcal AGN.

2. Toxicity of Specific Streptococcal Components and Extracellular Products

Streptococcus related toxicity has been extensively explored and this search has yielded a variety of substances which are biologically active. Beginning with the cell wall itself, the group specific C polysaccharide was shown to induce lesions of dermal connective tissue when it was present as a complex with protein (Schwab, 1962). Goldstein and Trung (1966) examined the kidneys among other organs following intravenous injections of various preparations of the C polysaccharide into rabbits. In general, the paramount reaction was vascular inflammation. The kidney glomerular changes were "subacute", with lobulation, cellular proliferation and irregular thickening of the walls of capillary loops. Purified polysaccharide does not lead to these changes (Rotta,

1969). In Maki *et al.*'s experiments which were previously mentioned, a sonicated preparation of type 25 streptococci was used to trigger kidney disease in rabbits presensitised with a vaccine. Further purification of the sonicated preparation revealed that the nephritogenicity resided in fraction with a high content of protein and carbohydrate. The mucopeptide or peptidoglycan itself has an intrinsic toxicity similar to that of the gram negative bacterial endotoxins (Rotta and Bednar, 1969; Heymer *et al.*, 1971) and is therefore not specific for streptococci. Cell wall techoic acid was found to bind spontaneously to mammalian tissues (Waltersdorff and Jackson, 1970). When it was injected into rabbits for over 30 days, 75% of the animals' kidneys showed calcification and tubular necrosis, which again is not typical of poststreptococcal AGN.

The M protein is the type specific antigen of group A streptococci and is only found occasionally in other streptococci (Maxted and Potter, 1967). It is associated with virulence of the organisms (Lancefield, 1962; Krasner *et al.*, 1964). Consequently, there has been much speculation concerning its role in AGN. Kaplan (1958) found that M protein localised in the glomerular tufts of mice injected with it. However, he observed localisation of the M protein of all types, both nephritogenic and non-nephritogenic. Furthermore, there was no difference in the duration of persistence among the different types. Kantor and Cole (1959) discovered a "fibrinogen precipitating factor" that was inseparable from the M protein of group A streptococci which could precipitate fibrinogen *in vitro*. *In vivo* (Kantor, 1961) it led to acute renal lesions

with thrombi presumably consisting of fibrin in the glomerular capillaries. Although the lesions were not typical of human AGN, it was suggested that the lesion might be an initiating point for further nephritic processes. In further experiments, Kantor (1964) found that M protein first localised in rat and mouse kidneys as parts of M-fibrinogen complexes, with simultaneous proteinuria and urea retention seen in the animals which disappeared by the sixth day but reappeared with the appearance of anti-M antibodies. The implication here is that non-renal antigen-antibody complexes might be instrumental in the induction of the eventual lesions. This hypothesis concerning immune complex mediated renal injury will be discussed more fully.

Individual streptococcal extracellular products have been found to affect a variety of animal organs *in vivo*. At least 20 to 24 distinct extracellular substances were found to be antigenic and to precipitate with antibodies found in normal pooled human gamma globulin (Halbert, 1966). Although the heart, liver, kidney, spleen, diaphragm, and tonsil were all examined after the intravenous injection of streptococcal extracellular products, the lesions found were mainly myocardial, hepatic, and diaphragmatic (Spira *et al.*, 1968). As early as 1945, streptolysin O (SLO) was mentioned as possessing *in vitro* cardiotoxic properties (Bernheimer and Cantoni, 1945). As it has already been noted, streptolysin S (SLS) was likely responsible for the renal tubular lesions in mice (Tan and Kaplan, 1962). Both SLO and SLS are cytotoxic for a variety of cells (Bernheimer and Schwartz, 1960). *In vivo* hemolysis was found following intravenous injection of sufficient doses of SLO

(Halbert *et al.*, 1965) and SLS (Ginsburg *et al.*, 1966). The latter found only few of the rabbits having mild proliferative glomerulitis 3 days following injection. The erythrogenic toxin is not related to nephritogenicity but is noted mainly for pyrogenicity (Watson, 1960), skin reactivity, and cytotoxicity (Kim and Watson, 1970). Among the other extracellular streptococcal products, nicotinamide adenine dinucleotidase (NADase) was proposed as being related to nephritogenicity of streptococci since type 12 organisms are good producers of this enzyme (Bernheimer, 1960), but later Gonzaga and Rammelkamp (1962) found that anti-NADase failed to protect against nephritis.

To conclude, it appears that no single streptococcal product by itself has been unequivocally shown to induce poststreptococcal AGN as it occurs in man.

3. Immunological Hypothesis for the Pathogenesis of Acute Glomerulonephritis

It was noted that the interval between the onset of streptococcal infection and acute glomerulonephritis varied between 7 to 21 days with an average of 10 to 11 days (Winkenwerder *et al.*, 1935). Others reached similar conclusions (Rammelkamp, 1964). Because of this time lapse, the fact that the kidneys in AGN are not infected, and that no obvious toxins have been discovered, it has been postulated that the pathogenesis might be immunological in origin.

The extent of the patient's antibody response to extracellular streptococcal products did not seem to be related to disease. Lyttle

et al., (1938) could not make a correlation between the titer of anti-streptolysin O to the severity of renal lesions. Similar conclusions were reached by others (Stetson *et al.*, 1955). Thus there has been no obvious indication that a good humoral antibody response meant more severe lesions.

a. Autoantibodies

The possibility that the kidney damage might be due to auto-antibodies to kidneys has been explored. Lange *et al.* (1949) found anti-kidney antibodies in 68% of AGN cases by using collodion particles coated with kidney antigens but stated that the method was questionable. Liu and McCrory (1958) found hemagglutination titers of 1:20 or higher in 71% of AGN cases using polystyrene latex suspensions coated with human kidney antigen. Kramer *et al.* (1961) found antibodies in 923 AGN cases. It is possible that for autoantibodies to appear, an alteration of normal kidney tissue may have to occur first (Lange *et al.*, 1965). In any case, the amount and the extent of occurrence of autoantibodies so far detected in humans are probably not sufficient to be pathogenetic.

b. Experiments with Anti-Kidney Antibodies

Experimentally, variable success has been achieved in the induction of glomerulonephritis through the presence of antibodies to kidneys.

i. Heterologous anti-kidney antibodies

In nephrotoxic serum nephritis (Lindemann, 1900; Masugi, 1934), anti-kidney antibodies produced in another species to kidney antigens of

the same species as the first animal were injected back into this latter animal, with consequent severe glomerulonephritis. The antigens responsible were shown to be of glomerular basement membrane (GBM) origin (Steblay, 1962). The pathogenesis of this type of kidney lesion was shown to be due to a combination of the heterologous phase in which the passively administered antibody fixes promptly to the kidney GBM (Unahue and Dixon, 1965*a*) and an autologous phase in which the host produces antibodies against the foreign gamma globulin, with the newly formed antibodies reacting with the already bound foreign gamma globulin (Unahue and Dixon, 1965*b*). Following the heterologous phase, there is polymorphonuclear cell infiltration and GBM thickening. In the autologous phase proliferation of endothelial and mesangial cells predominate (Smadel, 1937; Piel *et al.*, 1955; Feldman *et al.*, 1963). The deposits of both heterologous and autologous antibodies lie linearly along the endothelial side of the GBM.

ii. Autologous anti-kidney antibodies

What appears to be very similar to the nephrotoxic serum nephritis just described was seen in "autologous allergic nephritis" which has been produced with variable success by different investigators.

Cavelti and Cavelti (1945) observed that 127/207 of their rats developed renal lesions, 85 of which were glomerular, after intraperitoneal injections of streptococci in combination with rat kidney. Humphrey (1948) could not confirm this observation. Peck and Thomas (1948) injected rabbits subcutaneously with homologous kidney together with heat-killed group A and C hemolytic streptococci and Freund's adjuvant, and could not

detect either kidney lesions or antibodies to kidney.

Later Heymann *et al.* (1959) were successful in producing "nephrotic syndrome" in rats by intraperitoneal injections of a supernatant of a saline extract of rat kidney suspension with Freund's adjuvant. The lesions included GBM thickening, endothelial swelling, and rare epithelial crescents. They stressed the importance of using the soluble portion of the kidney suspensions. Smith *et al.* (1964) found by contrast that a combination of homologous kidney tissue, type 12 streptococci, and Freund's adjuvant was necessary, and that either component alone with adjuvant was not effective. Steblay and Rudofsky (1968) were able to induce a similar autologous renal disease by using a non-organ specific antigen, the human lung, with Freund's adjuvant, when injecting sheep intradermally, intramuscularly, and subcutaneously. Markowitz *et al.*(1967) injected alum precipitated soluble antigens from human glomeruli into 3 rhesus monkeys and induced glomerulonephritis in all of them. The monkeys exhibited proteinuria and slightly increased levels of blood urea nitrogen. Autologous gamma globulin was shown to localise in the glomeruli.

At first glance, autologous nephritis appears to be very similar to nephrotoxic serum nephritis in that the linear deposition of gamma globulin along the endothelial side of the GBM is noted in both cases. However, closer study with immunofluorescent staining of the glomeruli in autologous nephritis reveals that what seemingly is a linear deposition of gamma globulin and $beta_{1c}$ globulin along the GBM is actually confluent granular material (Okuda *et al.*, 1965). In addition, Feldman (1963) found that the deposits of electron dense material seen in the electron microscope

is usually found on the epithelial aspect of the GBM. All this led Edgington *et al.* (1967) to propose that autologous nephritis should actually be referred to as "autologous immune complex nephritis" since the sensitising antigen instrumental in the induction of host antibody probably originates non-glomerularly in the proximal renal tubular brush border.

Antigens from urine were also found to be able to initiate autologous nephritis (Dinh and Brassard, 1967; Lerner and Dixon, 1968). These antigens were later found to cross-react with antigens from solubilised GBM components even though the source of these urinary antigens may not be confined to the kidney (McPhaul and Dixon, 1969). In the induced AGN in rabbits, host gamma globulin deposits in a true linear glomerular pattern.

c. Immune Complexes Unrelated to the Kidney

The widely accepted experimental model for acute glomerulonephritis in man is the glomerulonephritis occurring in serum sickness (Germuth, 1953; Dixon *et al.*, 1958). There has been extensive study of this model in which antigen-antibody complexes exogenous to the kidney are involved in the induction of renal lesions (McCluskey *et al.*, 1960; Dixon *et al.*, 1961; Fish *et al.*, 1966).

Serum sickness glomerulonephritis can be induced by a single large dose of heterologous serum protein, small multiple doses of the same, or by injections of soluble antigen-antibody complexes. The nephritis following a large intravenous injection of foreign serum

protein is self-limiting and the immune complexes are quickly cleared (Germuth, 1953). Daily injections of foreign serum protein can lead to acute, progressive, or chronic glomerulonephritis. Animals which are good antibody producers do not develop the progressive or chronic forms of nephritis (Dixon *et al.*, 1961). Soluble antigen-antibody complexes in moderate antigen excess are the most toxic (Dixon, 1965). McCluskey *et al.* (1960) were able to see kidney lesions in their mice within 48 hours of the first of 3 intravenous injections of antigen-antibody complexes in antigen excess.

The biological activity of soluble antigen-antibody complexes and gamma globulin aggregates has been elucidated. They can bind complement (Ishizaka *et al.*, 1959) and produce Arthus-like reactions (Ishizaka *et al.*, 1961). This means that once the complement components are activated, a host of events is triggered, including the infiltration of polymorphonuclear leukocytes, liberation of vasoactive factors, fibrin formation, smooth muscle contraction, platelet and leukocyte agglutination, and increased vascular permeability. It has been shown specifically that one of the mechanisms which leads to immune complex entrapment in vessel walls involves immune complex mediated basophil release of a soluble factor which in turn causes clumping of platelets and their vasoactive amine release, thereby leading to increased vascular permeability (Cochrane, 1971). The tissue responds to the deposition of complexes with cellular proliferation and degenerative hyaline changes (Dixon, 1965).

Immunofluorescence microscopy reveals these complexes and

together with complement in granular deposits in the glomeruli. Electron microscopic studies indicate that they may be parts of electron dense deposits which are found in the glomeruli, particularly subepithelially (Andres *et al.*, 1963; Fish *et al.*, 1966).

Of the experimentally induced glomerulonephritides just described, the kidney lesions of immune complex (serum sickness) nephritis seem to resemble human poststreptococcal AGN most closely. However, the large quantity of antigen required to initiate such lesions, 250 mg/kg of bovine serum albumin for the one-shot type and 10 to 200 mg per rabbit per day for the chronic type of serum sickness (Wilson and Dixon, 1971), means that a different type of antigenic stimulation may be operative in man. By contrast, in autologous immune complex nephritis, only a small amount of the proximal tubular antigen is required to initiate what appears to be a self-perpetuating process of antigen-antibody deposition in the glomeruli, although the amount of deposition is much more confluent here. Poststreptococcal AGN is probably the result of a process which is similar to but not identical with the two above processes.

d. Identity of Extraneous Substances Localising in Kidneys of Cases of Poststreptococcal Acute Glomerulonephritis

Thus far it can be seen that the development of the immune complex nephritis gives rise to subsequent kidney and clinical changes similar to those seen in human poststreptococcal AGN. What remains undefined is the composition of the deposits seen in the kidneys of AGN. These deposits may be pathogenetic or they may be a consequence of a

prior damage, such as that induced by a toxin, but which would still serve to aggravate the damage.

From previous discussion in this review, it can be seen that most kidney biopsies from cases of poststreptococcal AGN in man contain glomerular deposits of gamma globulin and $beta_{1c}$ globulin. By using immunofluorescence techniques, Seegal *et al.* (1965) attempted to identify the immune specificity of the gamma globulin and of the unknown antigen. In the earlier study in which kidney sections from 26 cases of varied glomerulonephritides were stained with antiserum to whole streptococcal cells, 5 were found to be positive. Later, in a study which included 10 well documented cases of AGN suspected to be poststreptococcal, fluorescein labelled antibody localised 7S gamma globulin in 8 of 10 cases, $beta_{1c}$ globulin in 9 of 10, and type 12 antigen in 6 of 10. In their subacute (progressive) cases, no type 12 antigen could be localised although the kidneys were positive for 7S gamma globulin and $beta_{1c}$ globulin. Labelled antibodies to type 12 and 49 streptococci, their cell walls and membranes did not stain any of the kidney glomeruli. Moreover, fluorescein labelled type 12 M protein could be localised in 2 of 5 acute cases, and 4 of 5 subacute ones. When the immunoferritin technique was applied to the more severe cases, ferritin labelled antibodies to gamma globulin, $beta_{1c}$ globulin, and type 12 streptococcus were observed to localise in the glomeruli in electron dense precipitates in the endothelial and mesangial cells, the GBM, and on either side of it, and even in the capillary lumens and in Bowman's space (Seegal *et al.*, 1965; Andres *et al.*, 1966).

An earlier hypothesis by Markowitz and Lange (1962) stated that nephritis prone individuals possess glomerular antigens which cross-react with those of nephritogenic streptococci. They showed that normal, pooled glomeruli cross-reacted with nephritogenic streptococcal cytoplasmic components by passive hemagglutination and passive cutaneous anaphylaxis. The chemical composition of the glomerular extract and the streptococcal cell membrane were fairly similar (Markowitz and Lange, 1964). A more detailed account was presented later (Lange, 1969). Rapaport *et al.* (1969) induced nephritis in 13/16 dogs by unilateral perfusion of their kidneys with sera against a nephritogenic type 12 streptococcus prepared in sheep. The kidney lesions included glomerular swelling and hypercellularity, as well as infiltration of glomerular loops with polymorphonuclear leukocytes. There was evidence of sheep gamma globulin in 8 of the 16 dog kidneys. In a set of preliminary experiments in which antiserum prepared against a soluble extract of group A type 12 streptococcal membrane were used to perfuse kidneys unilaterally in 9 dogs, 8 of these developed microscopic evidence of nephritis, 5 of which had heavy albuminuria as well. However, sheep gamma globulin could only be detected in 2 kidneys. A similar experiment in rhesus monkeys was reported by Markowitz (1969). Glomerulonephritis was induced by one small dose of rabbit gamma globulin prepared against solubilised streptococcal cell membrane injected into the abdominal aorta of the monkey. Proliferative lesions were seen by light and electron microscopy. Deposition of gamma globulin in granular fashion in the glomeruli was noted. However the incidence and intensity of renal

damage was not noted.

Speculations were raised concerning the significance of a fraction of streptococcal membrane sharing characteristics with a similar extract of human glomeruli and mouse H-2 transplantation antigens (Lange and Markowitz, 1969). More recently Hirata and Terasaki (1970) found that type 1 streptococcal M protein has a structure common to human histocompatibility antigens as demonstrated by its ability to inhibit antilymphocyte serum. Holm *et al.* (1968) in turn found cross reactions between Lancefield extracts of type 3 and type 12 streptococci and human kidney. Each type shared two antigens with the kidney, but only one antigen was common to both types.

In experiments with the sera of 16 patients with poststreptococcal AGN, Treser *et al.* (1968) found that their gamma globulin fractions labelled with fluorescein stained the GBM and the mesangium of kidney tissue biopsied at the early nephritic stage, but not with those of normal kidneys. Interestingly enough, 11/15 normal sera when treated in this manner and applied to similar kidney sections also showed positive but weaker staining. Inference could be drawn that normal adults possess anti-streptococcal antibodies which can react with kidneys of AGN cases. Streptococcal plasma membranes completely absorbed the staining ability of the labelled sera. Rabbit antisera against the same streptococcal membranes when used to stain the sections first completely blocked this staining by fluorescein labelled human sera. The authors concluded that "plasma membrane constituents are present in the glomeruli of patients with acute poststreptococcal glomerulonephritis" but that

these sites are available only in early disease. Only streptococci isolated from AGN could specifically inhibit the staining property of kidneys from AGN cases (Ty *et al.*, 1970).

From the above studies on the nature of reactants in the glomeruli of nephritic kidneys it can be seen that there is yet no clearcut trend in their identification. On one hand, Seegal *et al.* found type 12 antigens, possibly M protein, in the glomeruli. On the other hand, Lange *et al.* found that it was the plasma membranes of nephritogenic streptococci which localised in the kidney. Even if it is proven that one substance localised and not the other, the question remains: why does it preferentially localise in the kidneys over and above all the other antigens man is exposed to? If indeed the GBM shares antigenic determinants with nephritogenic streptococcal cell membranes, the answer appears more obvious. The experiments of Rapaport *et al.* support this view although their high rate of success in obtaining GN by perfusing the kidneys of dogs with anti-streptococcal membrane sera is unlike the incidence of human AGN even in epidemic circumstances. More evidence in these areas is required.

e. Cell-Mediated Immune Response in Glomerulonephritis

As it has been discussed, the most highly favored theory on the pathogenesis of acute glomerulonephritis is the immune complex theory, supported by experimental and histological evidence. Confirmation of such a theory required identification of the immune reactants in the glomeruli of nephritic kidneys. Humoral antibody response by the host has been then the only type of immune response under serious consideration

by most workers in the field.

In recent years, few reports have been presented regarding the presence of cell-mediated immune response to streptococcal or kidney antigenis in patients with glomerulonephritis. Most of the cases under study were of the progressive and chronic variety, with very few cases of acute glomerulonephritis.

An earlier study of cases of diffuse glomerulonephritis using skin tests yielded equivocal results when normal kidney extract was used as the skin sensitising antigen (Wagner and Rokop, 1956). In the more recent studies, leukocyte migration inhibition tests alone or in conjunction with skin tests have been used as an index of cell-mediated immunity.

In a study of "leukocytic hypersensitivity" induced experimentally in rabbits by skin infection with group A streptococci, Lau and Scherago (1967) found that the skin tests remained positive longer than did the leukocyte immunity which only reappeared with the reinfection with a living heterologous type of streptococcus. Skin tests therefore cannot always be correlated with leukocyte studies.

Bendixen (1968) examined migration inhibition of leukocytes from cases of active glomerulonephritis, terminal nephropathia and pyelonephritis in comparison with normal controls, using fetal kidney homogenate as the antigen in the *in vitro* test. It was found that most of the active glomerulonephritides fell outside of the normal range of leukocyte reactivity, with the two cases of AGN showing leukocyte migration inhibition to hepatic as well as to renal homogenates.

In another study by Rocklin *et al.* (1970) a variation of the *in vitro* leukocyte migration inhibition technique was used. They incubated their antigen, solubilised GBM, with the lymphocytes of nephritic patients. The supernatant was collected and incubated with normal guinea pig peritoneal exudate cells whose migration would be inhibited if any migration inhibitory factor (MIF) had been released into the supernatant. The authors only found MIF present when the lymphocytes were from those nephritic patients whose glomeruli had linear anti-gamma globulin staining. In AGN where the gamma globulin deposits are granular, no MIF was produced. These results do not agree entirely with Bendixen's. However one must take into account the slightly different antigens and the techniques used.

Still another study by Zabriskie *et al.* (1970) used two parameters to measure lymphocyte response to streptococcal antigens in patients with progressive glomerulonephritis. In addition to using migration inhibition to measure the lymphocyte reactivity, they also quantitated DNA synthesis of the lymphocytes when the latter were exposed to antigen by measuring C^{14} thymidine incorporation. Group A streptococcal membranes and cell wall preparations inhibited the migration of lymphocytes from all but one of the nephritic cases. Both antigenic preparations also increased C^{14} thymidine incorporation into DNA 4- to 10-fold in the lymphocytes from nephritic cases. Patients with unrelated renal diseases such as pyelonephritis had no cellular reactivity toward the streptococcal antigens.

Indeed so far these workers have shown that cell-mediated

immunity exists in streptococcal infections and glomerulonephritis, against streptococcal antigens, and in addition against kidney components in the latter instance. The pathogenetic importance of this phenomenon is another matter. Much more evidence is required to show that cell-mediated immunity is as important as the humoral one in the initiation of the nephritic process in glomerulonephritis. At the same time, one cannot exclude the possibility that cell-mediated immunity may be vital in maintaining the kidney damage which had been initiated by a humoral mechanism, since experimentally it has been shown that with a "good" humoral antibody response, the damage is acute and resolves within a very short time (Dixon *et al.*, 1961). Then if such is the case, cell-mediated immunity probably plays a greater role in progressive and chronic forms of glomerulonephritis rather than in AGN.

4. Bacteriophages and Bacteriocines in Relationship to Acute Glomerulonephritis

a. Bacteriophages

Isolated reports have dealt with the possible association of lysogeny and nephritogenicity of streptococci. That erythrogenic toxin production is due to the lysogenic state of the group A streptococcal cell (Zabriskie, 1964) indicates that this might be possible.

Earlier Rammelkamp (1957) had observed that the nephritogenic types 12, 4, and 25 were more susceptible to lysis following infection with bacteriophage. Krause (1957), Kjems (1960), and Maxted (1964) all

found a high rate of lysogeny among streptococci. However, Maxted could not find any significantly higher incidence of susceptibility to lysis by virulent bacteriophage among the nephritogenic streptococci as compared to the non-nephritogenic ones when he scanned 179 strains, 78 of which were from nephritic cases.

In the largest survey of different strains of group A streptococci to date, Wannamaker *et al.* (1970) screened mainly type 12 and type 49 strains and also found that these nephritogenic streptococci are "frequently lysogenic and often release phage spontaneously". However they could correlate any lytic bacteriophage pattern or serological make-up with either type, nor could then find a common lytic pattern for all nephritogenic strains.

While working with freshly isolated strains from the epidemic area in Trinidad, Potter *et al.* (1968) made the interesting observation that only one of the 27 strains from nephritic cases was lysogenic while 33% of the strains from non-nephritic sources were. Outside of the nephritic area, only 6% of the strains were lysogenic. It was speculated that the nephritogenic strains were "cured" of bacteriophage.

b. Bacteriocines

Kuttner in 1966 discovered that some strains of group A streptococci produced "bacteriocines" which diffuse into the medium of an agar plate and can inhibit the growth of other strains of group A organisms as well as non-group A organisms. Attempts to isolate the bacteriocines were unsuccessful. Although few strains could serve as indicators, many

strains could produce these inhibitory factors. By using two indicator strains, she noted that the nephritogenic type of streptococci, 12, 4, and 49, produced them more frequently.

Since the above observation could be quite significant, Overturf and Mortimer (1970) tried to repeat Kuttner's experiments as closely as possible. No significant differences in bacteriocine production were noted by them between nephritogenic and non-nephritogenic group A streptococci. Sixty-two % of the former produced bacteriocines while 54% of the latter did. However the nephritogenic strains displayed larger and clearer zones.

It can be seen from these studies that there is no obvious association between lysogeny and nephritogenicity. As for the bacteriocines, not enough is known about them to draw any conclusions. One report noted that bacteriocines may be parts of incomplete bacteriophage as has been found with those of other bacterial species (Read, 1970).

5. L-Forms

That L-forms of streptococci may play a role in group A streptococcal sequelae remains a fascinating possibility. Streptococcal L-forms are readily induced *in vitro* under conditions of increased salt concentration, the addition of serum, and a penicillin gradient (Sharp, 1954), or by using phage-associated lysin in place of penicillin (Gooder and Maxted, 1958; Freimer *et al.*, 1959). The L-forms continue to produce M protein and hyaluronic acid (Freimer *et al.*, 1959). Mortimer (1965) was able to induce L-forms in mice by inoculating group A streptococci

intraperitoneally and then looking for L-forms at death. He did not mention within how many days the animals usually died. Various animal experiments were done to see how long L-forms could persist *in vivo*. Schmitt-Slomska *et al.* (1967) found that intraperitoneal inoculation of L-forms led to their persistence of at least 25 days whereas intravenous inoculation led to quick disappearance. However, L-forms of group A streptococci labelled with fluorescein injected into the thigh muscles of mice could not be detected beyond 3 days (Rickles *et al.*, 1969). Others also failed to keep L-forms viable *in vivo* (Clasener *et al.*, 1970).

In humans, Klodnitskaya (1962) was able to find L-forms in the blood of 28/42 scarlet fever patients in all stages of disease if penicillin was given early in disease. Otherwise the L-forms could only be detected in the very early stages of disease. Wittler *et al.* (1962) found transitional forms of growth which may be related to L-forms in blood cultures of 5/6 cases of rheumatic fever. Kagan (1962) reported similarly.

D. Summary of the Review

From the foregoing review one can see that poststreptococcal acute glomerulonephritis is a well established entity. Of the various theories formulated to explain its pathogenesis, the one involving an immune response on the part of the host is favored. However, the exact role of nephritogenic streptococci in such a system needs to be clarified.

III. MATERIALS AND METHODS

A. Strains of *Streptococcus Pyogenes*

	Designation	Group	Type
Nephritogenic strain	12-35	A	12
Non-nephritogenic strain	C-2	A	6

The strains of streptococci used in the preparation of nephrotoxin and associated preparations as listed above were isolated in an epidemic of nephritis in Nova Scotia in 1952 (Reed, 1953). For some preparations, M enriched streptococci of strain 12-35 were used (Becker, 1967).

B. Preparation of the Semi-Purified Extracts

1. Media

a. Todd-Hewitt Broth

This was either reconstituted from Difco dehydrated Todd-Hewitt broth, lot No. 049201, control No. 499080 and No. 537399, or was "home-made" according to the same formula as was used in the original experiments (see Appendix). The broths were dispensed as follows for each batch of growth:

i. Five test tubes each containing 5 ml.

ii. Four 125-ml Erlenmeyer flasks each containing 25 ml.

iii. Two 4-liter Erlenmeyer flasks each containing 1500 ml.

b. Sheep Blood Agar Plates

These were used for checking the purity of broth cultures and consisted of sheep red blood cells in nutrient base (5% v/v) (see Appendix).

2. Growth

Each batch of broth culture was propagated according to the following protocol:

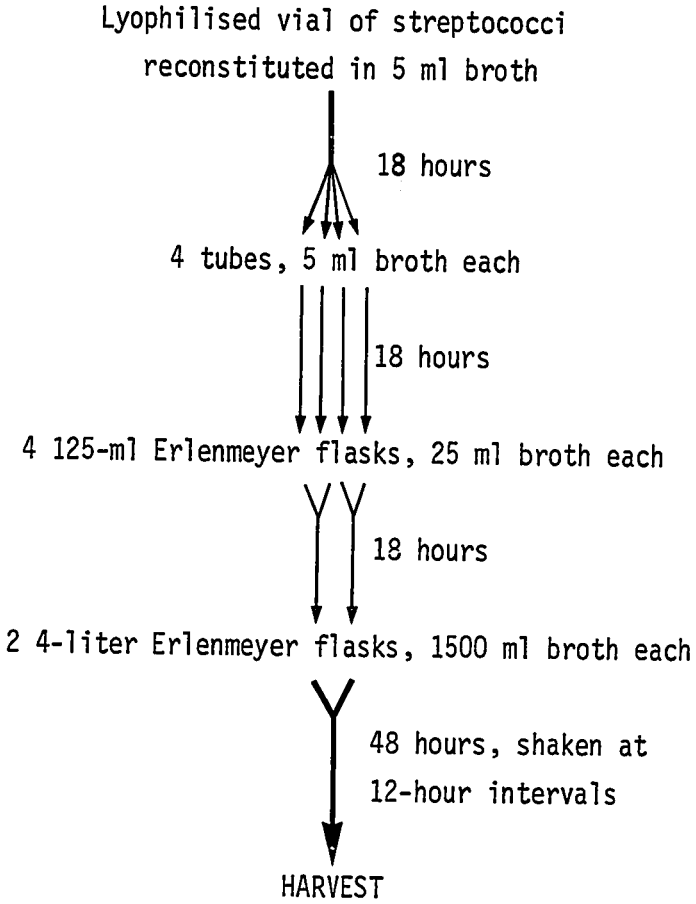


Fig. 1. Growth of streptococcal broth cultures.

All growth was at 37 C. Sheep blood agar plates were streaked at each stage of transfer to ensure purity of culture.

3. Harvest of Cultures

The broth cultures were allowed to sediment by standing. The supernatants were decanted and filtered through a membrane filter (0.45 μm porosity, Millipore) under positive pressure.

4. Dialysis

The 2.5 to 3.0 liters of culture filtrate were dispensed into cellulose dialyser tubing (1 1/8" inflated diameter, Visking). Each dialyser bag contained approximately 150 ml of filtrate. Dialysis was carried out in 10 volumes of prechilled distilled water at 8 C for 48 hours with constant stirring. The dialysate, approximately 25 to 30 liters, was concentrated in a flash evaporator ("Precision" Laboratory Evaporator, Precision Scientific Company) to 1000 ml. In this flash evaporator, the liquid to be concentrated is subjected to vacuum, fluxed through a jacket containing circulating steam and air mixture, and the vapor condensed over water cooled coils and discarded. The temperature of the dialysate was maintained at less than 30 C.

5. Ammonium Sulfate Precipitation

All ammonium sulfate precipitations were performed at room temperature. Two 10 ml samples were taken from each batch of the concentrated dialysate and placed in separatory funnels (50 ml capacity). Ammonium sulfate was added in small quantities with intermittent shaking until the saturation point was reached (i.e. when one crystal of the salt remained

and could not be dissolved). The amount of salt required to bring each 10 ml sample to saturation was noted and the duplicate values averaged. This was usually between 6 and 7 g per 10 ml.

The remaining concentrated dialysate was placed in a 3 liter beaker over a magnetic stirrer. Ammonium sulfate was first added in small amounts to bring the concentration up to 60% of saturation. Stirring was continued for at least 2 hours. Fine granules of precipitate were either in suspension or deposited in a thin film on the wall of the beaker. The supernatant was filtered through a fritted glass funnel (medium grade). The precipitate was discarded.

The clear supernatant was then placed in a clean beaker and enough ammonium sulfate was added in the same manner as previously to bring the solution up to 75% of saturation with ammonium sulfate. Following another 2 hours of stirring, the supernatant was again filtered through a fritted glass funnel (medium grade). This supernatant was now discarded, and the "60-75%" precipitate was retained.

An excess amount of aqueous ammonium sulfate solution (100% saturation) was used to wash the precipitate on the wall of the beaker and on the fritted glass funnel. The process was repeated using another aqueous ammonium sulfate solution (80% saturation).

This washed, "60-75%" precipitate was then redissolved in approximately 20 ml of distilled water. This will be referred to as the semi-purified extract, or "60-75%" nephrotoxin.

6. Storage of the Semi-Purified Extract

For use in further purification and chemical analyses, the semi-

purified extract was stored in the -20 C freezer.

For animal injections the semi-purified extract was adjusted to 20 mg/ml according to a standard curve derived from a plot of the values of $O.D._{276\ nm}$ versus the amount of 4X crystalline bovine serum albumin (Nutritional Biochemicals). According to previous data, spectrophotometric determination of sample concentration provides a non-destructive method of quantitation which yields results similar to those obtained by dry weight determinations (Ng, 1969). Following the adjustment of the concentration of the semi-purified extract, it was sterilised by membrane filtration (0.22 μm porosity, Millipore) and dispensed into sterile, rubber capped serum vials. The solutions were stored at 4 C. Sterility was tested by plating a small sample of the extract on a blood agar plate which was then incubated at 37 C.

C. Further Purification of the Semi-Purified Extract

1. Electrophoretic Separation of the Semi-Purified Extract

a. Paper Electrophoresis

The method used was essentially the same as Matheson and Reed's (1959) except the buffer strength was decreased: 0.01 M sodium borate-0.005 M sodium chloride, pH 9.2 (see Appendix). Either 3 MM or No. 1 chromatographic grades of paper were used. The electrophoretic runs were carried out for 3 hours at 10 v/cm.

b. Thin-Layer Electrophoresis

Electrophoretic separation of the semi-purified extract was carried out on thin-layer cellulose plates for the collection of the "nephrotoxic" bands. One advantage over paper electrophoresis was the reproducibility of electrophoretic separations as well as the ease with which the nephrotoxic bands could be eluted from the cellulose.

i. Preparation of the layers

The MN 300 cellulose powder (Macherey, Nagel & Co.) was washed with an excess of aqueous ethanol (50%) followed by an excess of distilled water, usually at least 2 liters of the latter per 30 g of powder. The washings were done over a fritted glass funnel (coarse grade). The washed cellulose was dried in a 50-60 C oven overnight. To spread 4 glass plates (20 x 40 cm), 14 g of washed MN 300 cellulose powder were homogenised in 90 ml borate buffer (one to be used in electrophoresis) by using an electric mixer (Virtis Research Equipment) at medium speed for 2 minutes. The slurry was spread onto the glass plates with a Desaga spreader. For preparative work the layers were spread to 375 um thickness but for analytical work the layers were spread to 250 μm. The plates were partially dried at room temperature until excess moisture had evaporated, for 20 to 40 minutes, depending on humidity of the room.

ii. Electrophoresis

The plates were placed in plexiglas electrophoresis tanks (custom-made, modified from Matheson, 1957) containing the same borate buffer as used above. Paper end flaps (7 x 20 cm, Macherey, Nagel & Co.) were used to bridge the void between the plate and the buffer troughs. The plates were equilibrated for 15 minutes at 10 v/cm. For preparative

separation, about 0.3 ml of the semi-purified extract was applied in a streak across the plate, 10 cm from the end near the cathode. A reference dye mixture (RBY dye, Gelman) of three dyes with different charges, apolon yellow, brilliant blue and red amaranth, was used to extimate the speed of separation. The plates were run for 3 hours at 10 v/cm.

iii. Drying and detection

The plates were dried for 10 minutes in a 90 C oven and examined in an ultraviolet viewing box (Chromato-Vue, Ultra-Violet Products, Inc.).

iv. Elution of nephrotoxin associated bands

The three fast migrating bands adjacent to the anode were separately scraped and collected by the use of a micro-preparative vacuum zone collector (Desaga). The collected cellulose powder was eluted with distilled water through an Allihn tube with a fritted disc (20 mm diameter x 100 mm height, fine porosity). The eluates were lyophilised and stored over a dessicant.

2. Column Chromatography of the Semi-Purified Extract

a. Concurrent Fractionation and Desalting Using Sephadex G-25 Gel

i. Preparation of columns

Sephadex G-25 medium gel (1,000 to 5,000 mw fractionation range, Pharmacia) was swollen in distilled water at room temperature for a minimum of 3 hours. Eight g dry gel were used for a 15 mm x 30 cm column and 40 g for a 25 mm x 45 cm column (Pharmacia). The fines were decanted off in repeated washings. The gel was finally equilibrated in the eluant

to be used, 0.02 M NaCl. The columns were packed by gravity. After washing with two volumes of eluant, a small amount (1 or 3 ml) of 0.1% blue dextran (Pharmacia) was placed on the column to check for proper column packing and function as evidenced by discrete banding and an expansion factor of less than 5X.

ii. Elution of samples

The samples were the semi-purified extracts of either type 12 or type 6 streptococcal culture filtrate origin. Approximately 1.5 ml were placed on the small column and 3 ml on the large column. The flow rate was maintained at 20 ml per hour. Three ml fractions were collected and read at 276 nm. Usually 10X to 20X dilutions were necessary for obtaining optical density values within a readable range. The ammonium sulfate eluted with the fractions was localised and quantitated by adding 0.1 ml of the eluate in each fraction to 3 ml 5% $BaCl_2$, which was then mixed well and read at 400 nm.

b. Further Fractionation on Sephadex A-25 Anion Exchanger

i. Preparation of columns

The gel was prepared in a similar way as the G-25 gel had been. Distilled water was used throughout for washing and elution. Only the 15 mm x 30 cm column was used.

ii. Elution of samples

The samples which were to be fractionated consisted of the last peak obtained by fractionation of the semi-purified extracts on G-25 medium as described in the previous section. The flow rate was maintained at 20 ml per hour. Three ml fractions were collected and read at 276 nm.

Fig. 2 below is a summary of the purification procedures which were used to prepare nephrtoxin and associated preparations and which have just been discussed in the preceding section. The same procedures were followed in the preparation of control material from both type 6 streptococcal culture filtrates and from the Todd-Hewitt broth alone for *in vivo* studies as well as for chemical characterisation of the purified material.

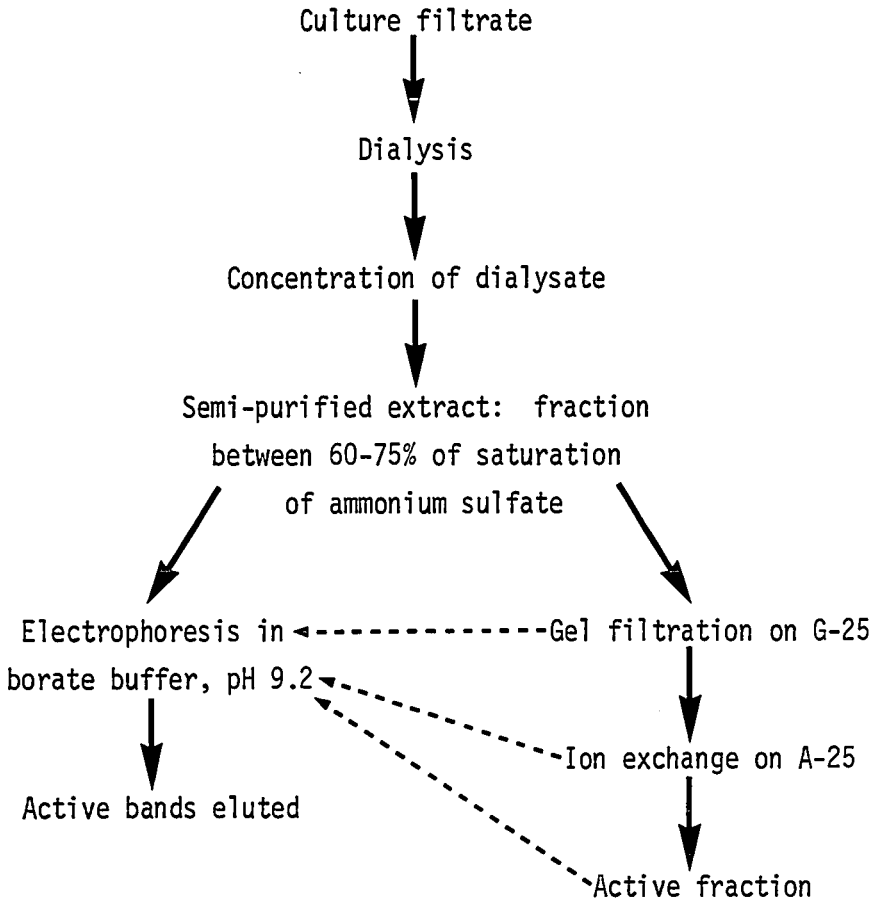


Fig. 2. Flow sheet for the purification of nephrotoxin.

D. *In Vivo* Studies Using Nephrotoxin Preparations

1. Animals

New Zealand white rabbits with an average weight of 2.5 kg (Canadian Breeding Association) were used. The rabbits were fed antibiotic free Purina rabbit chow, a complete ration. Fresh water was supplied daily.

2. Blood Pressure Measurements

The peripheral arterial pressure of the rabbit was determined by using the ear capsule method which was modified by Matheson (1953) from Reed and Kropp (1944). The capsule consisted of a brass tube (15 mm inside diam., 6 cm length) with a side arm to which was connected via a T tube a mercury manometer and a sphygmomanometer pressure bulb. The capsule was airtight, one end being sealed by a lucite window, and the other end by a condom rubber membrane. By attaching the capsule to a microscope barrel, its height could be adjusted.

When the blood pressure was measured, the rabbit's right ear was always used since the left one was saved for injections only. The rabbit was placed in a mold which had been shaped with plaster of Paris (Matheson, 1953). The ear was flattened out on a glass plate below which was illumination from a microscope lamp. The capsule was lowered so that its membrane was snug against the area of the ear where the central artery bifurcates. Care was taken to ensure that the capsule rim

did not press on the ear. The pressure bulb was pumped until the artery was occluded. The pressure was then released slowly until the blood could be seen to circulate again, at which point the pressure in mm Hg was read off the manometer. Six to ten consecutive readings at the minimum of 2 minute intervals were taken to yield an arithmetic mean for each day's blood pressure determination.

3. Urinalysis

a. Hematuria

The supernatant was tested with Hemastix reagent strips (Ames) for occult blood. The reaction is based on the peroxidase action of hemoglobin which in the presence of cumene hydroperoxide and orthotolidine forms a blue oxidation product (Elder *et al.*, 1962).

b. Albuminuria

A preliminary screening of the urine samples was performed with Combistix strips (Ames). Any urine sample giving a recording of "trace" or more was subjected to the qualitative sulfosalicylic acid test which was performed by adding a few drops of sulfosalicylic acid (20% w/v in distilled water) to the supernatant of the centrifuged urine. A positive test for albumin is indicated by clouding of the urine. Positive qualitative samples were subjected to the quantitative Kingsbury-Clark test (Kingsbury *et al.*, 1926). To 2.5 ml of urine supernatant were added 7.5 ml sulfosalicylic acid (3% w/v in distilled water). The tube was mixed and allowed to stand for 10 minutes before it was compared to a --

set of permanent albumin turbidity standards (0.01 to 0.1%, Harleco).

c. Urinary Casts

When albuminuria or hematuria was found, urinary casts were looked for in the urine sediment. The Sternheimer-Malbin stain (1951) was used for staining (see Appendix). The presence of any hyaline, granular, cellular or waxy casts were noted.

4. Injections

All injections were made intravenously via the marginal ear vein. For each experiment, the nephrotoxin containing preparations and the control preparations were injected in 3 to 5 divided doses at 2 to 3 day intervals.

5. Autopsies

All rabbits were bled by intracardial puncture before sacrifice. Approximately 50 to 100 ml blood were collected. The bloods were allowed to clot at room temperature in half of an hour, rimmed, and placed in the refrigerator for 2 hours for clot retraction. They were then centrifuged at 1000X G in the cold for 15 minutes. The sera were aspirated off, lyophylised, and stored.

The rabbits were killed by air embolism by the intravenous injection of 10 cc air. A midline ventral incision was then made. All organs were examined and the animal's general state of health was noted. Any ascitic fluid was noted, as was cardial valvular damage. The kidneys

were removed, decapsulated, and examined for petechial hemorrhages, paleness, enlargement, and other gross abnormalities.

Then the kidneys were bisected medially. The medial sides were examined for gross abnormalities and discoloration. Immediately photographs were taken of the medial and peripheral views of the kidneys.

6. Light Microscopy

a. Fixation

The kidneys which had been bisected medially were sectioned further. Each half kidney was sliced into two pieces by an incision made parallel to the plane of the medial cut. The bigger slice with the two exposed sides was cut into three wedges along the rays of the pyramids. The center wedge was fixed overnight in two changes of formalin (10% of a 38% stock formaldehyde Analar reagent, in saline).

b. Embedding

The fixed tissue was placed in stainless steel capsules, which were then all placed into a stainless steel basket and positioned onto the automatic Tissuematon (Fisher). The protocol for dehydration and paraffin infiltration is presented in the Appendix. The tissue sections from the liquid paraffin were embedded in fresh liquid paraffin in an embedding mold fitted with a plastic disposable embedding ring (Tissue-Tek, Canlab). The paraffin mold was allowed to solidify partially at room temperature and then hardened in cold water.

c. Sectioning

The paraffin blocks were sectioned at 4 μm on a rotary microtome (American Optical Company). The cut sections were floated over a 40 C waterbath which contained a small amount of gelatin (0.5 g/liter). The sections were dried on slides overnight in a 37 C incubator.

d. Staining

Kidney sections from all animal experiments were stained with both the hematoxylin-eosin stain, a general stain, and the periodic acid-Schiff stain which brings out the mucopolysaccharide containing membranes. In one experiment the alcian blue-periodic acid-Schiff stain was also done on the sections (Spicer and Warren, 1960). The alcian blue is specific for acidic mucopolysaccharides. All staining procedures are described in the Appendix.

7. Immunofluorescence Microscopy

a. The Freezing of Tissue

Kidney tissue pieces 0.5 cm^3 in size were cut from the cortex and quick-frozen in 2-methylbutane which had been precooled to -70 C over a dry ice-acetone mixture. Frozen tissue was stored in a -70 C freezer.

b. Sectioning

The frozen blocks were cut at 4 μm thickness on a cryostat (American Optical Company) which was maintained at -15 C. The cut sections were placed on clean slides kept at room temperature.

c. Fixation

The slides were pre-rinsed in 3 changes of cold saline within 5 minutes and then fixed in cold acetone for 1 to 2 minutes. Then the slides were briefly rinsed in cold phosphate buffered saline (PBS) (0.01 M, pH 7.2, see Appendix). The slides could be stored for a short period of time in this cold PBS without affecting the tissue.

d. Preparation of Staining Reagents

i. BSA-rhodamine

The BSA-rhodamine (lissamine rhodamine RB 200 conjugated bovine serum albumin, Microbiological Associates Inc.) was used as reconstituted from the lyophilised commercial product as a counterstain to decrease the amount of non-specific staining of the tissue by fluorescein labelled reagents (Smith *et al.*, 1959). For use, the BSA-rhodamine was diluted 20X with PBS.

ii. Normal rabbit gamma globulin-FITC

Fluorescein isothiocyanate (FITC) labelled normal rabbit gamma globulin was used as a control staining reagent to see that the non-specific staining of tissue was minimal.

Normal rabbit globulins were precipitated from whole serum with ammonium sulfate (adapted from Kendall, 1937). One volume of serum was added to 1 volume of saturated aqueous ammonium sulfate with stirring in the cold. After a few hours, the mixture was centrifuged. The precipitate was dissolved in a known amount of saline and the precipitation procedure was repeated. The precipitate was redissolved and dialysed against cold running water overnight, and then against cold saline for

24 hours. The contents of the dialysis bag were centrifuged to remove any non-specific precipitate that might have formed.

A sample was taken from the above globulin fraction and run against whole rabbit serum on cellulose acetate electrophoresis. The cellulose acetate strips (2.5 x 30 cm, Millipore) were moistened by being floated on the surface of barbital buffer (pH 8.2, see Appendix), and then slowly immersed. The strips were blotted briefly and placed in the electrophoresis chamber (Gelman) which contained the same buffer. The strips were equilibrated for 15 minutes at 10 volts/cm. The samples were applied about one quarter the way from the cathode. After a 90-minute run, the strips were taken out and placed directly into 0.02% nigrosin in 5% acetic acid for 30 minutes. The strips were decolorised with 5% acetic acid and rinsed with water before being dried between a filter paper and a covering glass plate at room temperature. In the purified globulin sample, very little contaminating albumin was seen.

To label the globulins with FITC, the dialysis technique of Clark and Shepard (1963) was used. The purified globulin fraction was adjusted to 5 to 10 mg of protein per ml by using the Lowry method of protein estimation, with 4X crystalline BSA as a standard (see Appendix). The final dilution was made up in 0.5 M carbonate-bicarbonate buffer, pH 10, and placed in a dialysis bag suspended in a cylinder containing cold fluorescein isothiocyanate (FITC, General Biochemicals) solution (0.1 mg/ml 0.05 M carbonate-bicarbonate buffer, pH 10) and allowed to label with stirring for 18 hours at 5 C.

The labelled globulins were separated from free FITC by chroma-

tography of the contents of the dialysis bag on a Sephadex G-25 (coarse) column (K 25/45). The conjugated globulin fraction came down with the void volume whereas the FITC was retarded. PBS (0.01 M, pH 7.2, see Appendix) was used for elution. Usually 10 to 15 ml of crude conjugate were applied. The flow rate used was 40 ml/hour.

The ratio of dye to protein in the conjugate was determined according to Wells *et al.* (1966) by reading the absorbance of the pooled fractions at 276 nm and 493 nm, the absorbance peaks for protein and FITC respectively. These absorbance values were aligned on a nomograph for the determination of the FITC/protein (F/P) ratio which should be between 1.5 and 4 for optimal staining of tissue with minimal non-specific fluorescence. The F/P ratio in the case of the prepared FITC labelled normal rabbit globulin was 4 to 5.

For use, the rabbit gamma globulin-FITC was diluted 10X the original serum volume.

iii. Goat anti-rabbit gamma globulin-FITC

Whole goat antiserum to rabbit gamma globulin (Antibodies Inc.) was first checked for antibody titer and specificity against whole rabbit serum by Ouchterlony's double diffusion method and by immunoelectrophoresis respectively.

iii a. Ouchterlony's double diffusion. Each microscope slide was coated with 2 ml molten 1% agarose in water (L'industrie Biologique Française). After the slides had been placed in the cold for 1 to 2 hours, 2 mm holes were punched and removed from the agar. Different dilutions of goat anti-rabbit gamma globulin were placed in alternate

holes against rabbit gamma globulin. The titer of the purchased antiserum was found to be 1:40.

iii b. Immunoelectrophoresis (Clausen, 1969). For each run 6 microscope slides were arranged on a rack and coated with 20 ml 1% agarose in barbital buffer (0.05 M, pH 8.6, see Appendix). After the gel had hardened in the cold for 1 to 2 hours, the holes and troughs were punched. The gel in the holes were first removed and replaced with whole rabbit serum which was then subjected to electrophoresis for 70 minutes at 10 volts/cm. Then the slides were taken out and the gel in the troughs replaced by goat antiserum. Diffusion was allowed to take place for 16 hours at 21 C in a moist atmosphere. The goat antiserum was found to be quite specific against rabbit gamma globulin, with only faint precipitin lines visible against rabbit albumin. It was considered specific enough for labelling with FITC for use.

iii c. Washing and staining of the double diffusion and immunoelectrophoresis slides. The slides were washed in 2 changes of saline over a period of 2 days, followed by distilled water for 1 hour. Then they were stained with azocarmine solution (0.05% in acetate buffer which consisted of 20 ml acetic acid and 150 ml glycerol added to 830 ml water) for 3 hours. Decolorisation took place for 2 hours in 2 changes of acetate buffer. The buffer was rinsed off with distilled water and strips of moistened filter paper placed on the gel. The gel was allowed to air dry overnight.

iii d. Purification and labelling of the goat antiserum. The globulins were purified from the goat antiserum in the same way as the

rabbit serum was purified, as described in the previous section. Labelling of the purified goat globulins was also performed as had been done previously. The batch of the labelled goat globulins with an F/P ratio of 4 to 5 was made up in PBS, 10X the original serum volume for use in staining. Antibody titer of the labelled product was checked again by double diffusion against rabbit gamma globulin.

iv. Unlabelled goat anti-rabbit gamma globulin

The commercial goat antiserum to rabbit gamma globulin was used in a 10X dilution for the blocking control. For staining an amount of this dilution of antiserum was added to an equal part of BSA-rhodamine.

e. Staining

For each kidney specimen, the following immunofluorescence slides were made:

i. Tissue control

The fixed tissue was rinsed with PBS.

ii. Control for non-specific fluorescein staining

The fixed tissue was rinsed with PBS and stained with BSA-rhodamine for 20 to 30 minutes, rinsed with PBS for 5 minutes, and followed by staining with normal rabbit gamma globulin-FITC for 30 to 45 minutes.

iii. Specific staining for rabbit gamma globulin

The fixed tissue was rinsed with PBS and stained with BSA-rhodamine for 20 to 30 minutes, rinsed for 5 minutes with PBS, and followed by staining with goat anti-rabbit gamma globulin-FITC for 30 to 45 minutes.

iv. Blocking control for specific staining of rabbit gamma globulin

The fixed tissue was rinsed with PBS, stained with unlabelled goat anti-rabbit gamma globulin + BSA-rhodamine for 45 to 60 minutes, rinsed for 5 minutes with PBS, and followed by goat anti-rabbit gamma globulin-FITC for 30-45 minutes.

f. Washing and Mounting of Immunofluorescence Slides

All staining solutions were removed by washing the slides with 3 changes of saline in a period of approximately 15 minutes, and briefly in distilled water before mounting with either 0.5 M carbonate-bicarbonate buffered glycerol (1 part buffer to 9 parts glycerol, Pital and Janowitz, 1963), or by glycine buffered glycerol (Roitt and Doniach, 1966; see Appendix).

g. Observation of the Immunofluorescence Slides

An IQ Fluorescence Conference Microscope (Gillett & Sibert) was used. The slides were viewed without the barrier filter. The background tissue autofluorescence was blue and the specific stained parts green-yellow.

Photographs were taken on Kodak Ektachrome X with the barrier filter D in place at 3 to 5 minutes of exposure.

8. Electron Microscopy

a. Primary Fixation

Tissue pieces no larger than 1 mm^3 were cut from the kidney

cortex and fixed immediately in 1.5% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4, see Appendix) for 1 to 1½ hours. The tissue was "minced" while it was immersed in the fixative. The fixed tissue was rinsed well in buffer and stored in the buffer for up to 2 weeks if necessary.

b. Post-Fixation

This took place for 1 to 1½ hours in 2% osmic acid in a veronal acetate buffer and sucrose solution (see Appendix). After post-fixation the tissue pieces were rinsed briefly in water.

c. Dehydration and Embedding

The fixed tissue was dehydrated by using gradually increasing concentrations of ethanol, from 20% to 100%, followed by 2 changes of propylene oxide. Spurr low-viscosity embedding medium (Polysciences, Inc.) was used to embed the tissue. (For details see Appendix).

d. Sectioning

Thick sections of 1 μm were first obtained and placed on a slide for staining with an aqueous toluidine blue solution (0.1% in 2.5% Na_2CO_3, pH 11.1) for 30 to 120 minutes (Trump *et al.*, 1961). The stain was washed off with tap water, rinsed briefly with 90% ethanol, followed by absolute ethanol, before being cleared in xylol and mounted in Permount. If glomeruli were found under the light microscope, thin sections were made from an adjacent portion of the same block.

e. Staining

The thin sections were placed on 400-mesh copper grids which had

been cleaned with 3% acetic acid, rinsed with water, and afterwards with acetone. Sequential staining was done with 4% aqueous uranyl acetate: ethanol (3:1) for 7 minutes (Watson, 1958), followed by Reynold's lead citrate (see Appendix) for 7 minutes.

f. Examination of Thin Sections

The sections were viewed on a Philips 300 electron microscope. For each section, 6 to 12 photographs were taken at random. Routinely the magnification was set at X3750.

9. Complement Assay

In one experiment the complement activity of the rabbit sera was assayed by measuring the hemolytic titers of the sera, expressed as 50% hemolytic units ($C'H_{50}$'s), in the presence of sensitised sheep erythrocytes. All complement titrations were performed in duplicate. Each time an assay was run, a duplicate set of titrations of a standard pooled guinea pig serum was also included. The large tube modification (total reaction volume 7.5 ml) of the standard method of hemolytic complement titration was used (Wadsworth *et al.*, 1931; Mayer *et al.*, 1945; Kabat and Mayer, 1961).

a. Buffers

Veronal buffer (pH 7.5) was used for all dilutions. It was made up as follows. These 2 solutions were first mixed together: 85 g NaCl + 3.75 g sodium 5,5-diethyl barbiturate in 1400 ml distilled water, and 5.75 g 5,5-diethyl barbituric acid in 500 ml hot distilled water. After

cooling to room temperature, 5 ml of a stock solution containing 1 M $MgCl_2$ and 0.3 M $CaCl_2$ were added. The whole mixture was made up to 2000 ml with distilled water. This stock veronal buffer solution was stored in the refrigerator and diluted 5X with distilled water before use.

Alsever's solution was used to dilute fresh sheep blood 1:1 for the preparation of sheep erythrocytes to be described later. In 1200 ml distilled water were dissolved 24.6 g glucose, 9.6 g sodium citrate dihydrate, and 5.04 g NaCl. The pH was adjusted to 6.1 with citric acid. The solution was sterilised by membrane filtration (0.22 μm, Millipore).

b. Centrifugation

Unless otherwise stated, all centrifugation in this section was done at 1000X G.

c. Collection of Rabbit Sera

The rabbits were bled from the marginal ear vein. Each time 2.5 ml were taken with a syringe fitted with a 22 gauge needle. The vein was dilated by rubbing the ear with xylol, but care was taken that no trace of xylol contaminated the blood. Afterwards the ear was cleansed with ethanol:water (1:1). As soon as the blood had clotted (15 to 30 minutes at room temperature), it was rimmed and centrifuged. The serum was aspirated off and frozen in a -70 C freezer. The maximum time interval between bleeding and the freezing of sera was 2 hours.

d. Collection of Standard Guinea Pig Sera

Ten guinea pigs were bled intracardially. Approximately 10 ml blood were collected from each. The bloods were treated the same way as

rabbit bloods were, except the whole procedure took slightly longer. The sera were pooled and treated with saline washed sheep erythrocytes (1.5 ml packed volume per 50 ml serum) to remove natural hemolytic antibody. The mixture was kept in the cold for 10 minutes and then centrifuged in the cold. The procedure was repeated once more. The serum pool was dispensed in 1 ml amounts for storage in the -70 C freezer.

e. Sheep Erythrocytes

These served as the antigen in the complement fixation system. The sheep erythrocytes were obtained from blood stored in an equal volume of Alsever's solution and allowed to age for 1 week before use. The sheep blood was centrifuged and the plasma and buffy coat removed. The packed erythrocytes were washed 3 times with 5 to 10 volumes of veronal buffer. They were finally suspended in approximately 18 volumes of veronal buffer to make a 5% suspension and filtered through 4 layers of cotton gauze. One ml of the filtered suspension was lysed with 14 ml of 0.1% $NaHCO_3$. The absorbance of this lysate was read at 541 nm in a Zeiss spectrophotometer using a cuvette of 1 cm light path. The erythrocyte suspension was adjusted to exactly 5% based on this reading according to the established value of the absorbance of a 5% erythrocyte suspension ($O.D._{541\ nm}$ of 10^9 erythrocytes/ml = 0.700).

f. Antiserum to Sheep Erythrocytes

The antiserum (Batch 99/1 from the Department of Microbiology and Immunology, McGill) had been prepared by immunising rabbits with erythro-

cyte stromata obtained from the acetic acid lysate of sheep erythrocytes (Kabat and Mayer, 1961). The hemolytic antibody titer of the antiserum was determined as follows: The antiserum's complement activity was first removed by heating the serum in a 56 C waterbath for 20 minutes. Halving dilutions of the antiserum were made from 1 x 400 to 1 x 51,200. One ml of each dilution was used to sensitise 1 ml of 5% erythrocyte suspension for 10 minutes at 37 C. Into the reaction tubes were placed 1 ml of sensitised erythrocytes, 1 ml of guinea pig complement in excess (serum diluted 1 x 100), and 5.5 ml buffer. The tubes were incubated in a 37 C waterbath for 90 minutes and placed at 4 C overnight. The next morning, the $O.D._{541\ nm}$ was determined following centrifugation. The degree of hemolysis at each antiserum dilution was expressed as % hemolysis based on the 100% hemolysis tube containing 1 ml of 2.5% sheep erythrocytes which had been lysed by the addition of 6.5 ml of 1% $NaHCO_3$. The values of % hemolysis were plotted against the dilution of antiserum on a graph. The greatest dilution of antiserum giving rise to complete hemolysis was considered 1 unit. Four units, or 1 x 800 dilution as found, were used routinely for all subsequent experiments for the sensitisation of an equal volume of a 5% erythrocyte suspension.

g. Standard Guinea Pig Complement

The pool of guinea pig sera was titrated each time an assay of rabbit complement was performed to ensure the uniformity of all the reagents so that the results are comparative. Eight dilutions of guinea pig serum, from 1 x 100 to 1 x 1000, were made in duplicate each time.

h. Titration of Rabbit Complement

All dilutions of rabbit serum were made with cold buffer and were made as quickly as possible, since the average $C'H_{50}$ titer of rabbit sera is low to begin with. For titration the rabbit sera were diluted from 1 x 10 through to 1 x 50. One ml of the rabbit serum was added to 1 ml sensitised erythrocytes and 5.5 ml buffer.

i. Controls

These were all made in duplicate:

i. Blank control. 1 ml sensitised erythrocytes + 6.5 ml buffer.

ii. Control of complete hemolysis. 1 ml 2.5% erythrocyte suspension + 6.5 ml 0.1% $NaHCO_3$.

iii. Control of complete hemolysis with sensitised erythrocytes. 1 ml sensitised erythrocytes + 1 ml guinea pig complement (1 x 50) + 5.5 ml buffer.

iv. Erythrocyte control, no hemolysis. 1 ml 2.5% erythrocyte suspension + 1 ml guinea pig complement (1 x 50) + 5.5 ml buffer.

j. Calculation of $C'H_{50}$'s in Rabbit Sera

The hemolysis of erythrocytes in the presence of antibody and complement follows a sigmoidal curve; therefore the $C'H_{50}$ cannot be arrived at if only limited number of points for plotting the amount of complement source used against % hemolysis obtained are available. Von Krogh (1916) devised an equation to express this phenomenon: $x = K(\frac{y}{1-y})^{1/n}$,

where x =the amount of complement, y = the degree of hemolysis (e.g. 50% = 0.5), and $1/n$ is affected by reaction conditions. The logarithmic derivation of this equation, $\log x = \log K + \frac{1}{n} \log (\frac{y}{1-y})$, yields a linear relationship between $\log x$ and $\log (\frac{y}{1-y})$, with the slope $1/n$, and the intercept log K. At 50% hemolysis, $\frac{y}{1-y} = 1$, and $x = K$, or in logarithmic terms, when $\log \frac{y}{1-y} = 0$, $\log x = \log K$. Therefore, for each rabbit serum, all points between 10% to 90% hemolysis were plotted against the amount of serum used. Either the log/log paper was used, or the values were converted to log functions, both according to von Krogh's equation, so that straight lines were obtained on graph plots for the extrapolation of the $C'H_{50}$ value.

k. Anticomplementary Action of Rabbit Sera

The sera were also checked for any factors which could be anti-complementary by the incubation of a 1 x 10 dilution of the rabbit serum with an equal amount of a 1 x 50 dilution of guinea pig serum for 10 minutes at 37 C before addition to sensitised erythrocytes. The degree of hemolysis obtained was expressed as the ratio of % change to the % hemolysis brought about by 1 ml of a 1 x 100 dilution of guinea pig serum in the usual test reaction performed on the same day.

E. *In Vitro* Tissue Culture Toxicity Studies

1. Media and Reagents

a. Growth Medium

To maintenance medium No. 3 (Gibco) was added heat inactivated

calf serum (Gibco) to bring the final serum concentration up to 10%. Antibiotics were added to these final concentrations: penicillin, 100 units/ml; streptomycin, 50 µg/ml; and fungizone, 0.5 µg/ml. (See Appendix).

b. Maintenance Medium

The same maintenance medium No. 3 as above was used without the additional calf serum.

c. Trypsin

For trypsinising chick embryos and monolayers, a 0.25% trypsin solution in 0.01 M phosphate buffered saline was used (see Appendix).

d. Glassware

All glassware were washed in 7X detergent solution (Linbro Chemical Company) and rinsed thoroughly before sterilisation. If the glassware had been previously used for tissue culture, they were pre-soaked in 0.25% trypsin.

2. Preparation of Primary Chick Embryo Cultures

The primary chick embryo culture was obtained from Mrs. Margit Homola and had been prepared as follows:

Eight 10 to 12 day chick embryos were eviscerated, minced, and trypsinised for 30 minutes with stirring at 37 C. After centrifugation at 1000X G for 5 minutes, 1.4 ml of packed cells were obtained. The cells were resuspended in a small amount of medium and filtered through

8 layers of gauze. The filtrate was centrifuged once, washed with medium, and centrifuged again. The cells were adjusted to 10^6/ml in growth medium and dispensed in 60 ml volumes into 8 1-liter Roux bottles. After 3 days of growth in a moist chamber in an atmosphere of 5% CO_2 with one change of medium in the interval, monolayers were obtained and ready for subculture.

To prepare the primary chick embryo subculture, the supernatant above the monolayer in each Roux bottle was replaced by 15 to 20 ml of the trypsin solution. After 1 minute, the trypsin solution was poured off. The layer was incubated for 10 minutes at 37 C, washed off with 10 ml medium, and centrifuged for 5 minutes to sediment the cells. The packed cells were resuspended in 20 to 50 volumes of growth medium. Viable count was determined by using the hemocytometer. For counting, 0.5 ml trypan blue solution (0.4% in Hanks' balanced salts solution, Gibco) was added to 1 ml of the cell suspension which had been diluted again 5X. All the unstained refractile cells were counted. The final cell concentration was adjusted to 10^6/ml with growth medium. Roller tubes with screw caps (16 x 125 mm) were each inoculated with 1 ml of the suspension and placed in tissue culture racks for incubation at 37 C in the moist 5% CO_2 chamber. After 48 hours, the roller tube cultures were ready for use.

3. Preparation of L Cell Cultures

Monolayers of L cells (derivative of the NCTC clone 929, mouse connective tissue, Sanford *et al.*, 1948) were provided by Mrs. Homola.

4. Prepared Cell Cultures

Commercially available cell strains and cell lines were obtained as roller tube cultures (16 x 125 mm) from Flow Laboratories.

Designation	Derivation
H Ep-2	Human carcinoma of the larynx, heteroploid (Moore *et al.*, 1955)
WI-38	Human embryonic lung, diploid (Hayflick and Moorhead, 1961)
LLC-MK_2	Rhesus monkey kidney, heteroploid (Hull *et al.*, 1962)
Vero	African green monkey kidney, heteroploid (Yasumura and Kawakita, exact ref. unable to locate)

F. Chemical Characterisation of Nephrotoxin Associated Preparations

1. Dansylation as a Means of Identifying N-Terminal Amino Acids

Gray's (1967) method for dansylation of the N-terminal amino acid of a peptide or polypeptide was followed. The reagent 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) reacts with the basic amino, phenolic, thiol, and imidazole groups of amino acid under alkaline

conditions. However only the alpha amino acid of a peptide so labelled is stable to acid hydrolysis of the labelled peptide. Separation of the dansylated amino acids were achieved by ascending chromatography or by high voltage electrophoresis, or by a combination of both (Arnott and Ward, 1967).

a. Materials for Dansylation

i. Standard known di- and tripeptides

The following peptides were used in amounts of 0.3 to 0.5 μM, or approximately 0.1 mg each: Glycyl-D-asparagine, glycyl-L-leucine, histidyl-histidine, DL-leucyl-glycine, and L-leucyl-glycyl-glycine.

ii. Standard polyamino acids

One mg each was used: Poly-DL-alanine, mw 1700; poly-L-aspartic acid, mw 3300; and poly-L-glutamic acid, mw 38,000. All polyamino acids were obtained from Yeda Research & Development Co.

iii. Unknown samples

Approximately 0.1 to 1 mg of samples eluted from thin-layer electrophoretic separations of the "60 to 75%" semi-purified extracts were used.

b. Procedures for Dansylation

i. Method A

Into a small test tube were placed the dry sample, 25 μl 0.2 M $NaHCO_3$ and 25 μl DNS-Cl (5 mg/ml acetone). The mixture was capped and allowed to stand at room temperature for 3 hours with occasional agitation.

ii. Method B

Into a small test tube were placed the dry sample, 25 µl 0.5 M $NaHCO_3$ and 8 M urea, and 25 µl DNS-Cl (10 to 20 mg/ml acetone). The mixture was capped and allowed stand at room temperature overnight with occasional agitation.

c. Hydrolysis

The labelled samples were dried *in vacuo* in a rotary flash evaporator. The dried sample was dissolved in 0.5 ml of 6.7 N HCl and transferred to a 2-ml freeze-drying ampoule which was then sealed under vacuum. Hydrolysis was performed at 105 C for 20 hours. Upon completion of hydrolysis, the ampoule was opened and the contents dried *in vacuo* in the flash evaporator at temperatures not exceeding 40 C. In order to drive off all the HCl, the evaporation procedure was repeated twice following the addition of small amounts of water. The dried samples were made up in a minimal amount of pyridine/water (50%, v/v).

d. Separation of Dansyl Amino Acids

Both electrophoresis (Gray and Hartley, 1963) and thin-layer chromatography (Mesrob and Holeyšovský, 1966; Arnott and Ward, 1967) were used to separate the hydrolysis products. A standard set of dansyl amino acids (Pierce Chemical Company) was used to identify the unknown ones.

i. High voltage electrophoresis

The buffer was 0.8% acetic acid-0.4% pyridine, pH 4.4. The samples were spotted on No. 1 paper (20 x 40 cm) which had been moistened in

buffer, and run in a water-cooled high voltage apparatus (Camag) at 80 volts/cm for 2 hours.

ii. Ascending thin-layer chromatography

For one system, the 20 x 20 cm glass plates were coated with 250 um layers of MN 300 cellulose powder (Macherey & Nagel Co., 1:6 w/v slurry) and dried at room temperature. The samples were spotted 1 cm from the bottom edge and the plates were run in the 0.8% acetic acid-0.4% pyridine system for 60 minutes. An alternate system was useful in separating DNS-leucine from DNS-isoleucine and consisted of plates coated with silica gel H:kieselgel G, 5:1 (30 g slurried in 75 ml water) which were dried and activated at 100 C for 15 minutes before development in petroleum ether:2,4,6-trimethylpyridine:methyl ethyl ketone:glacial acetic acid, 75:5:24:3 for 75 minutes.

iii. Detection of dansyl amino acids

After fan-drying, both the electrophoretic and chromatographic separations were examined at 360 nm (Chromato-Vue, Ultra-Violet Products, Inc.).

2. Amino Acid Analysis of the Semi-Purified Extracts

The Sephadex G-25 column separated frations of the "60 to 75%" semi-purified extracts of group A type 12 and type 6 streptococcal broth cultures and of a Todd-Hewitt broth alone were acid-hydrolysed. The amino acid composition of the hydrolysates were examined by two-dimensional thin-layer chromatography. (The elution profile of the semi-purified extract from a Sephadex G-25 column is shown in *Fig. 8.*)

a. Hydrolysis

All sample fractions were pooled and lyophilised. For hydrolysis, 1 ml of 6.7 N constant boiling HCl was added to a 2 to 3 mg dried sample in a small, narrow-necked freeze-drying ampoule, sealed *in vacuo* with a propane flame, and hydrolysed for 18 hours at 105 C.

b. Thin-Layer Chromatography

The hydrolysates were dried *in vacuo* in a flash evaporator under 40 C. The procedure was repeated 3 times after the addition of a small amount of water each time. Finally the dried sample was dissolved in a minimal amount of 50% pyridine/water (v/v).

i. Preparation of plates

A slurry of 29 g silica gel G:60 ml water was spread on glass plates (20 x 20 cm) at 250 µm thickness with the Desaga spreader. Before use, the plates were air-dried completely.

ii. Development of plates

Approximately 10 ul of sample were spotted in the lower left corner of a plate 1 cm from each edge. The plate was developed for 1 hour in the first solvent (chloroform:methanol:17% ammonia, 2:2:1) after which it was air-dried completely for 2 hours, or fan-dried for half of an hour. The plate was then developed in the second solvent (phenol:water, 3:1, w/v) for 2½ hours. After the second development the plates were fan-dried for half of an hour. (Fahmy *et al.*, 1961).

iii. Detection of amino acids

The Moffat-Lytle (1959) polychromatic stain for amino acids was used as a spray reagent. To make the reagent, 50 ml of solution A (50 ml

0.2% ninhydrin in ethanol:10 ml glacial acetic acid:2 ml 2,4,6-trimethylpyridine) were mixed with 3 ml of solution B (1% $Cu(NO_3)_2.3\ H_2O$ in ethanol) just before use. After the plates were sprayed, they were dried at room temperature and then at 95 C for 10 minutes. The amino acids which became visible at room temperature and after heating were both noted.

3. Gas-Liquid Chromatography

The unknown samples, Bands 1, 2 and 3 which are the fastest migrating bands obtained from electrophoretic separation of the semi-purified extracts of both type 12 and type 6 streptococcal broth cultures, were examined by gas-liquid chromatography. These bands are associated with nephrotoxicity. The control material was the eluate of blank portions of the cellulose thin-layer electrophoresis plates which had been run in the same borate buffer as the semi-purified extracts. Although the cellulose powder had been pre-washed with ethanol and water before being plated onto glass plates, there was a possibility that some impurities remained.

a. Hydrolysis

The samples were subjected to either hydrolysis in 2 N HCl at 100 C for 1 hour or in 6.7 N HCl at 105 C for 20 hours.

Following hydrolysis the samples were dried *in vacuo* on the flash evaporator as in Section III-F2. Finally, the samples were each dissolved in a small amount of water in a 5 ml vial and freeze-dried. If the

samples were not used immediately, they were stored in a dessicator.

b. Preparation of Trimethylsilyl Derivatives

Bis-(trimethylsilyl)acetamide (BSA) with acetonitrile as solvent was used throughout for the preparation of trimethylsilyl (TMS) derivatives (Klebe *et al.*, 1966). The other silylating agents tried included hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in a 2:1 ratio with toluene as solvent, and trimethylsilyldiethylamine (TMSDEA) with tetrahydrofuran as solvent (Birkoffer and Ritter, 1960; Rühlmann, 1961; Pierce, 1968; Gehrke and Leimer, 1970; Pacáková *et al.*, 1971).

In addition to the unknown samples, various standard compounds such as amino acids, purines and pyrimidines and monosaccharides were silylated. For each sample, the procedure was as follows: To 1 mg of a very dry sample in a 5 ml very dry disposable glass vial were added 200 µl BSA and 100 µl acetonitrile. The vial was covered with a foil-lined cap. The reactants were allowed to reflux over a sandbath at 80 to 90 C until all the solid sample had dissolved, in 10 to 15 minutes.

c. Chromatographic Column

The glass column (6' length, 3.5 mm i.d.) was packed with the solid support chromosorb W (acid washed, dimethyldichlorosilane treated, 100/120 mesh) coated with 15% SE-52, a phenyl-, methyl-silicone liquid phase, all of which were obtained from Applied Science Laboratories, Inc. To coat the chromosorb W, 10 g of it were slurried with 1.5 g SE-52 which had been dissolved in 50 ml chloroform. The chloroform was dried off by vacuum suction. The column was packed by gravity and plugged at the two ends with glass wool.

The column was conditioned before use by being bled at 210 C for 48 hours.

d. The Gas Chromatograph and Chromatographic Conditions

The Hewlett-Packard Model 402 gas chromatograph with a flame ionisation detector was used. The flash heater was set at 260 C and the detector at 275 C. The column was operated isothermally at 160 C. The flow rate of the carrier gas, dry nitrogen, was adjusted to 20 ml per minute which was obtained with the rotameter set at 1.2 and the gas gauge at 40 lb/in^2. For the flame, the hydrogen flow rate was adjusted to 40 ml per minute with the rotameter set at 3.5 and the gas gauge at 20 lb/in^2. The air supply for the hydrogen flame was adjusted to 300 ml per minute with the rotameter set at 3.0 and the gas gauge at 20 lb/in^2. Before use each day, the gas chromatograph was equilibrated for 1 hour with the flame lit. For overnight shutdowns, only the flame was turned off by cutting off its hydrogen and air supplies.

Approximately 0.2 to 0.5 μl of sample was injected each time into the gas chromatograph with a 1-μl Hamilton syringe. If samples were found to be too concentrated, carbon tetrachloride was used for dilution. The response was monitored at 10 x 8 or 10 x 4 attenuation.

4. Quantitative Chemical Analysis of Nephrotoxin Associated Preparations

a. Protein Estimation

i. By ultraviolet spectrophotometry

The optical density of the samples were read at 276 nm against

a bovine serum albumin (4X crystalline, Nutritional Biochemicals) standard (diluted from 0.1 to 1 mg/ml).

ii. By the Lowry method

The method according to Wu (1922) and Lowry *et al.* (1951) was used. Concentrations of standard bovine serum albumin from 0.05 to 0.5 mg/ml were within the range of accuracy. (See Appendix).

iii. By the biuret method

The method of Gornall *et al.* (1949) was used. Concentrations of standard bovine serum albumin from 1 to 10 mg/ml were within the range of accuracy. (See Appendix).

b. Determination of Hexosamines

The Davidson modification (1966) of Elson and Morgan's (1933) original method using 2,4-pentanedione (acetylacetone) and *p*-dimethyl-aminobenzaldehyde was used (see Appendix). Sensitivity of the method ranged from 0.05 to 0.1 μM of glucosamine.

c. Determination of Organic Phosphate

The micro phosphate method of Meun and Smith (1968) was followed with slight modification (see Appendix). The accuracy of the method was best at phosphorus concentrations of 1 to 6 μg/ml.

d. Determination of Nitrogen

The method of Jacobs (1959) using indanetrione hydrate (ninhydrin) was applied to the eluates of the fast migrating bands obtained by electrophoretic separations of the semi-purified extracts only. The accuracy of

this method was in the range of 0.5 to 5.0 μM of NH_3 (50 to 500 μg bovine serum albumin) of the original sample. (See Appendix).

5. Qualitative Spot Analysis of Nephrotoxin Associated Preparations

a. Preparation of Samples

The samples were either hydrolysed in 2 N HCl at 100 C for 1 hour or in 6.7 N HCl at 105 C for 16 to 20 hours and then flash evaporated as previously had been done to drive off all the HCl. The dried samples were made up in 50% ethanol.

b. Descending Paper Chromatography

The samples were spotted on No. 1 chromatography paper (20 x 40 cm) and developed in 1-butanol: acetic acid: water (4:1:5). The lower phase was used to saturate the atmosphere of the glass tank, and the upper phase was used as the developing solvent. After development, the paper was fan-dried, or was dried in an 80 C oven for 10 minutes.

c. Detection Methods

The chromatograms were examined by ultraviolet light (Woiwod, 1950) and by the spot tests listed in Table 1.

6. Ultrafiltration of the Semi-Purified Extracts

Ultrafiltration using Diaflo membranes (Amicon Corp.) was attempted as a means of desalting the semi-purified extracts which had been prepared by ammonium sulfate precipitation (Blatt *et al.*, 1965).

TABLE 1

Reagents Used to Treat Paper Chromatograms for the Detection of Hydrolysed Nephrotoxin Associated Substances

*Reagent**	*Substances tested for*	*Reference*
Moffat-Lytle	Amino acids	Moffat and Lytle (1959)
Aniline hydrogen phthalate	Reducing sugars	Partridge (1949)
Elson-Morgan	Hexosamines	Elson and Morgan (1933)
Quinine sulfate	Phosphate esters	Rorem (1959)
Silver nitrate-bromophenol blue	Purines, pyrimidines and derivatives	Wood (1955)
Sodium fluoresceinate	Aromatic and heterocyclic compounds	Waldi (1965)

* Methods used in the preparation of these reagents are presented in the Appendix.

In using the ultrafiltration apparatus, filtration was effected by positive pressure applied over the solution to be filtered with 45 lb/in^2 of nitrogen.

7. Ultraviolet Spectra of the Semi-Purified Extracts and Their Component Fractions

The ultraviolet spectra were obtained on the Beckman DK-2A scanning spectrophotometer from 200 nm to 340 nm of the semi-purified extract, its Sephadex G-25 column fractions, the DEAE-Sephadex column fractions of Fraction 4 from the G-25 column, and the eluates of the 3 fast migrating bands from electrophoretic separation of the semi-purified extracts. All of these preparations were of type 12 streptococcal origin. Whenever possible, comparative spectra were also made from preparations of type 6 streptococcal and Todd-Hewitt broth origin.

8. Fluorescence Spectra of Further Purified Component Fractions of the Semi-Purified Extracts

Fluorescence spectra of the DEAE-Sephadex fractions of Fraction 4 from the Sephadex G-25 column and of the 3 fast migrating bands of both type 12 and type 6 origin were obtained on the Aminco-Bowman spectrophotofluorometer (by courtesy of the Department of Pathology, McGill University). The fluorescence spectra were recorded at their excitation maxima.

9. Infrared Spectra of the Active Bands

In order that meaningful infrared spectra of the 3 fastest migrating bands of both type 6 and type 12 origin could be obtained, any salts (mainly borate) and trace impurities were removed by means of a weak cation exchanger, Bio-Rex 70 (Bio-Rad, Inc.). This resin was first washed with methanol, then with distilled water, followed by reactivation with 0.5 N HCl. Washing with distilled water was continued until the pH of the eluate approached that of the distilled water. The resin was then poured into a column. The bed size was 10 mm x 15 cm.

The first fraction, BR-1, consisted of the peak obtained with water as the eluant. The eluted fractions were monitored at 270 nm. The second fraction, BR-2, consisted of the peak obtained when the second eluant, 1 N HCOOH, was used. BR-2 was flash evaporated 2 to 3 times before it was freeze-dried along with BR-1.

Approximately 1 mg of the lyophilised material was pulverised with 300 mg dry potassium bromide (KBr) with an agate or a mullite mortar and pestle (Cook *et al.*, 1954). A compressed, transparent disc of this mixture was made on a 25 ton ring press (Research & Industrial Instruments Company) by applying 10,000 kg pressure for 3 minutes.

A Perkin-Elmer 457 infrared scanner (by courtesy of the Department of Epidemiology and Health, McGill University) was used to obtain the spectra.

IV. EXPERIMENTAL PROCEDURES AND RESULTS

A. *In Vivo* Activity of Nephrotoxin Preparations

The different terms used in designating the preparations which were injected into animals intravenously are listed in Table 2 below. The sequence of the production of these nephrotoxin preparations had been summarised in *Fig. 2* on p. 41.

TABLE 2

Preparations of Nephrotoxin Used in *In Vivo* Experiments

Preparation of Nephrotoxin	*Source of Preparation*
Culture filtrate	Todd-Hewitt broth which had been used in the cultivation of streptococci of a nephritogenic strain, filtered through Millipore membrane.
Semi-purified extract	Dialysate obtained from 48 hour dialysis of above culture filtrate, then precipitated between 60 to 75% of saturation with ammonium sulfate.
G-25 Fraction 1, 2, 3, or 4	One of the 4 fractions obtained by gel filtration of the semi-purified extract on Sephadex G-25 with 0.2 M NaCl as eluant.
Band 1, 2, or 3	Material eluted from any of the bands 1, 2, or 3, respectively the 3 fastest migrating bands obtained upon electrophoresis of the semi-purified extract in 0.01 M borate buffer.

1. Changes in Total Complement Activity Following Nephrotoxin Administration

Introduction - Lange *et al.* (1960) and Pickering *et al.* (1968) found that total hemolytic complement activity was uniformly decreased in the sera of cases of acute glomerulonephritis. In addition, the latter group found evidence of the presence of a heat labile complement inactivator in these sera which probably acts on one or more of the complement components from C'3 to C'9. In the sera from cases of persistent hypocomplementemic glomerulonephritis, Spitzer *et al.* (1969) could demonstrate the presence of a "C'3 nephritic factor" which could cleave C'3. Such factor(s) could possibly then activate the last components of complement in the absence of the initiating C'1,4,2 complex. The relationship of these factors to Götze and Müller-Eberhard's (1971) C'3 proactivator is not known. The importance of all of these complement inhibitor(s) or lytic factor(s) in glomerulonephritis is still unknown. Their role in the cause or effect of kidney damage is not yet clear.

The following experiment was designed to demonstrate whether nephrotoxin could initiate a similar complement inactivation process.

a. Animals and Treatment

Table 3 following summarises the treatment schedule of the rabbits used in Experiment 1. All sera from each individual rabbit were frozen at -70 C and later titrated on the same day. All rabbits were exsanguinated, killed, and autopsied 66 days following the first injection. Sections of the kidneys were fixed for light microscopy.

TABLE 3

Schedule of Experiment 1

Group	Type 6 control	Type 12
Treatment	Semi-purified extract of type 6 streptococcal origin; 50 mg per rabbit i.v.	Semi-purified extract of type 12 streptococcal origin; 50 mg per rabbit i.v.
Rabbits used	461, 462, 463, 464	465, 466, 467, 468
Injection schedule	At extract concentration of 20 mg/ml, 0.7 ml on day 0, 0.9 ml on day 2, 0.9 ml on day 7.	
Blood pressure measurements	Days 0, 3, 9, 11, 15, 23, 30, 35, 39, 50, 53, 56, and 64.	
Urinalyses	Days 10, 17, 32, 38, 45, 51, 58, and 65.	
Sera collection	Days 0, 7, 14, 22, 30, 38, 50, and 56.	

b. Results

i. Blood pressure measurements

Traditionally, elevation of blood pressure of the rabbits to 30 mm Hg and above for a period of a minimum of 10 days has been arbitrarily chosen as the criterion of hypertension (Matheson, 1957; Cheuk, 1960). According to this criterion, some hypertension was observed in both groups of rabbits in Experiment 1, as seen in *Fig. 3*, although the type 12 rabbits exhibited greater rise in pressure.

ii. Urinalyses

All urines were negative for occult blood. Some samples were positive for protein when Combistix was used for screening. However, when these positive urines were subjected to the sulfosalicylic acid test, only No. 467 from the type 12 group was positive on day 58, having the equivalent of 0.01% albumin.

iii. Total hemolytic activity of complement

All the sera were titrated for total hemolytic complement activity as outlined in Materials and Methods in the lysis of sensitised sheep red blood cells. *Fig. 4* shows that among the rabbits injected with the type 6 preparation, No. 463 showed very slight depression whereas No. 462 and No. 464 showed greater depression of total hemolytic complement activity. Among the rabbits injected with the type 12 preparation, as seen in *Fig. 5*, only No. 468 showed decreased activity.

iv. Anticomplementary activity of the rabbit sera

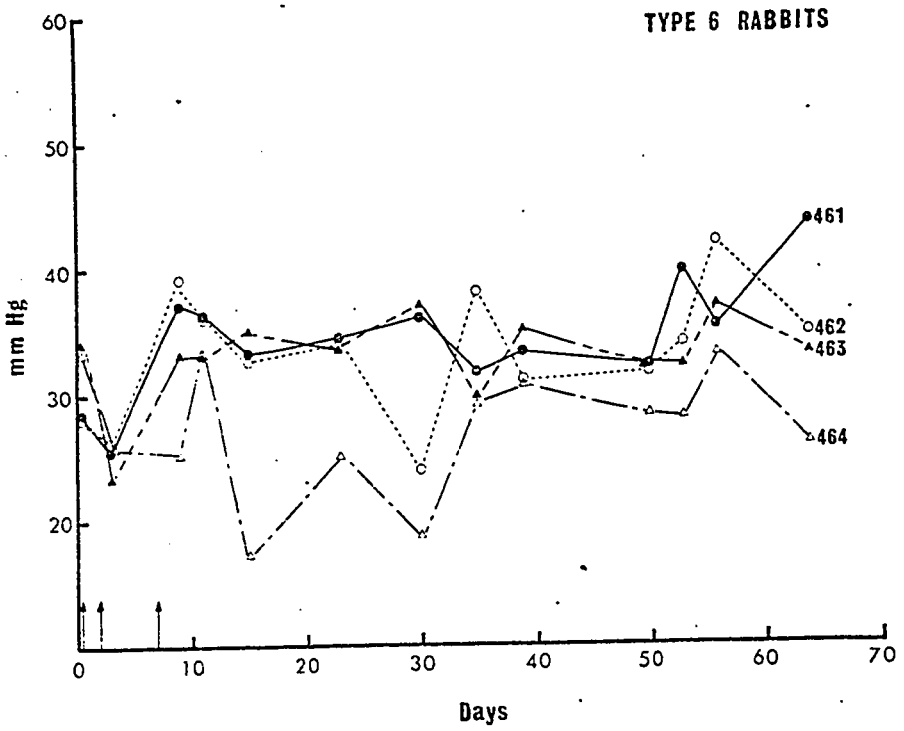
Among the rabbits injected with the type 6 preparation, only No. 462 exhibited the increasing ability to inhibit guinea pig complement

activity (*Fig. 6*). Among the rabbits injected with the type 12 preparation, No. 465 showed a transient ability to inhibit guinea pig complement activity (*Fig. 7*).

v. Kidney morphology

Table 4 on p. 85 summarises the kidney morphology observed among the rabbits in Experiment 1. From the gross morphology, the kidneys of rabbits which were injected with the type 12 preparation appeared to be affected. However upon microscopic observation, any characteristic lesions of acute glomerulonephritis (AGN) were not apparent, possibly due to the masking by the presence of lesions of unknown etiology or to the duration of the experiment.

Summary of Experiment 1 - Only minimal difference was obtained in the blood pressures of the two groups of rabbits. No significant changes were found in their urinalyses. The total hemolytic activity of complement and anticomplementary activity of the sera of rabbits injected with the type 12 preparation were not distinguishable as a whole from those of the animals injected with the type 6 preparation. Changes in complement activity therefore did not characterise the rabbits receiving the nephrotoxin containing semi-purified extract.



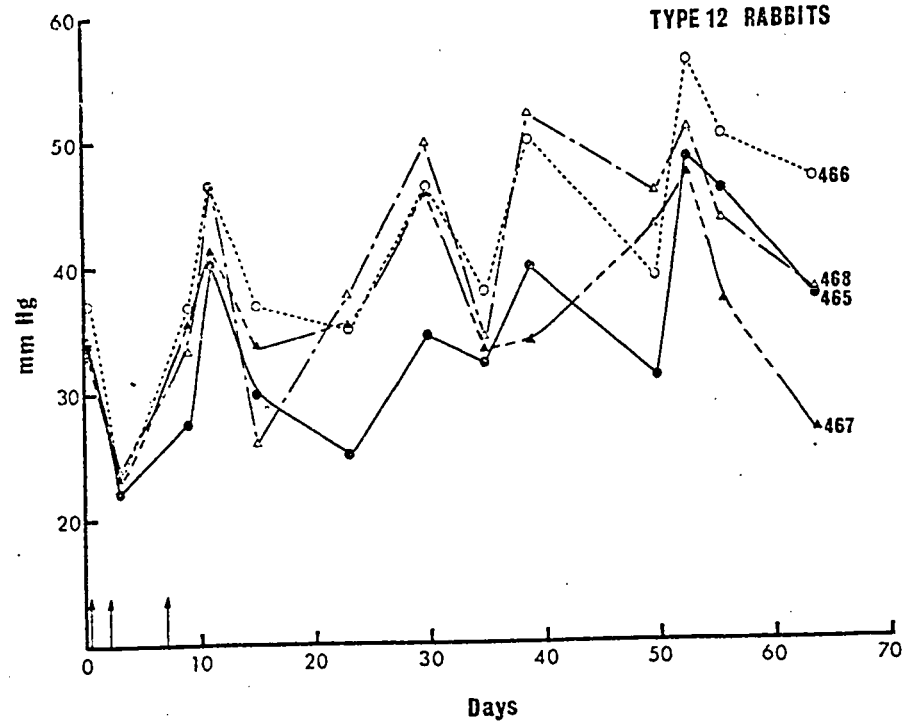


Fig. 3. Blood pressure readings of the rabbits from Experiment 1 *receiving the type 6 or the type 12 streptococcal semi-purified extract.*

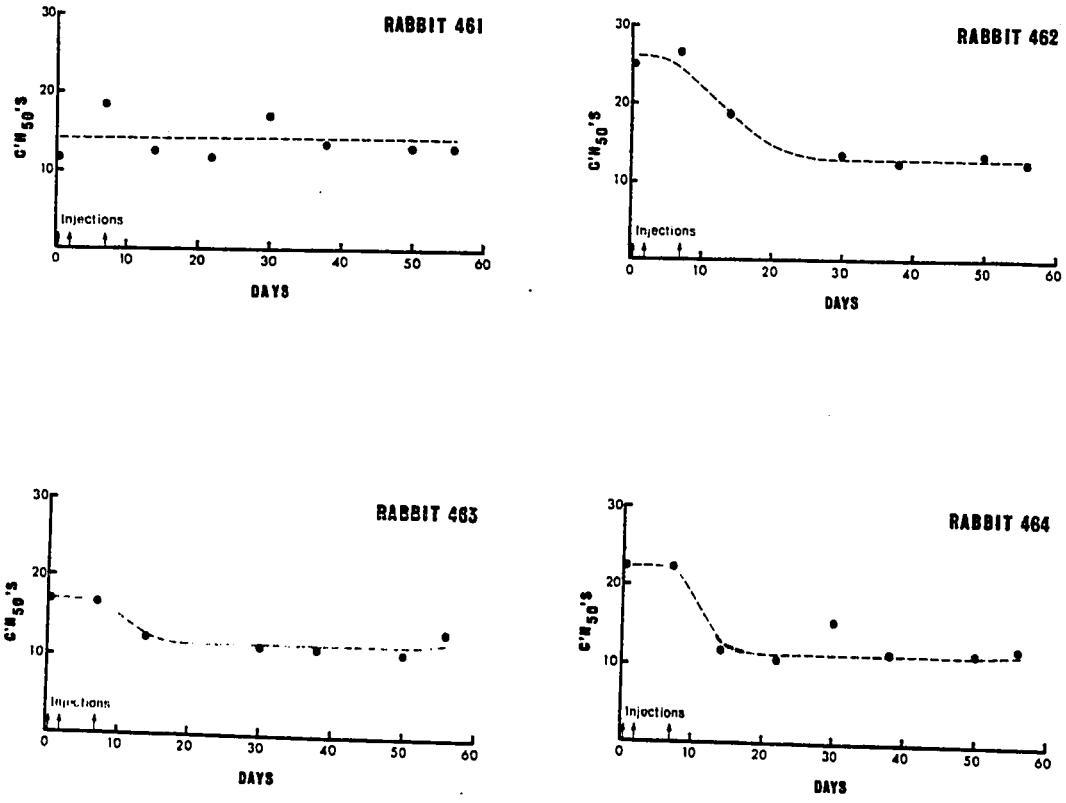


Fig. 4. Titers of total hemolytic activity of complement expressed as 50% hemolytic units of the rabbits injected with type 6 streptococcal semi-purified extract.

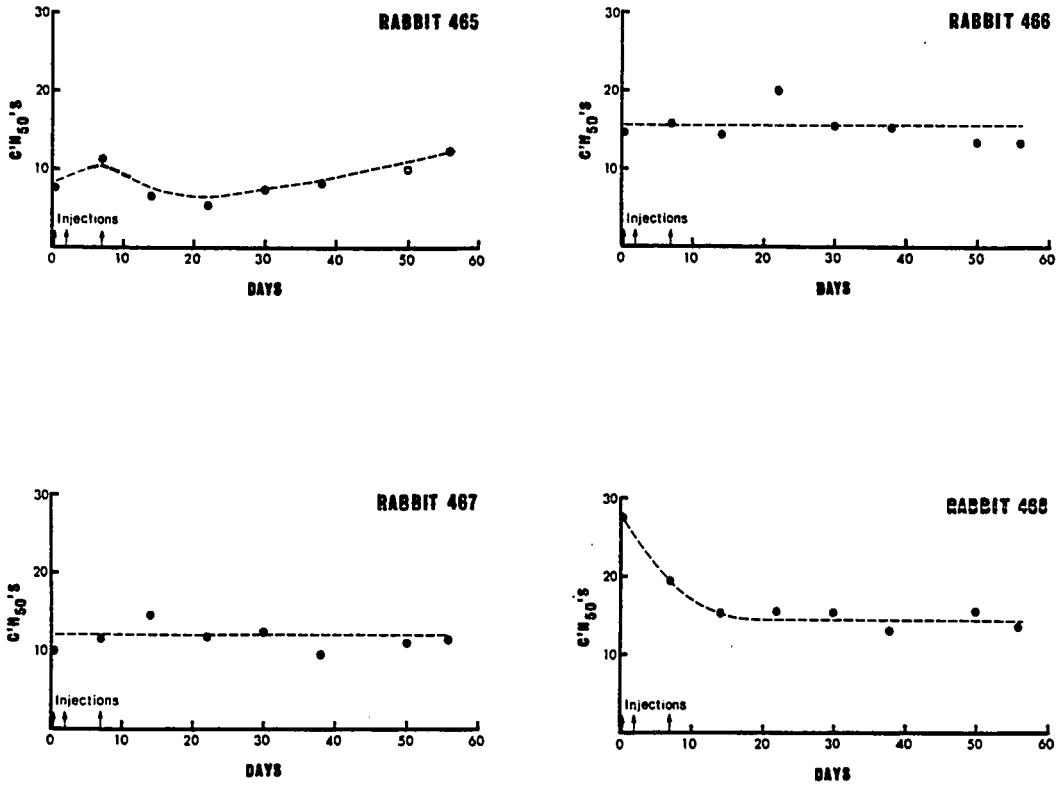


Fig. 5. Titers of total hemolytic activity of complement expressed as 50% hemolytic units of the rabbits injected with type 12 streptococcal semi-purified extract.

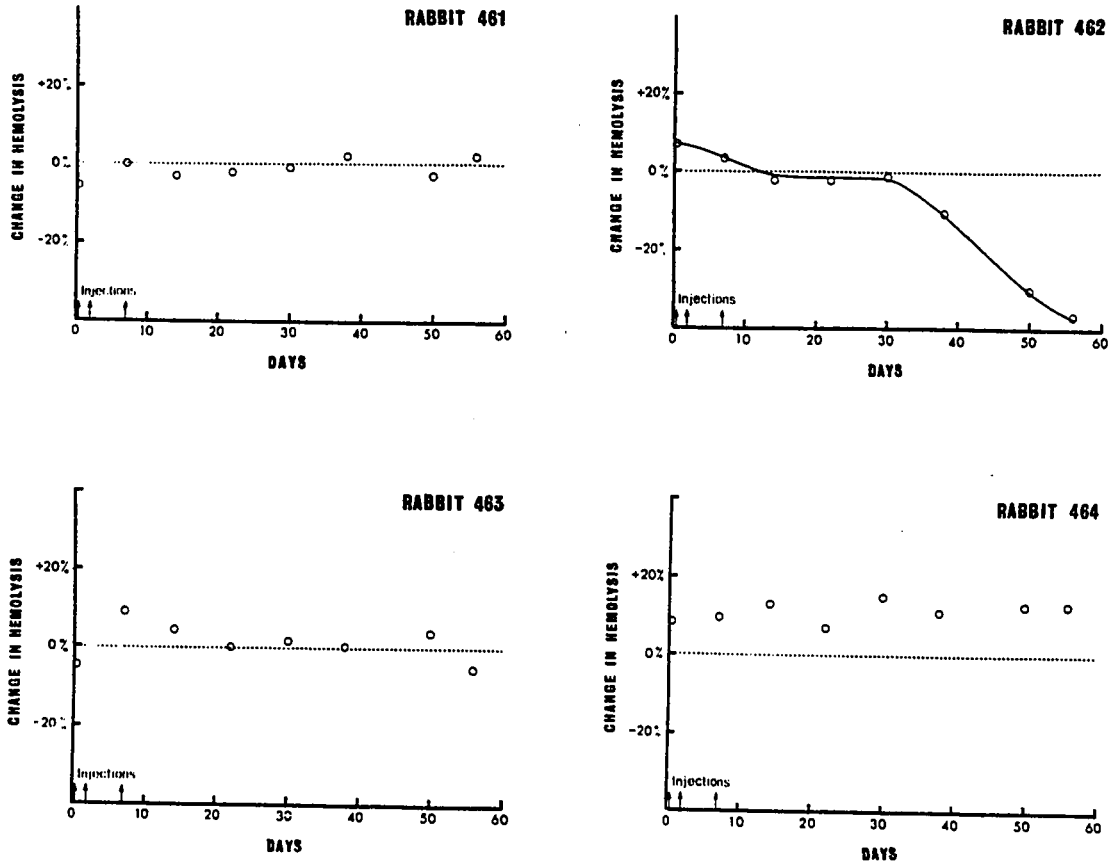


Fig. 6. Effect of the sera from rabbits injected with type 6 streptococcal semi-purified extract on the hemolytic activity of normal guinea pig complement.

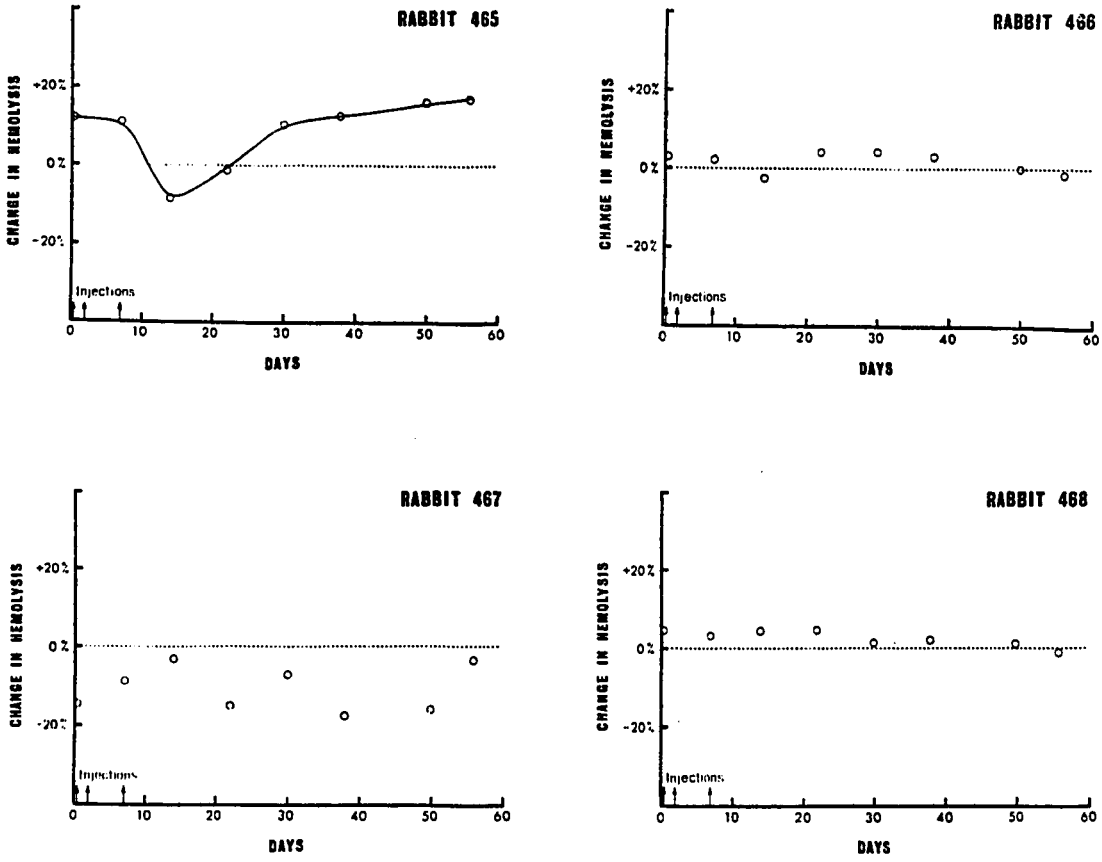


Fig. 7. Effect of the sera from rabbits injected with type 12 streptococcal semi-purified extract on the hemolytic activity of normal guinea pig complement.

TABLE 4

Kidney Morphology of Rabbits from Experiment 1

Group	*Rabbit*	*Gross Morphology*	*Microscopic Diagnosis*
Type 6 semi-purified extract	461	Normal	Early AGN*; pyelonephritis
	462	Pitted surface	Lesions atypical of AGN
	463	Slightly red	Lesions atypical of AGN
	464	Normal	Lesions atypical of AGN
Type 12 semi-purified extract	465	Pitted surface	Lesions atypical of AGN
	466	Petechial hemorrhages	Lesions atypical of AGN
	467	Slightly hemorrhagic	Lesions atypical of AGN
	468	Pale spots; slightly hemorrhagic	Lesions atypical of AGN

* AGN = Acute glomerulonephritis

2. Attempts to Locate Nephrotoxicity Among the Fractions Obtained by Gel Filtration of the Semi-Purified Extract

Introduction - Fardy (1967) had tried using column chromatography without success to purify the type 12 semi-purified extract further. Since it is very time consuming to obtain purified nephrotoxin by elution of electrophoretically separated bands of the semi-purified extract in borate buffer (Cheuk, 1960), further attempts were made in the present work to make use of column chromatographic separations. By using 0.02 M NaCl as the eluant (Section III C), the semi-purified extract was able to be separated into 4 overlapping peaks by gel filtration on columns of Sephadex G-25 medium gel (*Fig. 8*). The fourth peak, from here on referred to as Fraction 4, was of special interest since it gave rise to a pattern upon electrophoresis in 0.01 M borate buffer consisting principally of 3 fast migrating bands (see *Fig. 8*), one or more of which could correspond to the active band obtained by Cheuk (1960).

Efforts were therefore made to prepare the column purified fractions of the type 12 semi-purified extract in sufficient quantity for *in vivo* testing. It was hoped that in the following experiment, the nephrotoxicity of the type 12 semi-purified extract could be localised in one of the fractions, and that this would indirectly confirm Cheuk's work if activity could be demonstrated in Fraction 4.

a. Animals and Treatment

The rabbits were injected intravenously with the four fractions obtained by gel filtration of the type 12 semi-purified extract (see Table 5 below).

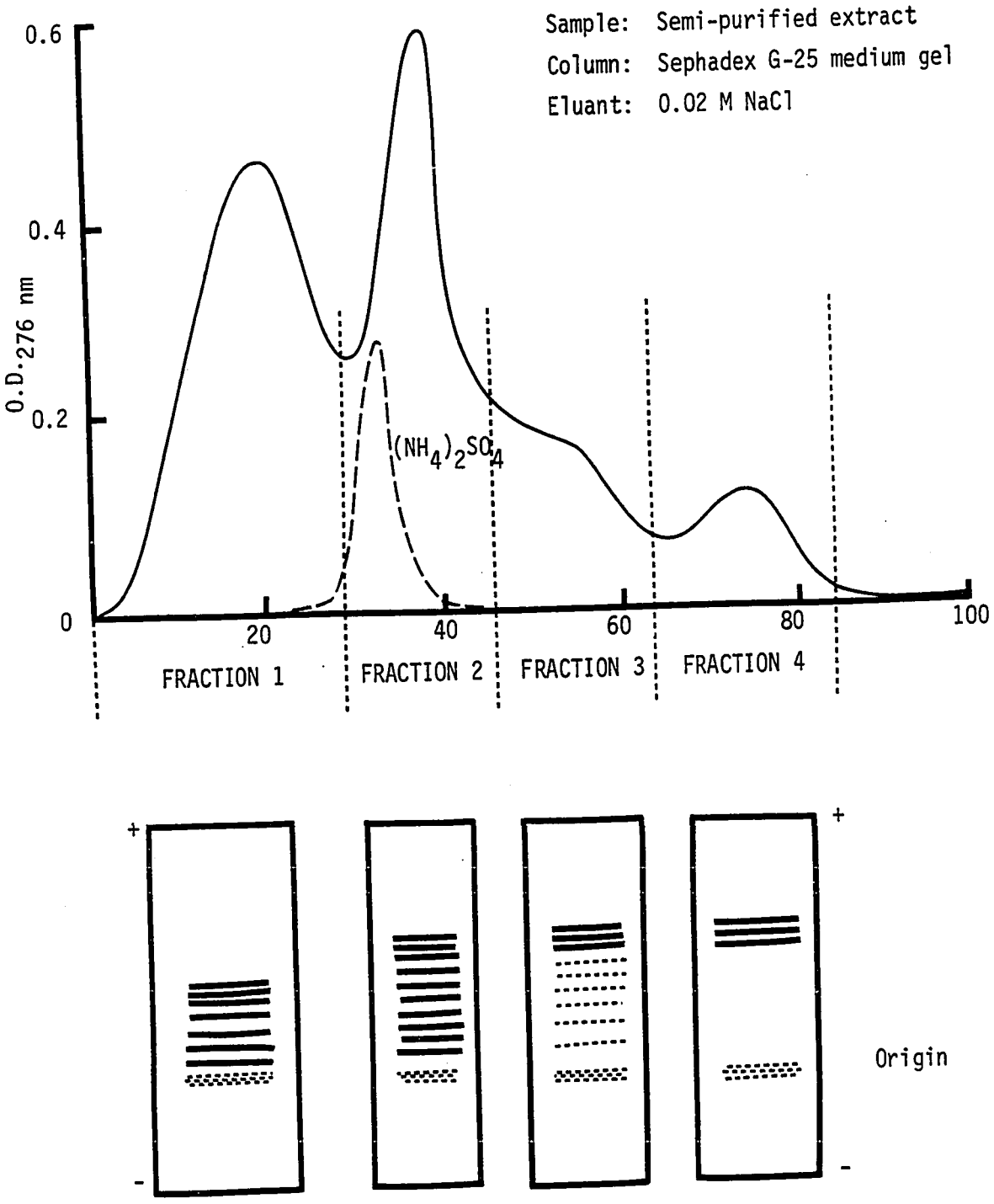


Fig. 8. Top: *Gel filtration pattern of the semi-purified extract which was precipitated from a culture filtrate between 60 to 75% of saturation with ammonium sulfate.* Bottom: *The corresponding electrophoretic patterns of the fractions on thin-layer electrophoresis.*

TABLE 5

Schedule of Experiment 2

Group	Saline Control	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Treatment	← 30 mg per rabbit i.v. →				
Rabbits used	523	525	522	528	533
	527	526	535	534	542
	529	541	538	536	543
	539	545	540	537	544
Injection schedule	At concentrations of 15 mg/ml, 0.6 ml on day 0, 0.7 ml on day 2, and 0.7 ml on day 5.				
Blood pressure measurements	Days -23, 1, 6, 14, 21, 27, 30, 41, 44, 47, 55, and 65.				
Urinalyses	Days -12, 8, 15, 23, 29, 36, 42, 49, 56, 63, and 69.				

All rabbits were exsanguinated and killed 78 days following the first injection. Rabbit No. 545 (Fraction 1 group) had died suddenly on the night of day 28. Autopsy findings revealed the presence of ascitic fluid, hemorrhagic lungs, and enlarged, pitted kidneys with petechial hemorrhages. Gram negative Pasteurella-like coccobacilli were isolated from the lung and the blood. All kidneys were fixed for light microscopy.

b. Results

i. Blood pressure measurements

The blood pressure recordings are shown in *Fig. 9*. All groups exhibited some degree of hypertension. However, the values of the groups which were injected with fractionated material did not differ significantly from the control values, the limits of which were plotted on each graph as solid bars.

ii. Urinalyses

All urines were negative for occult blood except for a small amount in rabbit No. 533 (Fraction 4 group) on day 15. Protein equivalent to 0.01% albumin was found in rabbit No. 541 (Fraction 1 group) on day 36, and in rabbit No. 537 (Fraction 3 group) on day 42. All other urines were negative for protein.

iii. Kidney morphology

Rabbits which were injected with Fractions 1 and 4 exhibited more severe kidney lesions. Both gross and microscopic observations are summarised in Table 6 on p. 91.

Summary of Experiment 2 - No significant differences were found in blood pressures among the various groups of rabbits, each having been injected with one of the four Sephadex G-25 column fractions of the type 12 streptococcal semi-purified extract. No gross urinary abnormalities were found. Histologically, rabbits from the Fraction 1 and Fraction 4 groups possessed more severe kidney lesions.

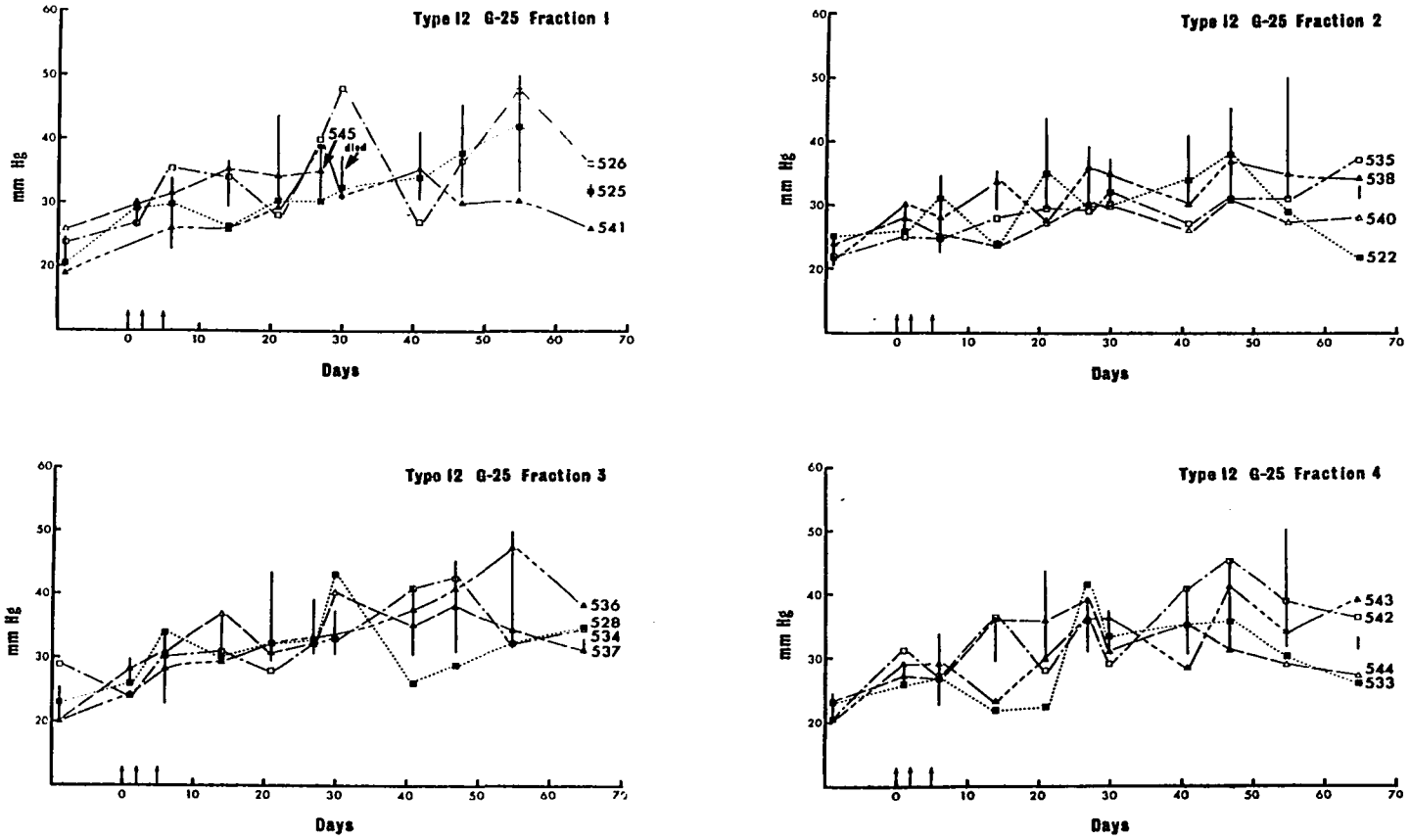


Fig. 9. Blood pressure readings of rabbits from Experiment 2. *Each group received a different fraction obtained by gel filtration of a type 12 streptococcal semi-purified extract on a Sephadex G-25 column.*

TABLE 6

Kidney Morphology of Rabbits from Experiment 2

Group	*Rabbit*	*Gross Morphology*	*Microscopic Diagnosis*
Saline control	523	Pitted, hemorrhagic	Pyelonephritis and glomerulitis
	527	Normal	Glomerulitis
	529	Normal	Normal
	539	Normal	Mild glomerulitis
Fraction 1	525	Normal	Mild glomerulitis
	526	Normal	Mild glomerulitis
	541	Abscessed, with hemorrhagic pits	Acute glomerulonephritis and pyelonephritis
	545	Enlarged, pitted, with petechiae	Intercurrent infection
Fraction 2	522	Pitted, abscessed	Essentially normal
	535	Normal	Pyelonephritis
	538	Normal	Normal
	540	Pitted, slightly hemorrhagic; some ascitic fluid	Essentially normal
Fraction 3	528	Normal	Normal
	534	Normal	Focal glomerulonephritis
	536	Normal	Normal
	537	Normal	Focal glomerulonephritis
Fraction 4	533	Slightly enlarged and pitted	Glomerulitis
	542	Slightly pitted	Normal
	543	Normal	Glomerulitis and pyelonephritis
	544	Normal	Unilateral glomerulonephritis

3. The Length of the Optimal Experimental Period

Introduction - Agnew (1955) had noted that rabbits which had received localised subcutaneous infections with nephritogenic streptococci showed no notable pathology at 1 week but exhibited "damage characteristic of lower nephron nephrosis" at 2½ to 3 weeks. Before and after that time, duration of the experiments involving rabbits had varied greatly, but were of usually more than 30 days (Matheson, 1953 and 1957; Holder, 1954; Cheuk, 1960; Day, 1967; Fardy, 1967; Micklea, 1968; Ng, 1969).

Since the latent period of poststreptococcal acute glomerulonephritis in man following respiratory infections is approximately 10 days (Stetson *et al.*, 1955) and following skin infections, approximately 20 days (Kaplan *et al.*, 1970*a*), and that the disease is usually self-limiting with very few cases becoming chronic, it was felt that the duration of the interval between injection of the nephrotoxin and the killing of the rabbits is crucial if maximal effects are to be seen. The most acute renal lesions may be more evident at less than the 35 to 40 day experimental period which has been required to see significant differences in the blood pressures between experimental and control groups of animals. Therefore rabbits were used in the following experiment to determine whether the lesions were more severe after 10 or after 20 days following the first injection of type 12 streptococcal semi-purified extract, with the appropriate type 6 and saline controls. This data is of prime importance to the immunofluorescence and electron microscopic studies to be undertaken later.

a. Animals and Treatment

The rabbits used and their treatment and experimental protocol are tabulated below.

TABLE 7

Schedule of Experiment 3

Group	Saline control		Type 6 control		Type 12	
Treatment	Saline		Type 6 semi-purified extract, 50 mg per rabbit		Type 12 semi-purified extract, 50 mg per rabbit	
	Killed 10 days	Killed 20 days	Killed 10 days	Killed 20 days	Killed 10 days	Killed 20 days
Rabbits used	595	597	596	599	600	602
	598	601	605	615	603	604
	606	609	616	617	607	608
	612	614	618	621	610	611
	620	628	624	625	613	619
	627	630	626	629	622	623
Injection schedule	At concentrations of 50 mg/ml, 0.5 ml on day 0 and 0.5 ml on day 3.					
Urinalyses	10 day groups: Day 4 and day 6. 20 day groups: Days 4, 6, 11, and 14.					

Blood pressure measurements were not taken on these groups of animals since in the most recent experiments (Fardy, 1967; Ng, 1969) hypertension was not seen until the 15th day following the first injection.

Kidney sections from all rabbits were fixed and processed for light microscopy.

b. Results

i. Urinalyses

All urines were negative for occult blood and protein.

ii. Kidney morphology

Among the type 12 rabbits, those killed at 20 days appeared to have more severe kidney lesions. However, type 6 rabbits also exhibited some kidney lesions. The results are summarised in Table 8.

Summary of Experiment 3 - The rabbits which were killed 20 days following the first intravenous injection of the type 12 semi-purified extract (nephrotoxin) appeared to have more severe renal damage as compared to those killed after 10 days. The rabbits injected with the corresponding type 6 semi-purified extract also exhibited some renal damage.

TABLE 8

Kidney Morphology of Rabbits from Experiment 3

Group	*Duration*	*Rabbit*	*Gross Morphology*	*Microscopic Diagnosis*
Saline control	10 days	595	Normal	Glomerulitis
		598	Normal	Possible acute GN
		606	Normal	Pyelonephritis
		612	Normal	Normal
		620	Normal	Essentially normal
		627	Normal	Minimal glomerulitis
	20 days	597	Pitted surface	Pyelonephritis
		601	Pale; some ascitic fluid	Pyelonephritis
		609	Some hemorrhages	Normal
		614	Normal	Essentially normal
		628	Pitted, with pete-hemorrhages	Essentially normal
		630	Normal	Glomerulitis
Type 6 semi-purified extract	10 days	596	Normal	Essentially normal
		605	Normal	Changes atypical of AGN
		616	Normal	Glomerulitis
		618	Normal	Glomerulitis
		624	One small red area	Minimal glomerulitis
		626	Normal	Glomerulitis
	20 days	599	Petechial hemorrhages	Possible AGN
		615	Small kidneys with some petechiae	Essentially normal
		617	Pitted	Pyelonephritis
		621	Pitted	Pyelonephritis
		625	Pockets of exudate	Minimal glomerulitis
		629	Normal	Minimal glomerulitis
Type 12 semi-purified extract	10 days	600	Pale, enlarged, with petechial hemorrhages	Glomerulitis, possible AGN
		603	Normal	Essentially normal
		607	Normal	Pyelonephritis
		610	Pitted, with pete-chial hemorrhages	Normal
		613	Normal	Pyelonephritis
		622	Slightly hemorrhagic	Normal
	20 days	602	Pale, rough textured, petechial hemorrhages	Toxic damage and pyelonephritis
		604	Petechial hemorrhages	Glomerulitis
		608	Severe petechial hemorrhages	Pyelonephritis and glomerulitis
		611	Normal	Pyelonephritis
		619	Pitted	Minimal glomerulitis
		623	Normal	Minimal glomerulitis

4. Kidney Changes in the Nephrotoxin Model Examined by Electron Microscopy and Immunofluorescence

Introduction - Electron microscopy has been shown to be a valuable tool in the study of acute poststreptococcal glomerulonephritis in man (Farquhar *et al.*, 1957; Vernier *et al.*, 1958; Kimmelstiel *et al.*, 1962; Movat *et al.*, 1962; Trump and Benditt, 1962; Strunk *et al.*, 1964; Herdson *et al.*, 1966). In addition to changes seen by light microscopy, more detailed structural alterations are revealed by electron microscopic observations. It will be remembered from the Review of the Literature that the "hump", a protrusion which may or may not be electron dense and usually found subepithelially with relationship to the glomerular basement membrane, has been shown to be characteristic of acute glomerulonephritis in both its exudative and proliferative forms (Kimmelstiel *et al.*, 1962; Herdson *et al.*, 1966) within 40 days of onset of disease. These humps have also been observed in experimental animals with glomerulonephritides of immunological etiology, such as those found in NZB/NZW F1 hybrid mice (Channing *et al.*, 1965; Hicks and Burnet, 1966; McGiven and Lynraven, 1968), thereby giving rise to the suggestion that they represent immune complexes. In the following experiment, the kidneys were examined ultrastructurally for these and other changes in the nephrotoxin rabbit model.

By immunofluorescence methods, kidneys from cases of acute glomerulonephritis in man have been shown to contain gamma globulin and $beta_{1c}$ globulin regularly. Day (1967) had found some evidence of glomerular localisation of gamma globulin in rabbits injected with

preparations of type 12 nephrotoxin. The kidneys in the following experiment were examined to see if this is so. The presence of glomerular gamma globulin would suggest that an immune process might be operative in the kidney, similar to that seen in man.

a. Animals and Treatment

Among the preparations given to the rabbits in this experiment, a distinction was made between the type 12 streptococcal cultures grown in the commercial, reconstituted Todd-Hewitt broth (Difco) and those grown in the McGill Homemade (HM) Todd-Hewitt broth which was prepared from fresh beef heart infusion. From more recent data (Day, 1967; Fardy, 1967; Ng, 1969; Friedlander, unpublished data), the renal lesions of rabbits receiving nephrotoxin have not appeared as clearcut as those from previous experiments. The commercial product had been introduced to replace the homemade broth to eliminate batch differences (Day, 1967; Fardy, 1967). The rabbits used in this experiment and their treatment are summarised in Table 9. The kidneys of the rabbits upon sacrifice were processed for the following observations.

i. Light microscopy

The routine stains, hematoxylin-eosin and periodic acid-Schiff, were performed. In addition, the alcian blue-periodic acid-Schiff stain (Spicer and Warren, 1960) was done to detect any redistribution of glomerular sialoprotein which is found on the epithelial aspect of the glomerular basement membrane, as had been found by Drummond and Chiu (1970).

ii. Electron microscopy

Samples from all kidneys were embedded. Only the left kidneys

were sectioned and examined due to the time consuming nature of the work involved. Aside from the necessary controls, if the diagnosis of a kidney based on gross and light microscopic observations was completely normal, it was not processed for electron microscopy.

iii. Fluorescence microscopy

The presence of rabbit gamma globulin was specifically stained for.

TABLE 9

Schedule of Experiment 4

Group	Saline control		Type 6 Difco		Type 12 Difco		Type 12 HM	
Treatment	Saline		Type 6 Difco TH* culture filtrate		Type 12 Difco TH culture filtrate		Type 12 McGill TH culture filtrate	
Day killed	22	23	22	23	22	23	22	23
Rabbits used	893	894	900	896	907	895	905	902
	897	899	912	901	914	898	908	906
	904	919	917	916	918	911	921	920
	922		909**			915		923
Injection schedule	Using 2X concentrated culture filtrates: 1 ml on day 0, 1.5 ml on day 2, 1.5 ml on day 7, 1.5 ml on day 9, and 1.5 ml on day 11.							
Blood pressure measurements	Days -6, 0, 7, 16, and 21.							
Urinalyses	Days 10 and 16.							

*TH = Todd-Hewitt; **No. 909 died on day 3 of unknown causes.

b. Results

i. Blood pressure measurements

The blood pressure measurements are plotted in *Fig. 10*. No significant differences were noted between the various groups.

ii. Urinalyses

No occult blood nor protein was detected from any of the urines tested.

iii. Kidney findings

The findings of the kidneys of the rabbits injected with filtrates of type 6 and type 12 streptococcal cultures as revealed by observations of their gross appearance, light microscopic, ultrastructural, and immunofluorescence microscopic examinations are shown in Table 10.

Fig. 11 shows a normal kidney and a kidney with petechial hemorrhages.

By light microscopy, the kidneys of rabbits in the two groups injected with type 12 culture filtrates had more severe lesions although some animals among the controls had minimal lesions. *Fig. 12* shows different views of glomeruli from rabbits with acute glomerulonephritis as compared to more normal sections. The kidney sections stained by the alcian blue-period acid-Schiff method did not differ from each other significantly as to the distribution nor the extent of uptake of the alcian blue stain.

When kidney sections were examined by the immunofluorescent technique for the presence of rabbit gamma globulin, 3 rabbits were positive in each of the type 6 and type 12 McGill Homemade Todd-Hewitt broth group. In the saline control group, kidneys from 6 out of 7 rabbits

were positive. These data indicate that the presence of gamma globulin cannot be correlated with the preparation injected. *Figs. 13 to 17* show some of the fluorescent kidney sections obtained in comparison to the controls.

Ultrastructural data from this experiment show rather striking differences between the control groups (saline and type 6) and the type 12 groups (both Difco and McGill Homemade Todd-Hewitt broth cultures). *Figs. 19 to 25* show the kidney glomeruli from rabbits injected with type 12 preparations to have many humps, hypercellularity, cellular edema, and some minimal glomerular basement membrane thickening as compared to the normal section seen in *Fig. 18.*

Summary of Experiment 4 - Significant differences in blood pressures were not found among the various groups of rabbits. Urines were all normal. The presence of rabbit gamma globulin in the kidneys could not be correlated with nephrotoxin treatment. Light microscopy of the kidneys revealed 4 of the 14 rabbits which had been injected with type 12 preparations as having acute glomerulonephritis. Electron microscopy revealed a much greater incidence of proliferative glomerulonephritis as compared to the isolated endogenous cases seen among the control animals.

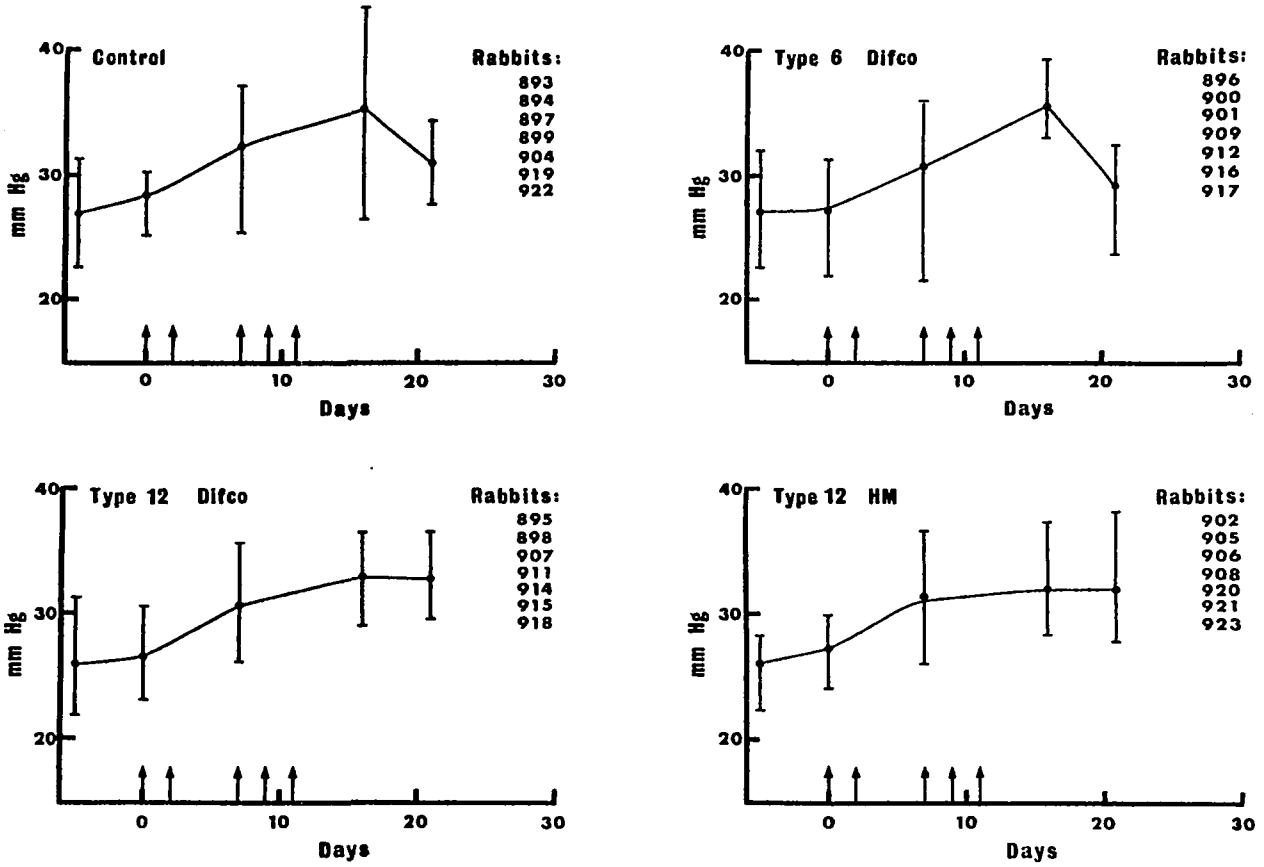


Fig. 10. Blood pressure readings of rabbits from Experiment 3. *The animals received the following i.v. injections:* Control - *Saline,* Type 6 Difco - *Type 6 semi-purified extract from Difco Todd-Hewitt broth base,* Type 12 Difco - *Type 12 semi-purified extract from Difco Todd-Hewitt broth base, and* Type 12 HM - *Type 12 semi-purified extract from McGill "Homemade" Todd-Hewitt broth base.*

TABLE 10

Kidney Morphology of Rabbits from Experiment 4

Group	*Rabbit*	*Gross Morphology*	*Diagnosis Based on Light Microscopy*	*Electron Microscopic Findings*	*Rabbit Gamma Globulin in Glomeruli*
Saline control	893	Petechial hemorrhages	Normal	No glomeruli found	+*
	894	Very small amount of ascitic fluid	Mild glomerulitis	Very mild inflammation; 2 humps seen	+++
	897	Purple colored	Mild glomerulitis	Epithelial proliferation; FP** fusion; GBM*** thickening	+
	899	Normal	Normal	Not done	+
	904	Normal	Pyelonephritis-glomerulitis	Normal	0
	919	Pale	Mild glomerulitis	Minimal abnormalities; some FP fusion	++
	922	Normal	Mild glomerulitis	Hypercellular; swollen cells	+
Type 6 (Difco)	896	Some pitting	Pyelonephritis	Not done	Not done
	900	Normal	Focal GN****	No glomeruli found	+
	901	Pitting	Pyelonephritis-glomerulitis	Not done	+
	912	Normal	Essentially normal	Normal	0
	916	Pitting; some petechial hemorrhages	Pyelonephritis	Essentially normal	+
	917	Pitting	Pyelonephritis	Hemorrhagic	0

* The glomerular fluorescence was graded from 0, no fluorescence, to +++, intense fluorescence.

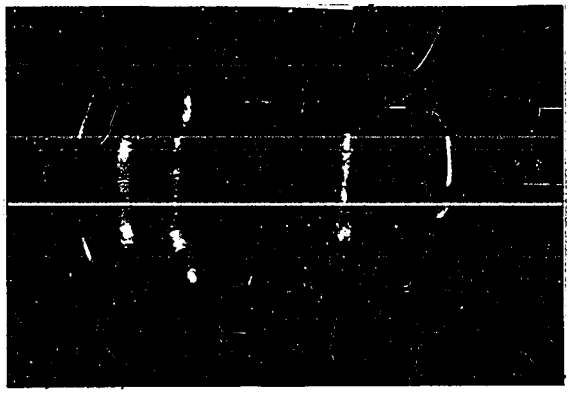
** FP = foot process.

**** GN = glomerulonephritis.

***GBM = glomerular basement membrane.

TABLE 10 (Continued)

Group	*Rabbit*	*Gross Morphology*	*Diagnosis Based on Light Microscopy*	*Electron Microscopic Findings*	*Rabbit Gamma Globulin in Glomeruli*
Type 12 (Difco)	895	Pitting	Pyelonephritis	GBM thickening; FP fusion; edematous cells	0
	898	Many petechial hemorrhages	Essentially normal	Normal	0
	907	Normal	Mild glomerulitis	Many humps; FP fusion	0
	911	Normal	Mild glomerulitis	Many humps and deposits	0
	914	Some ascitic fluid	Acute glomerulonephritis	Proliferation; edema; FP fusion	Not done
	915	Normal	Glomerulitis	Not done	0
	918	Slight amount ascitic fluid; petechial hemorrhages; pitting	Pyelonephritis-glomerulitis	Hypercellular, edematous; GBM thickening	0
Type 12 (McGill homemade)	902	Some petechial hemorrhages	Pyelonephritis-glomerulitis	Very minimal GBM thickening and FP fusion	++
	905	Slight amount ascitic fluid	Pyelonephritis-acute glomerulonephritis	Normal	0
	906	Normal	Normal	Not done	++
	908	Pitting; enlarged	Pyelonephritis	Normal	0
	920	Rough surface with capsular pockets of fluid	Pyelonephritis	No glomeruli found	0
	921	Slight amount ascitic fluid	Pyelonephritis-acute glomerulonephritis	One hump; minor FP fusion	+
	923	Normal	Acute glomerulonephritis	Humps and deposits; epithelial prolif.	0



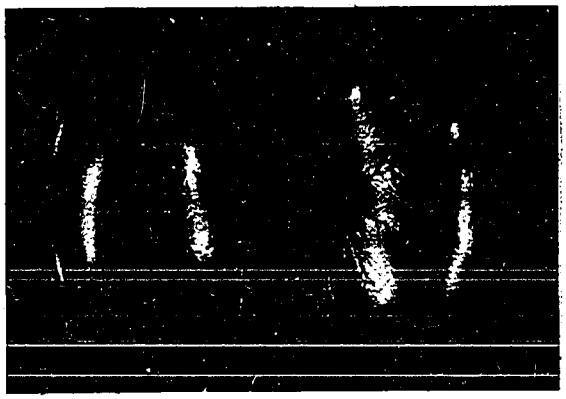


Fig. 11. a: *Normal kidneys.*

b: *Slightly enlarged kidneys with petechial hemorrhages.*

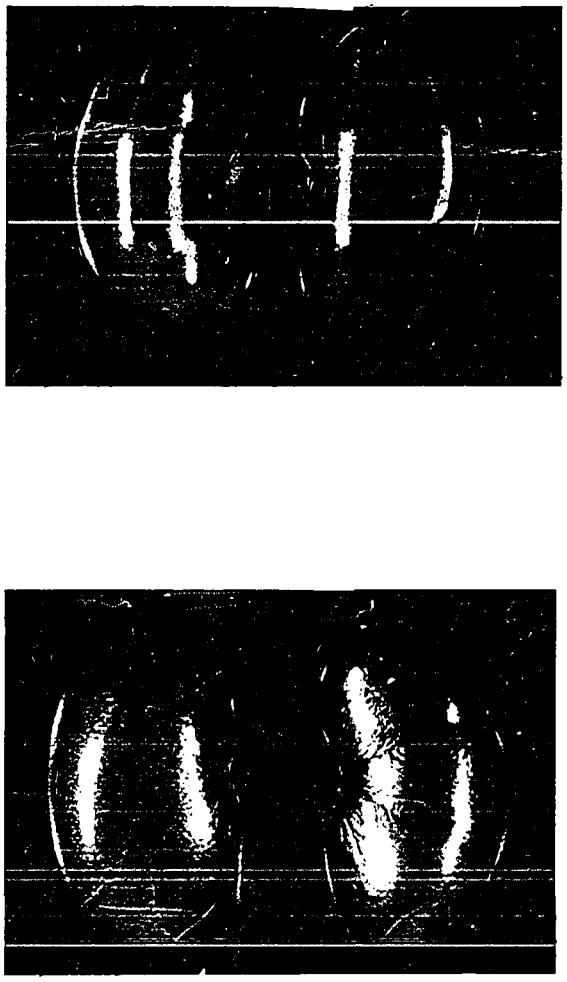


Fig. 11. a: *Normal kidneys.*

b: *Slightly enlarged kidneys with petechial hemorrhages.*

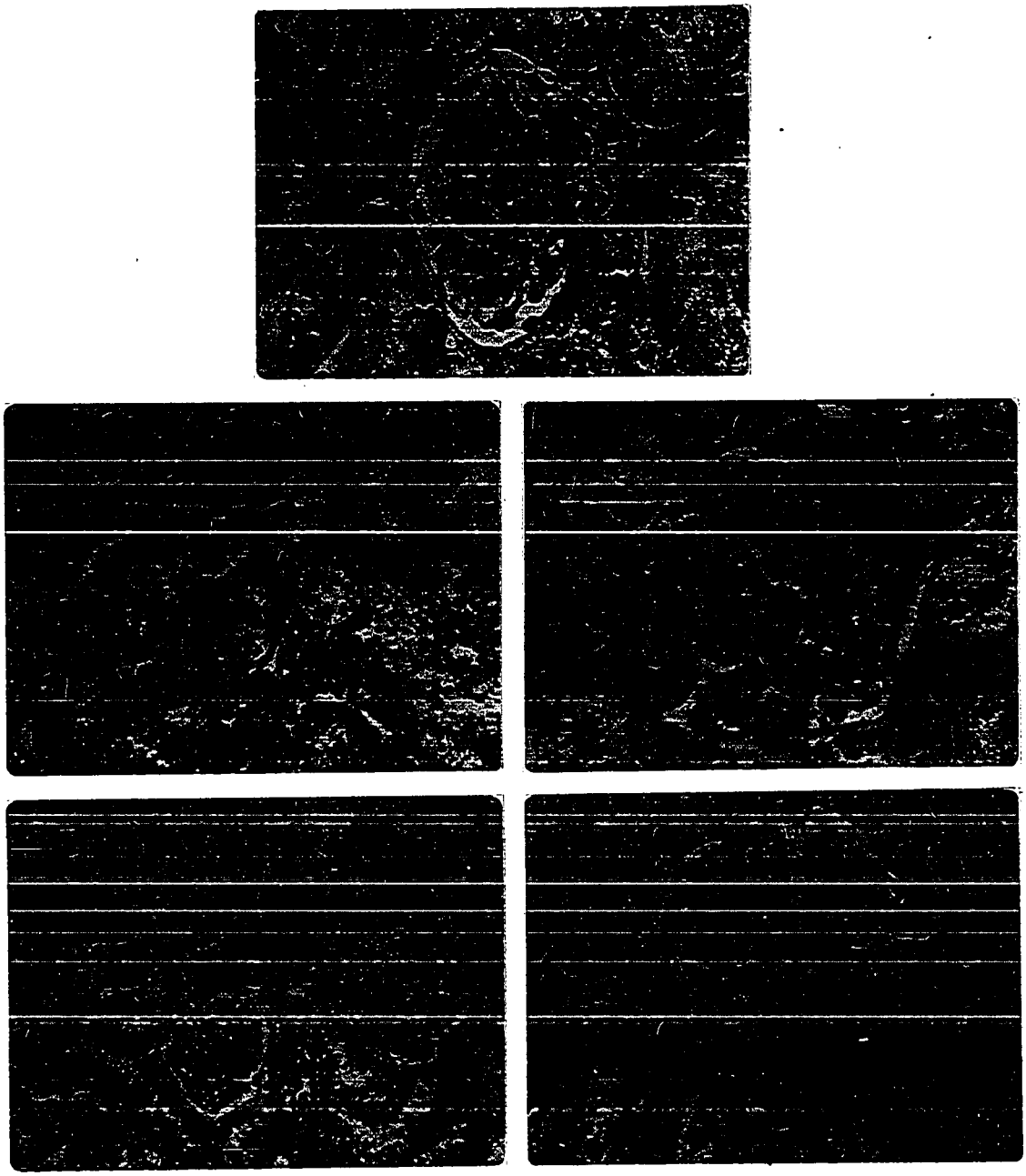


Fig. 12. a: *Normal glomerulus* (893L, X40, H-E).
b: *Enlarged glomeruli with cellular proliferation* (921L, X16, H-E).
c: *Glomerulus same as* b (921L, X40, H-E).
d: *Glomerulitis* (915R, X16, H-E).
e: *Hypercellular glomerulus* (921R, X40, PAS).

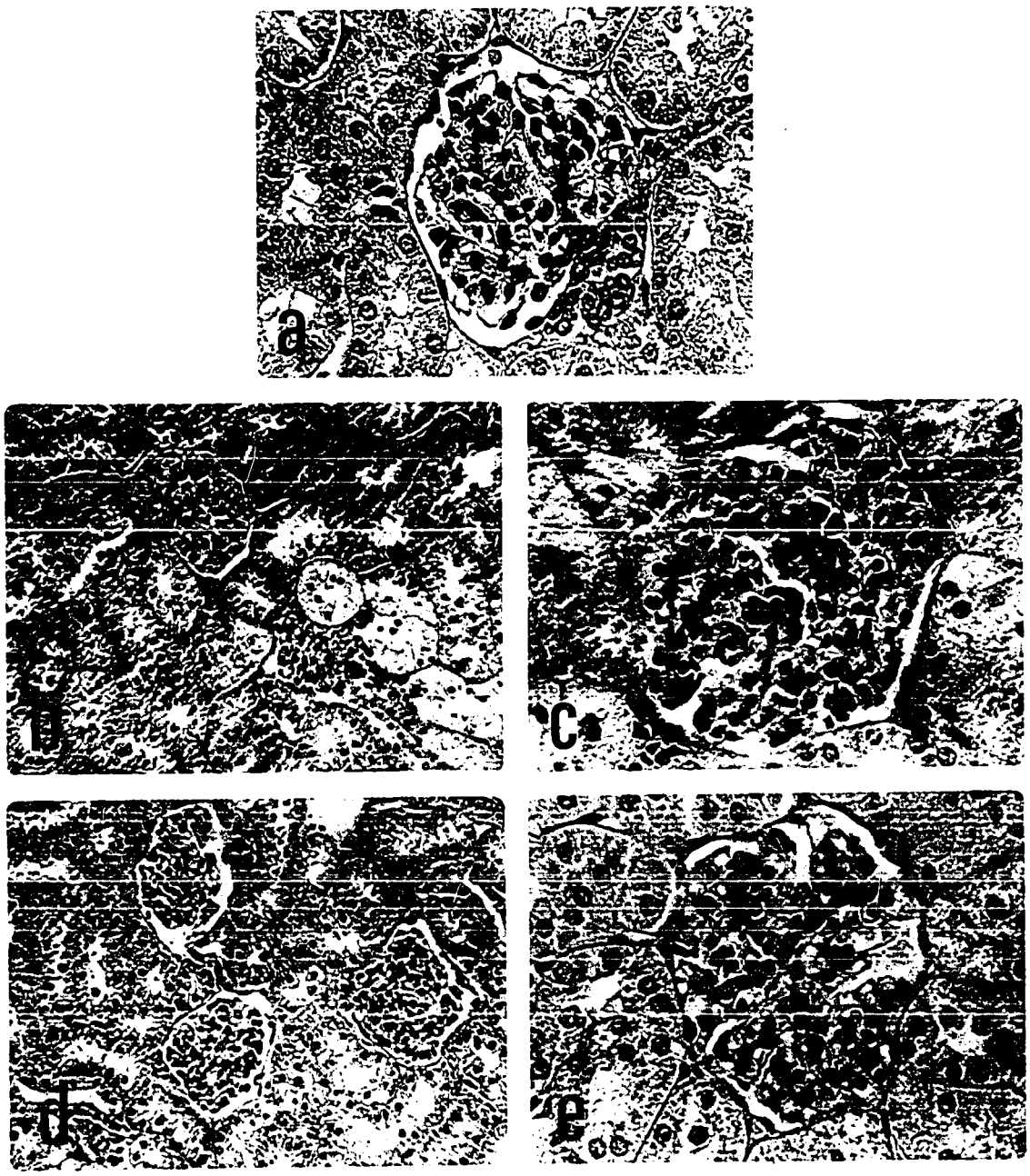


Fig. 12. a: *Normal glomerulus* (893L, X40, H-E).
b: *Enlarged glomeruli with cellular proliferation* (921L, X16, H-E).
c: *Glomerulus same as* b (921L, X40, H-E).
d: *Glomerulitis* (915R, X16, H-E).
e: *Hypercellular glomerulus* (921R, X40, PAS).

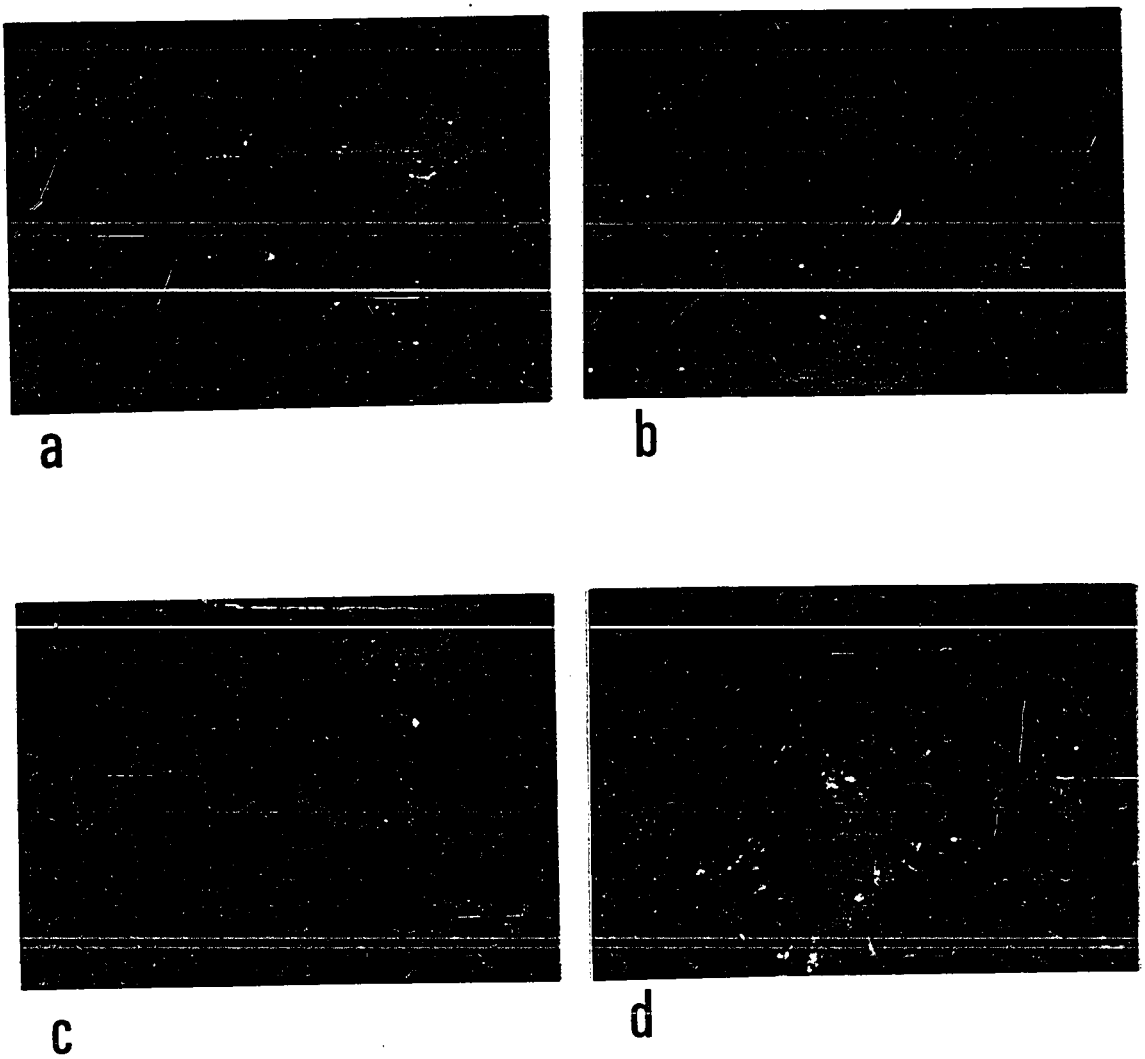


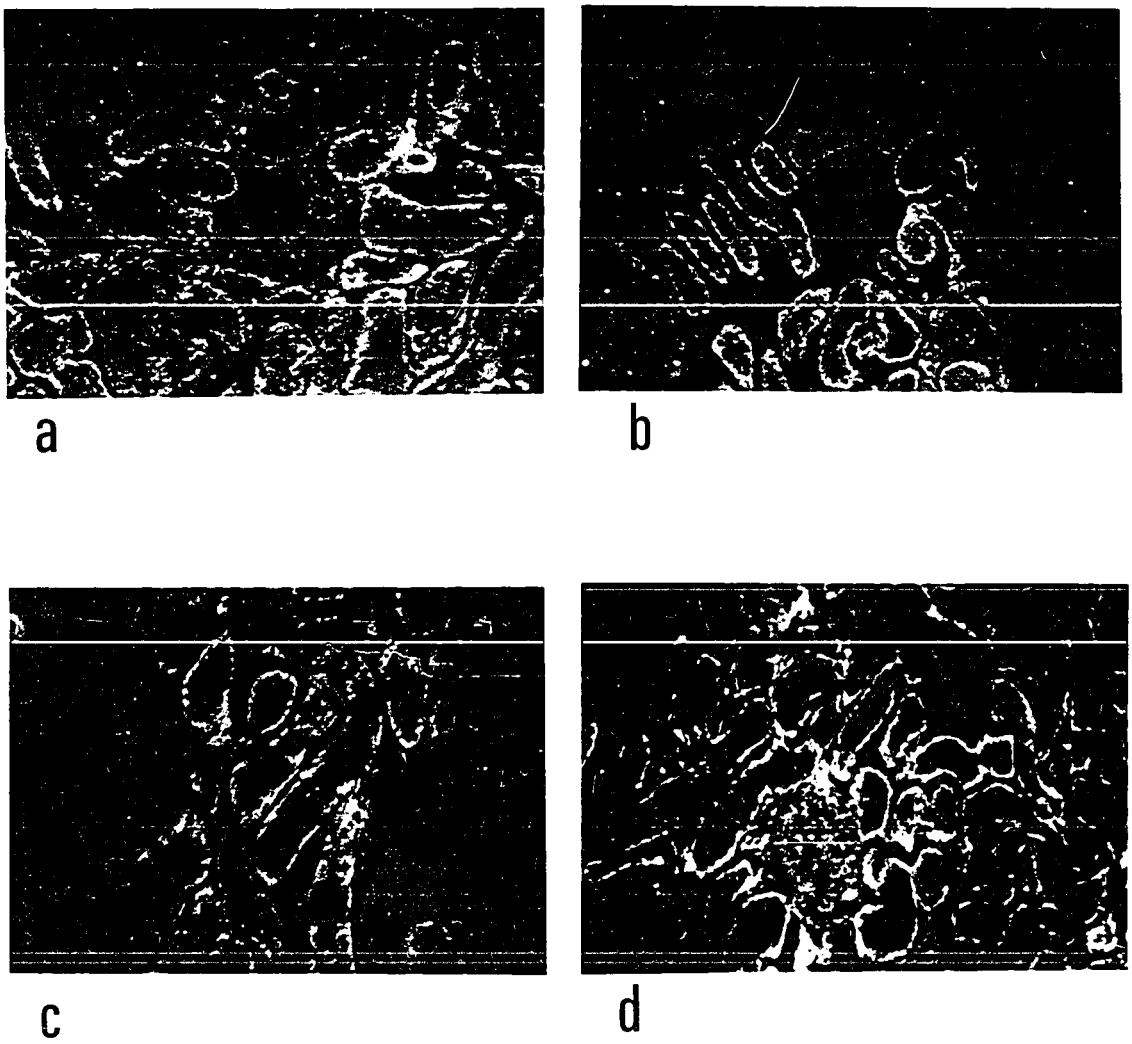
Fig. 13. *Immunofluorescent staining of kidney sections at low magnification* (X10).

a: *Control - unstained* (904).

b: *Control - stained with FITC-labelled normal rabbit gamma globulin* (902).

c: *Non-specific fluorescence - stained with FITC-labelled goat anti-rabbit gamma globulin* (903).

d: *Minimal fluorescence - stained with FITC-labelled goat anti-rabbit gamma globulin* (900).



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a: *Control - unstained* (904).

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c: *Non-specific fluorescence - stained with FITC-labelled goat anti-rabbit gamma globulin* (903).

d: *Minimal fluorescence - stained with FITC-labelled goat anti-rabbit gamma globulin* (900).

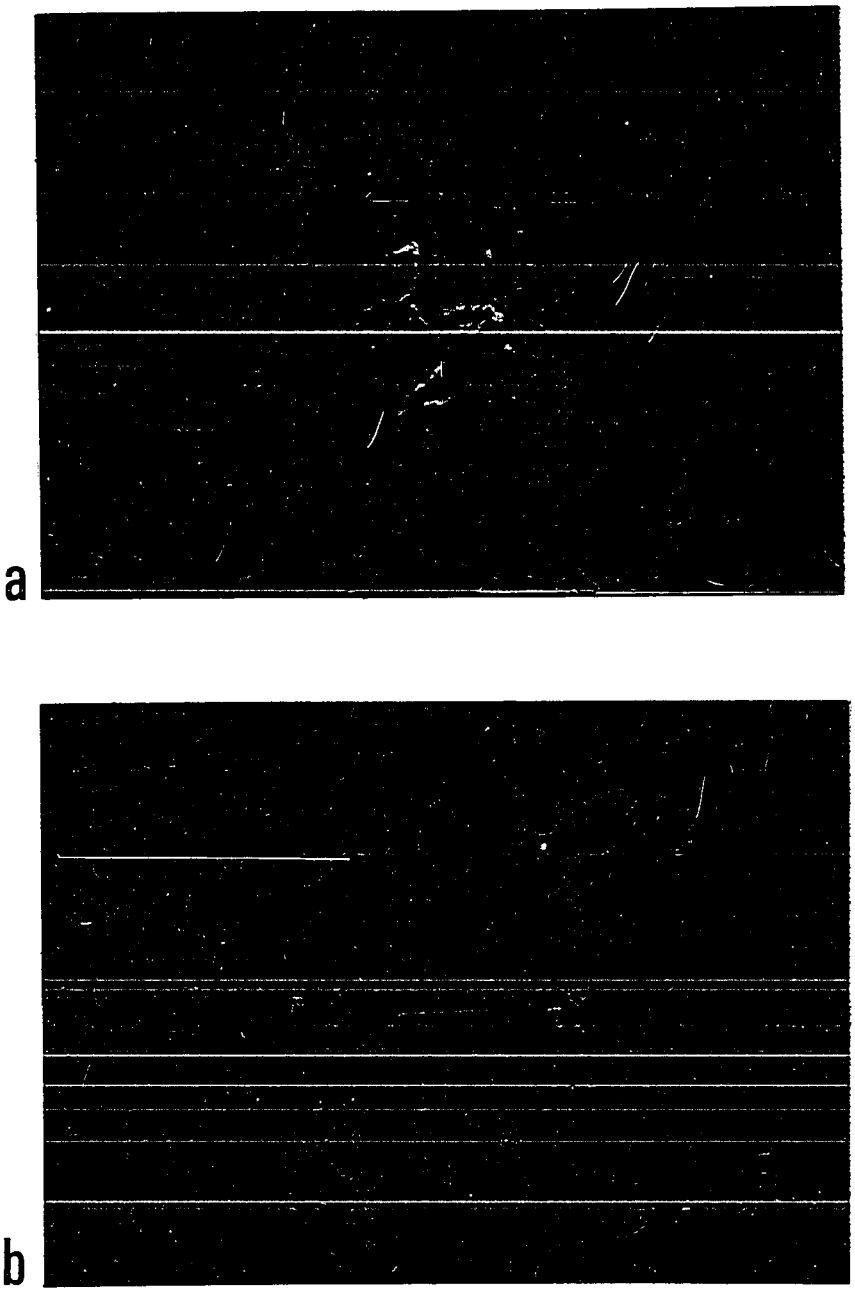
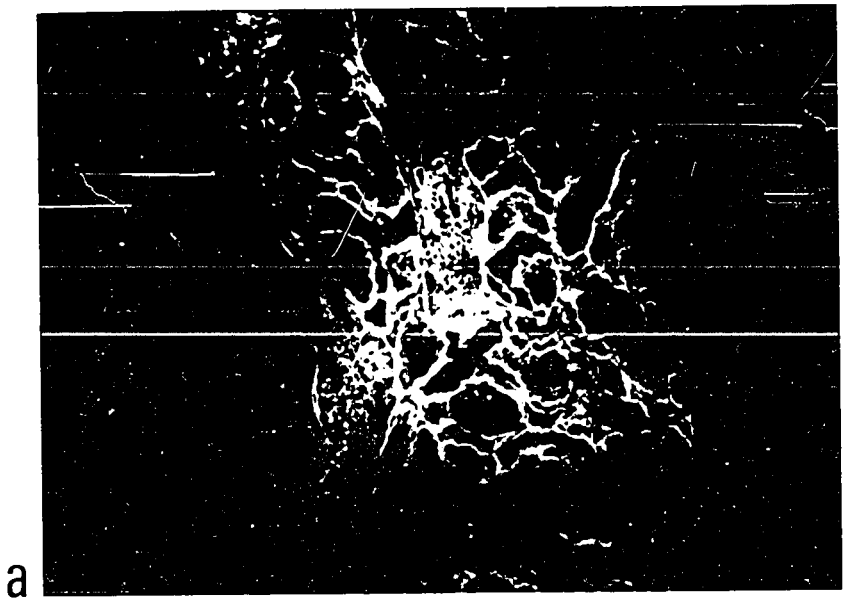


Fig. 14. Immunofluorescent staining of kidney sections at low magnification (X10).

a: *Glomeruli from section graded 1+ overall fluorescence* (897).

b: *Blocking control - stained with unlabelled anti-rabbit gamma globulin, followed by the labelled antiserum* (906).



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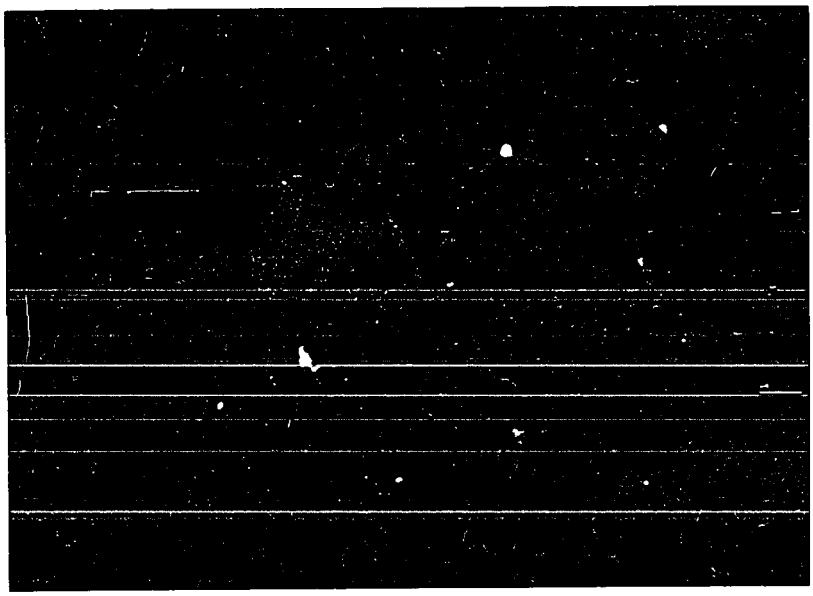

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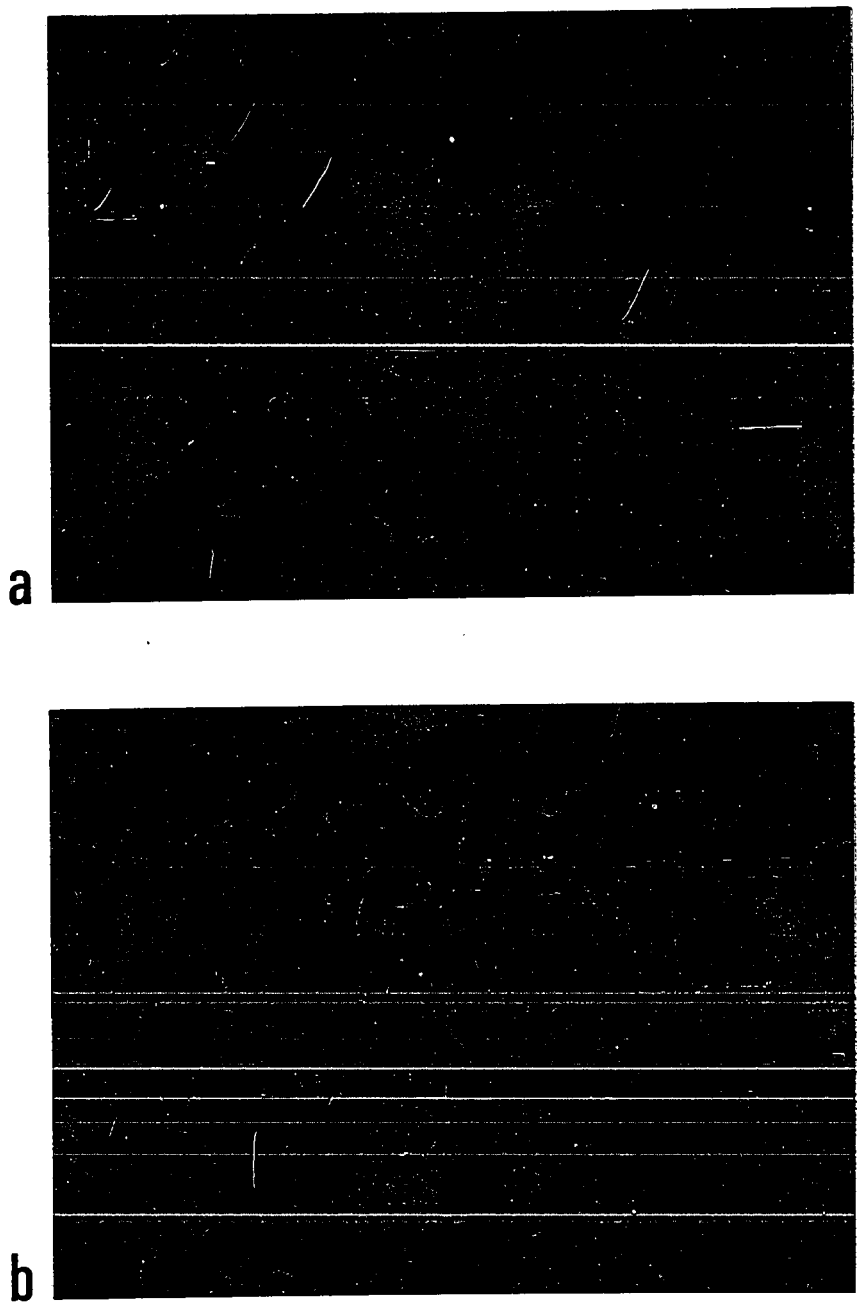
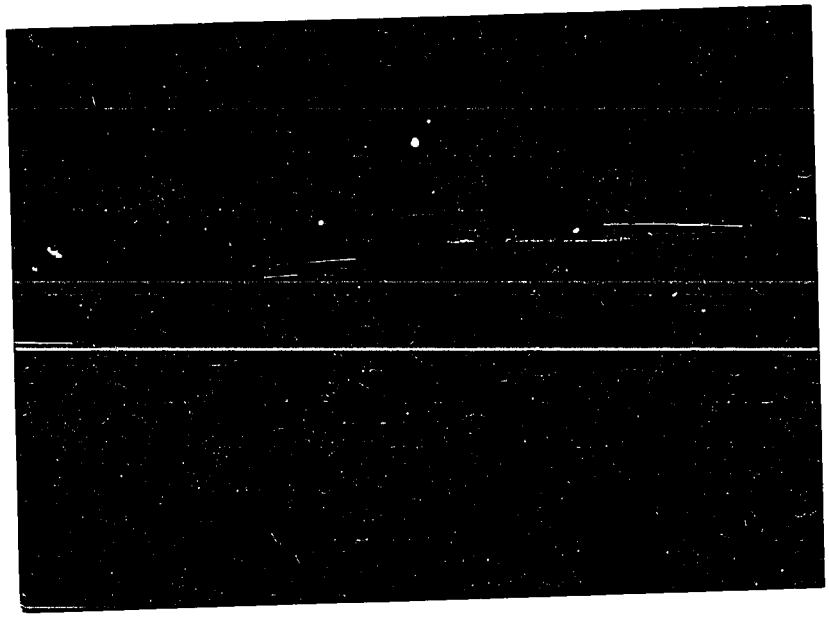


Fig. 15. Immunofluorescent staining of kidney sections at higher magnification (X40).

a: *Control - unstained* (900).

b: *Control - stained with FITC-labelled normal rabbit gamma globulin* (900).



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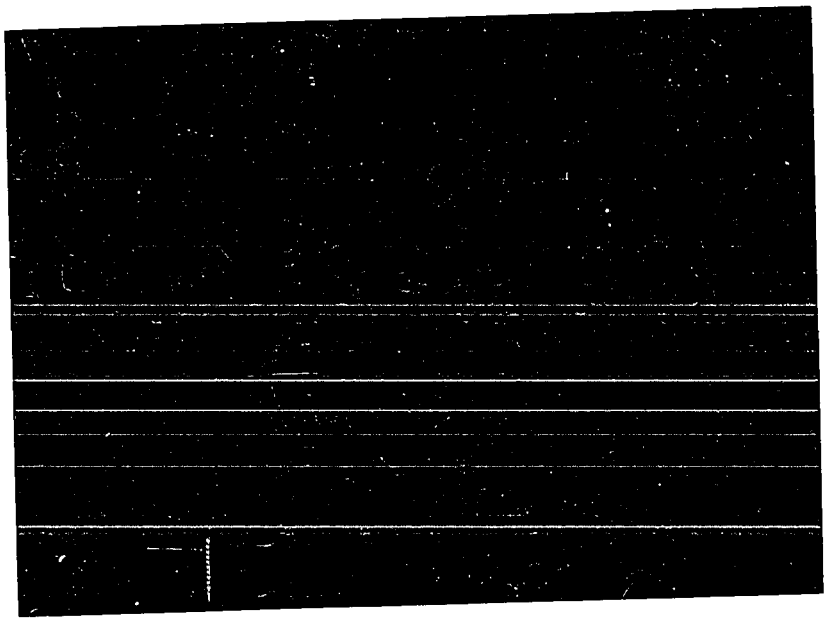

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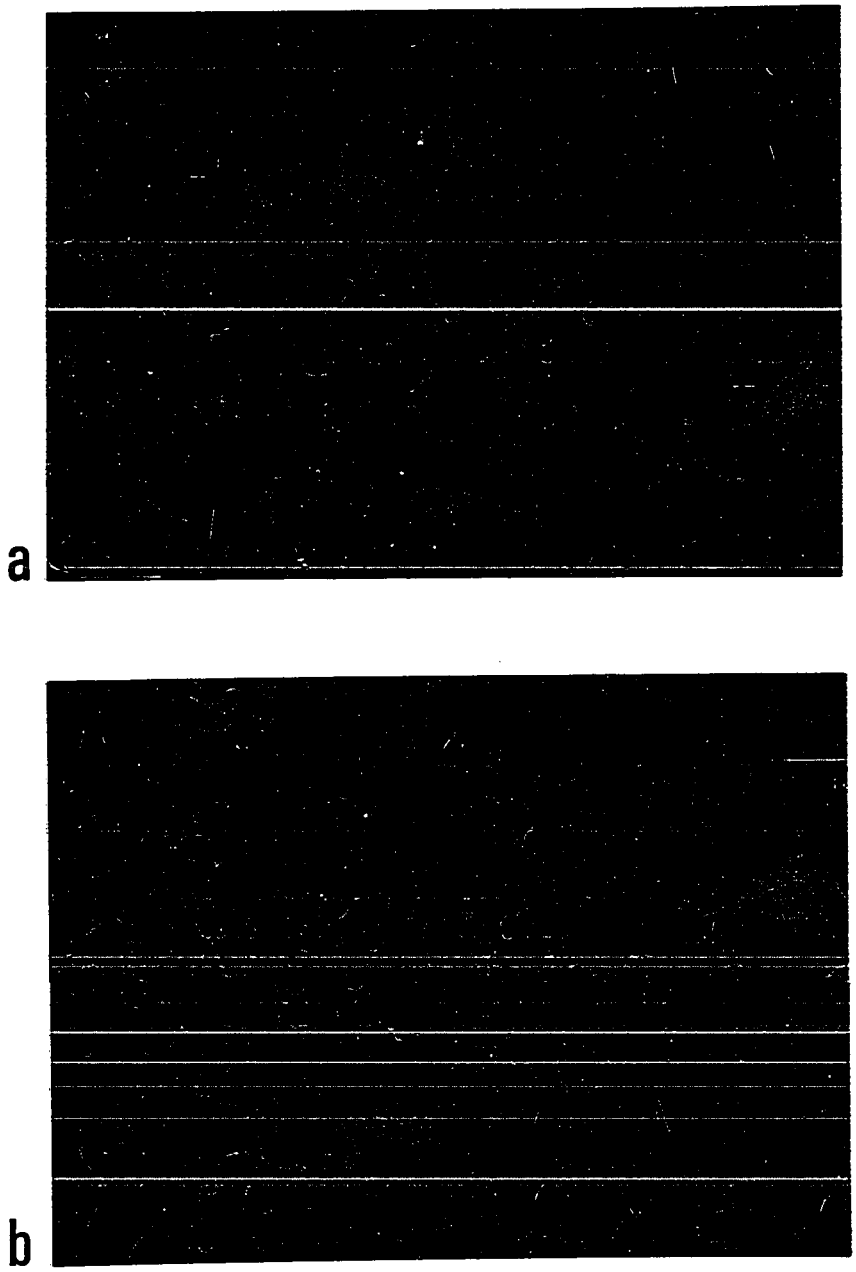
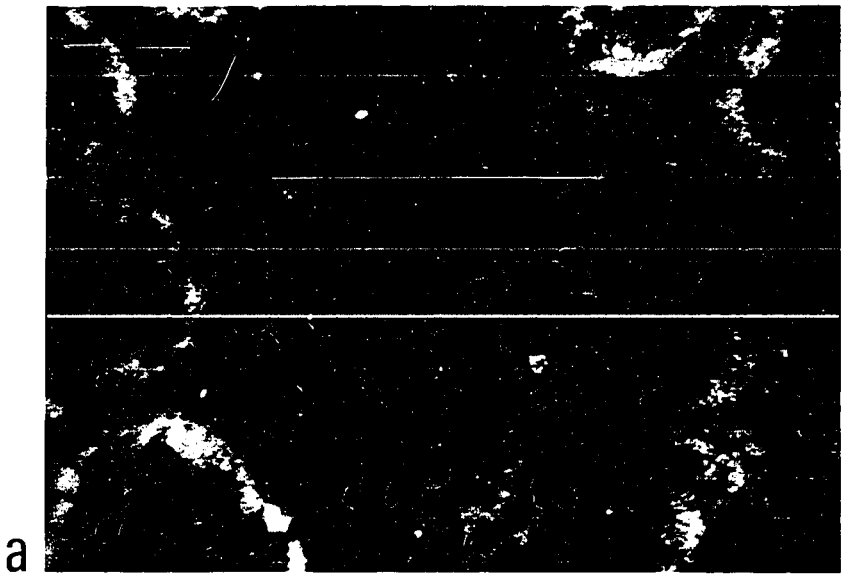


Fig. 16. Immunofluorescent staining of kidney sections at higher magnification (X40).

a: *Non-specific fluorescence - stained with FITC-labelled anti-rabbit gamma globulin* (903).

b: *Glomerulus from section graded 1+ overall fluorescence* (897).



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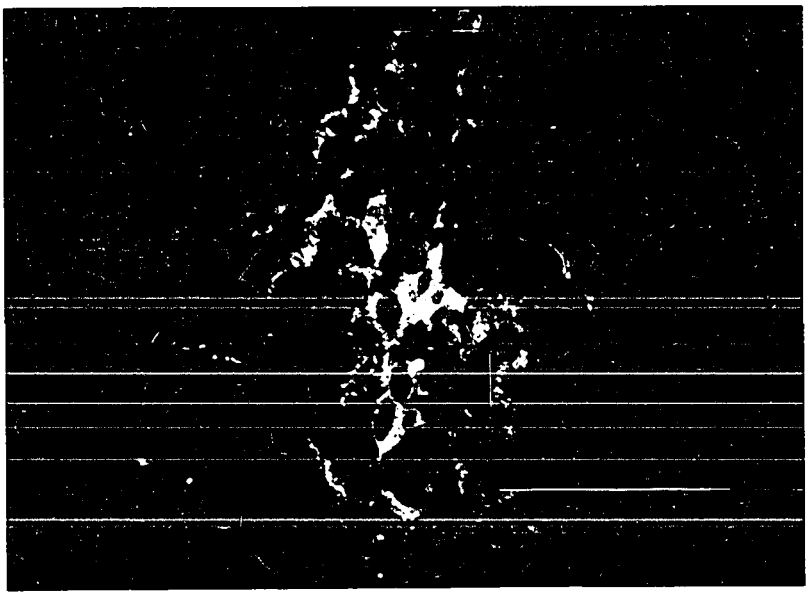

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Fig. 14. [illegible] (X40).

a: [illegible] (903).

b: [illegible] (897).

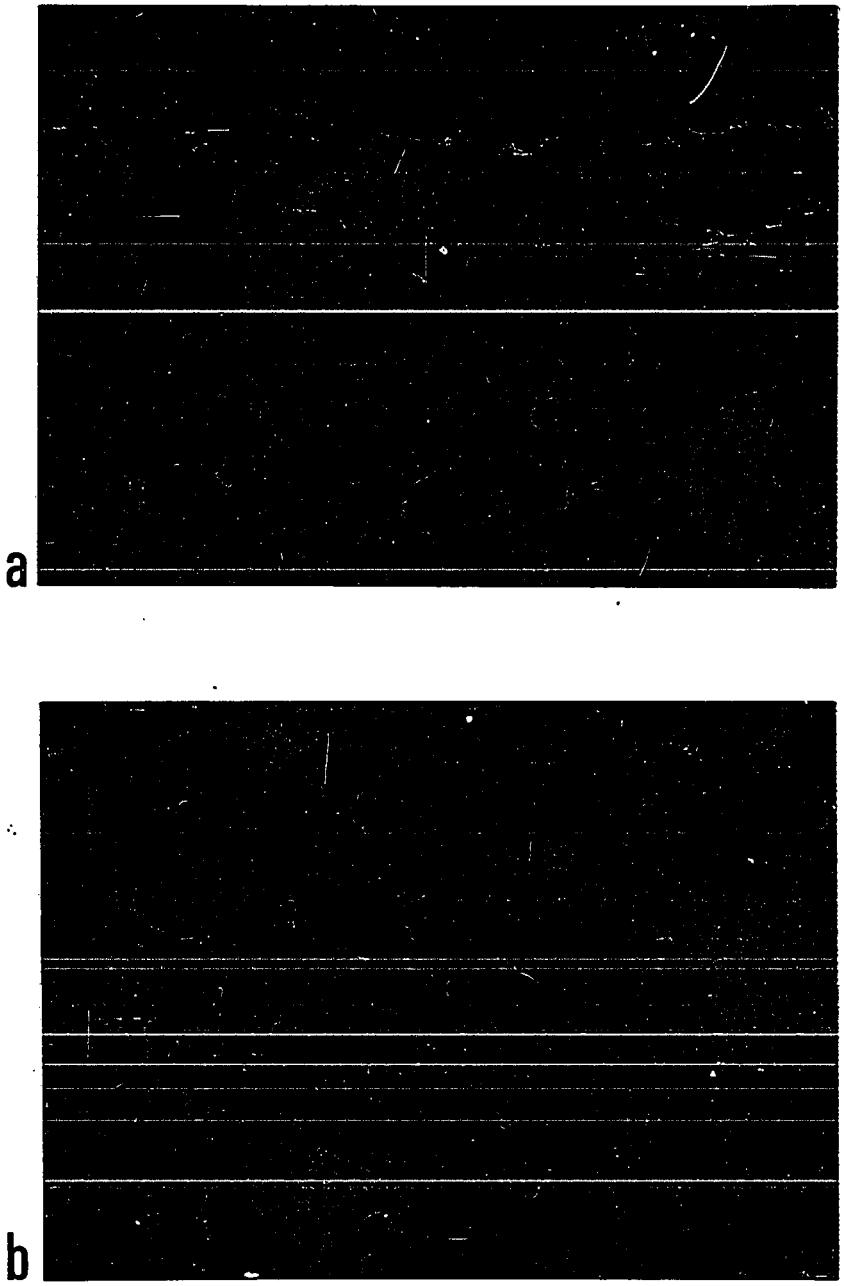
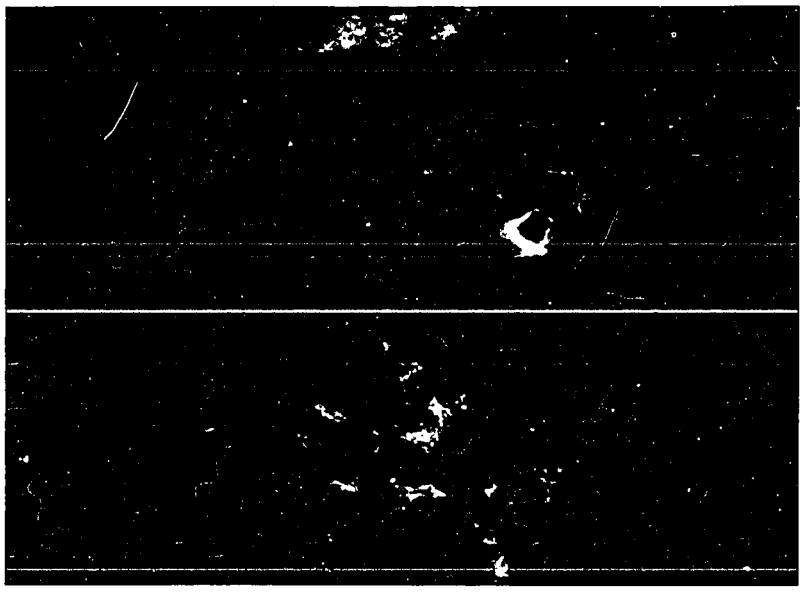


Fig. 17. Immunofluorescent staining of kidney sections at higher magnification (X40).

a: *Glomerulus from section graded 2+ overall fluorescence* (902).

b: *Blocking control - stained with unlabelled anti-rabbit gamma globulin, followed by the labelled antiserum* (902).



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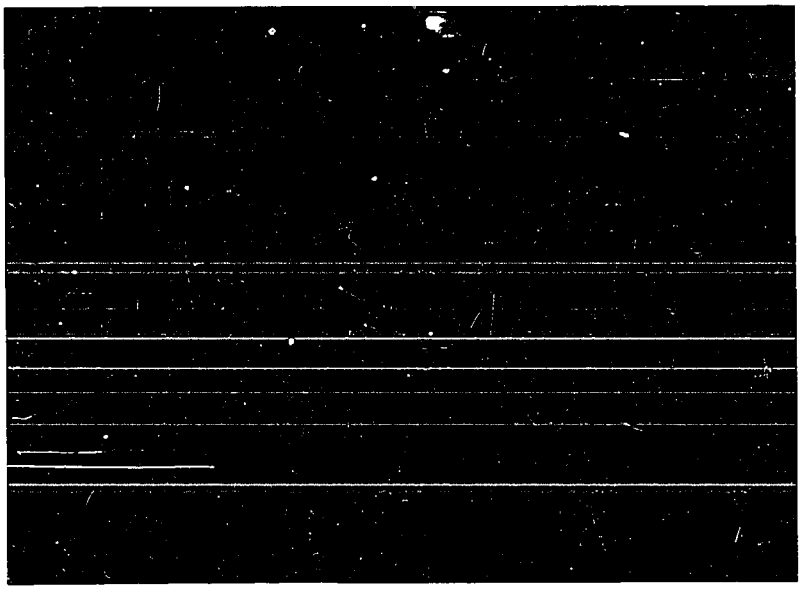

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Explanations for *Figs. 18 to 25* which follow.

Fig. 18. View of normal kidney glomerulus with inset of corresponding toluidine blue thick section. Rabbit No. 904L. X 7100.

Fig. 19. View of kidney glomerulus showing mesangial proliferation and endothelial edema. Rabbit No. 895L. X 6100.

Figs. 20 and 21. Views of kidney glomerulus showing humps. Rabbit No. 907L. X 6100.

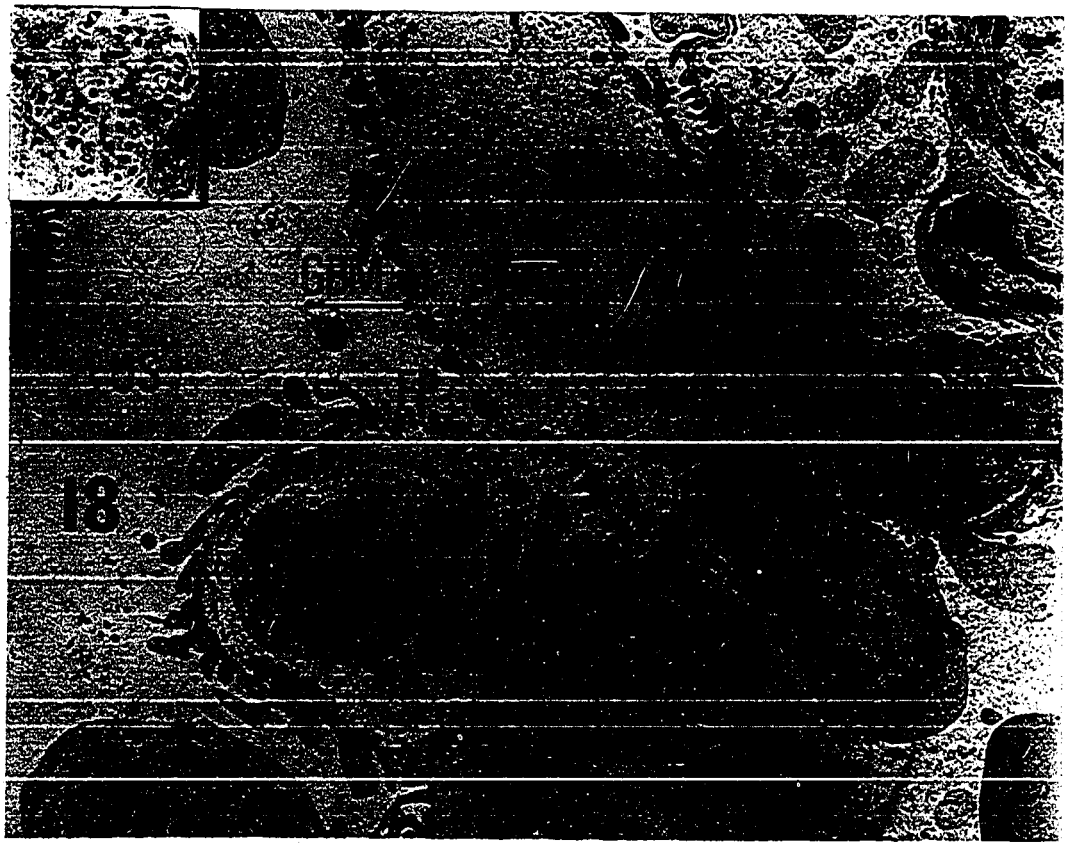
Figs. 22 and 23. Views of kidney glomerulus showing glomerular basement membrane thickening, foot process fusion, and cellular proliferation and edema, with inset of toluidine blue thick section of same glomerulus. Rabbit No. 918L. X 6100.

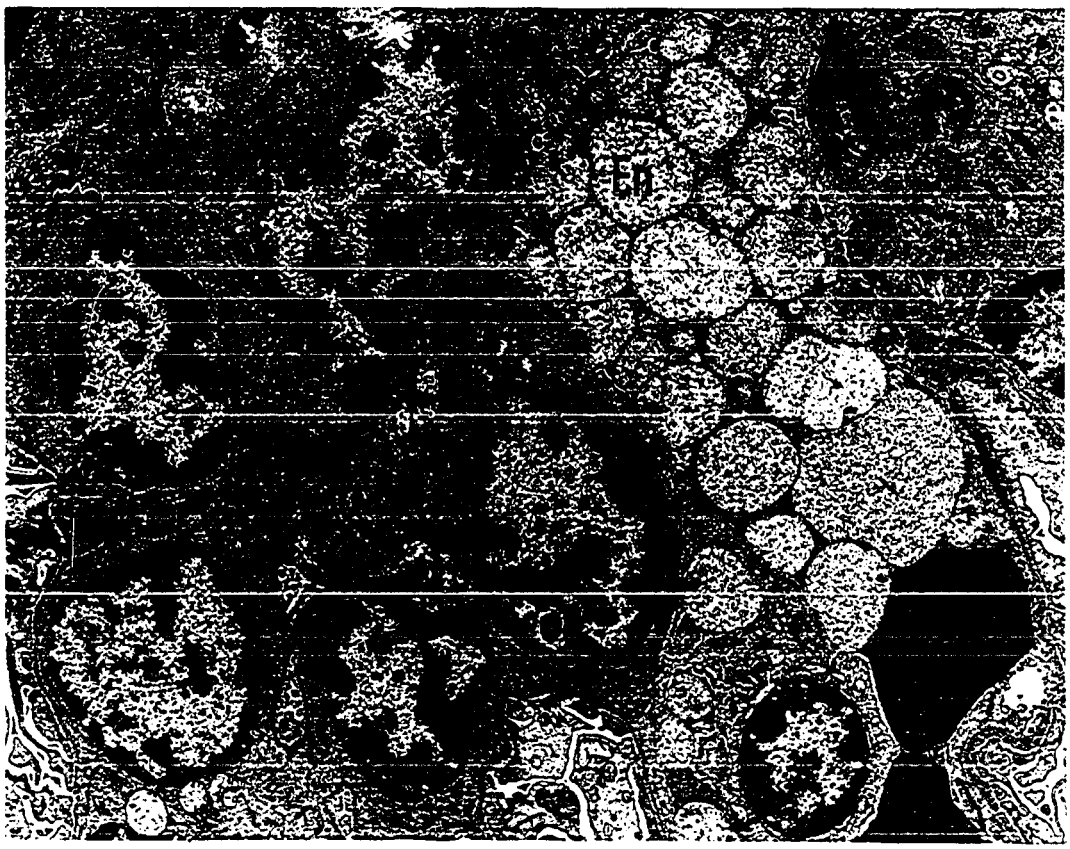
Fig. 24. View of kidney glomerulus showing endothelial proliferation and a circulating leukocyte. Rabbit No. 914L. X 6100.

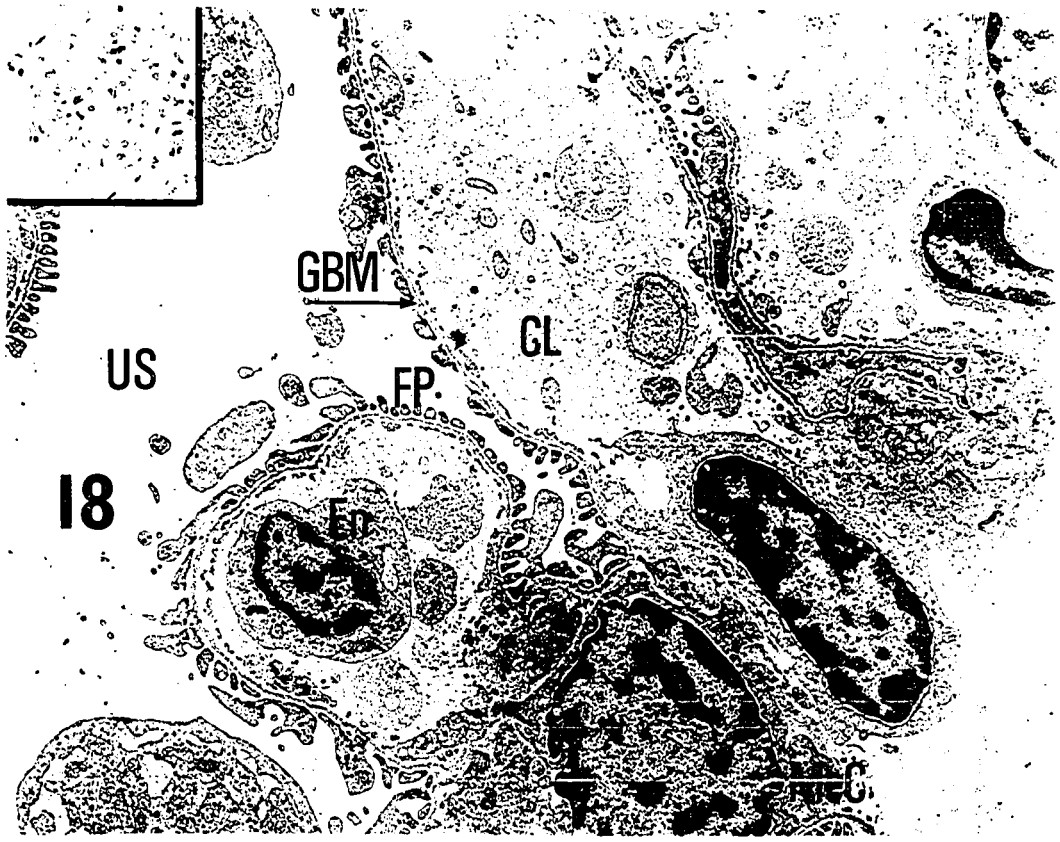
Fig. 25. View of kidney glomerulus showing hump, cellular congestion and endothelial and epithelial cells undergoing active metabolism. Inset shows toluidine blue thick section of same glomerulus. Rabbit No. 918L. X 6100.

Abbreviations:

CL = capillary lumen.
En = endothelial cell.
Ep = epithelial cell.
FP = foot process.
GBM = glomerular basement membrane.
Leu = circulating leukocyte.
MeC = mesangial cell.
US = urinary space.



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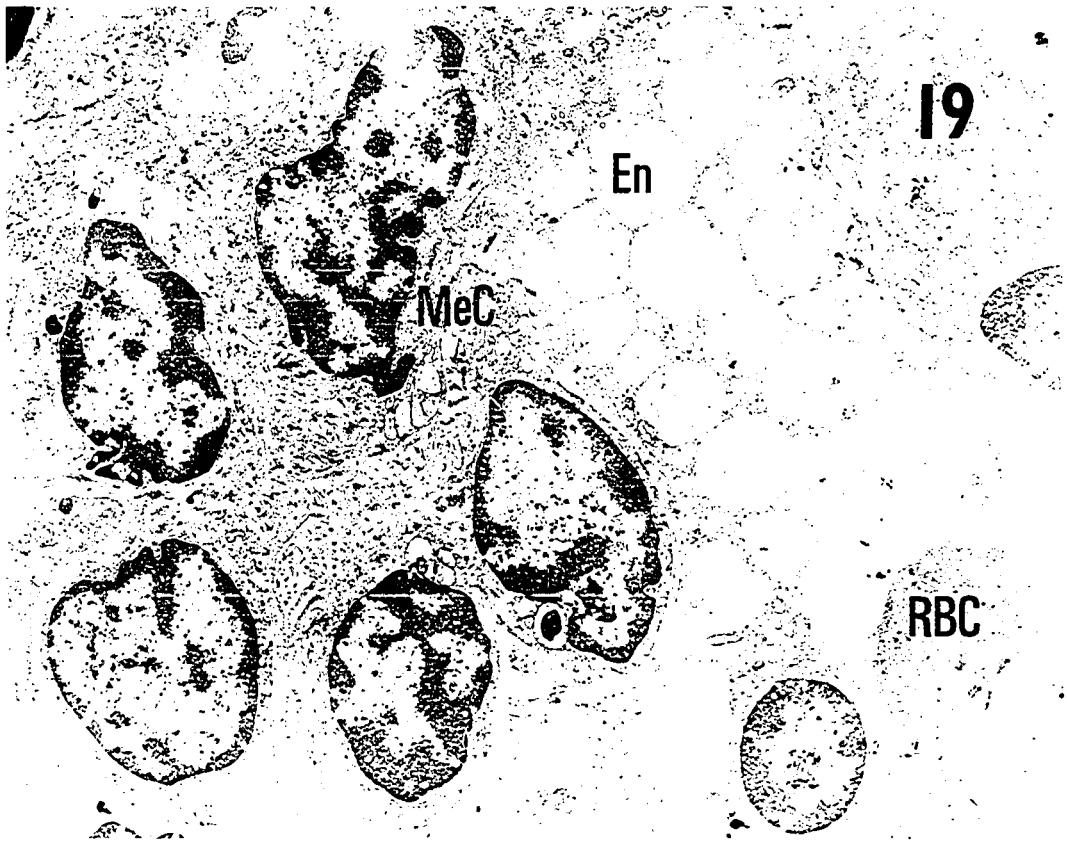
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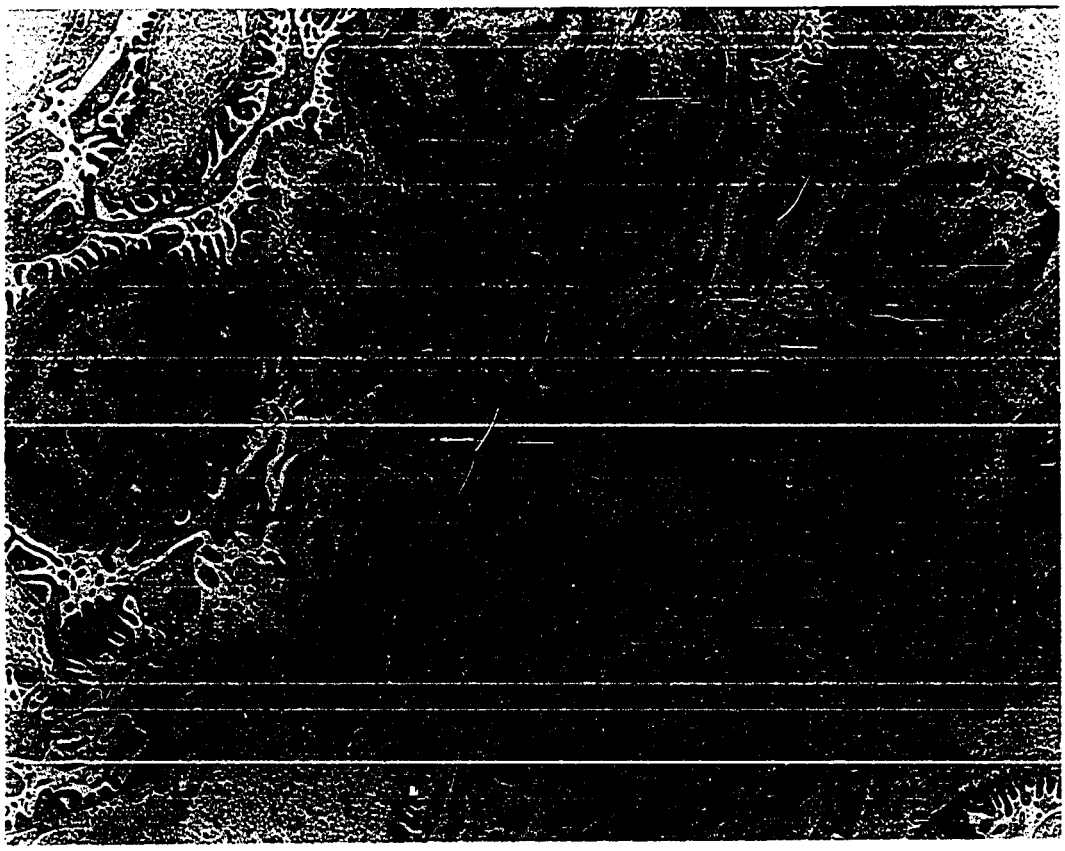


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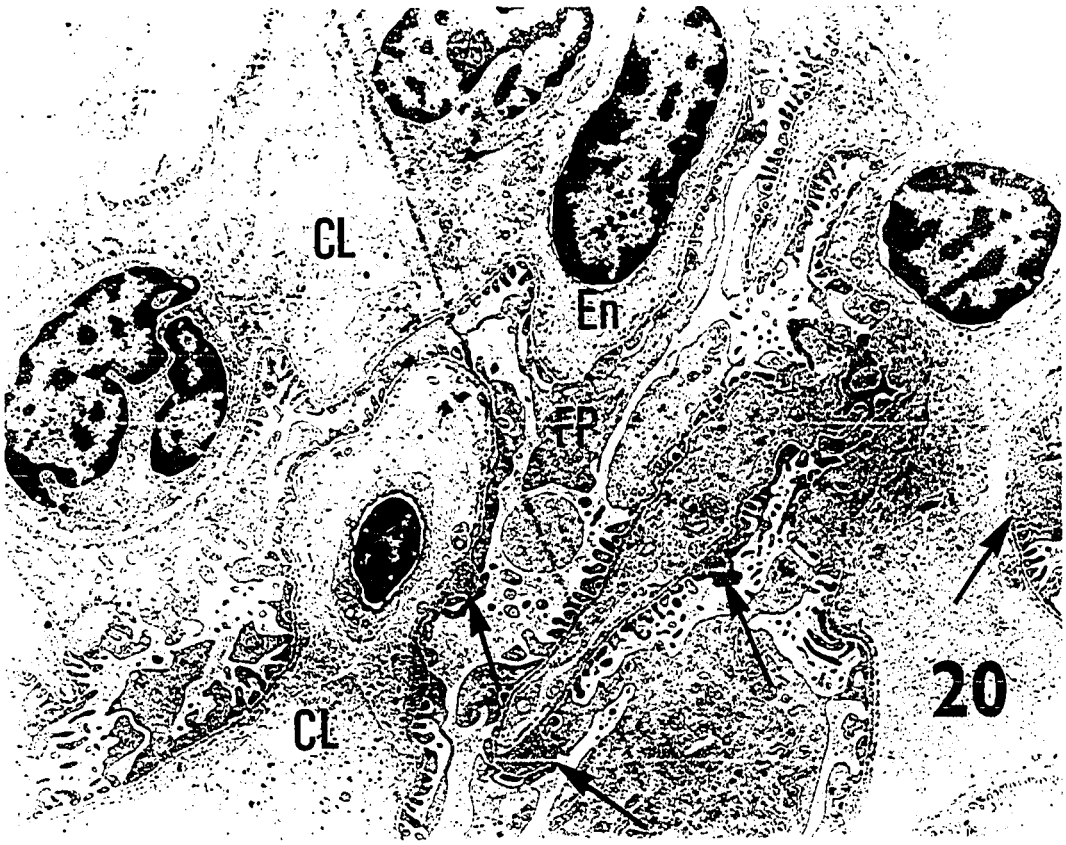
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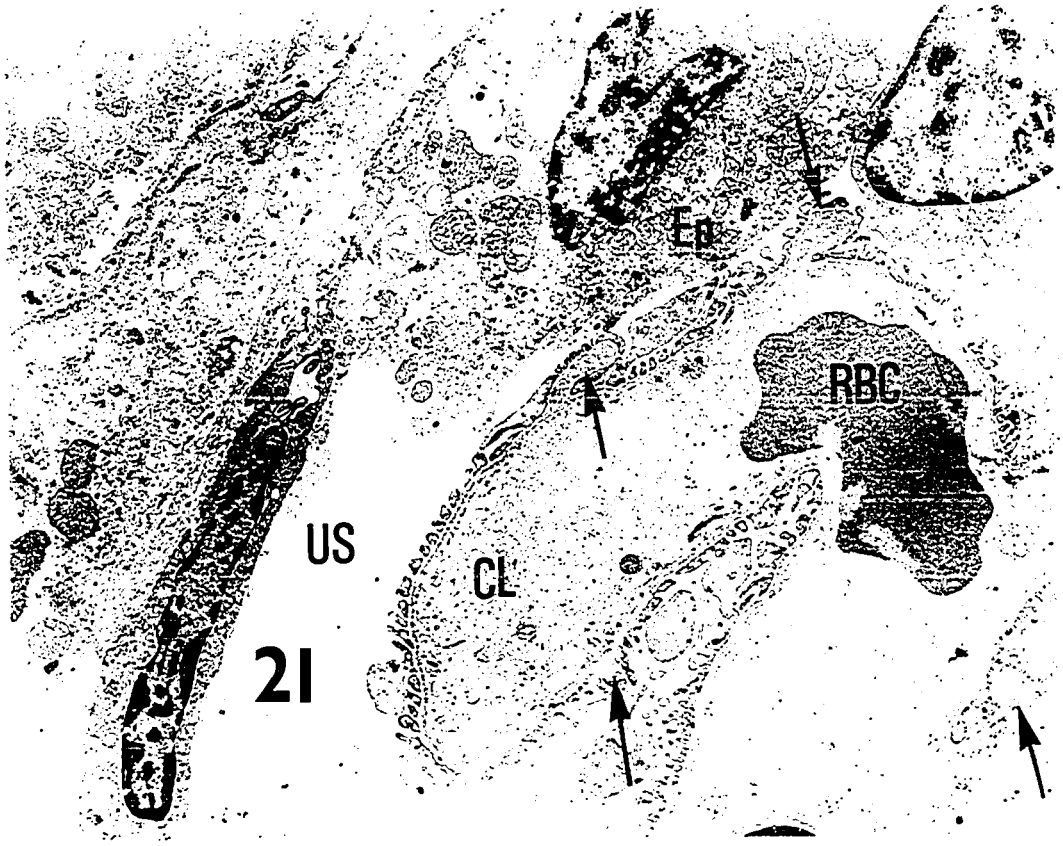
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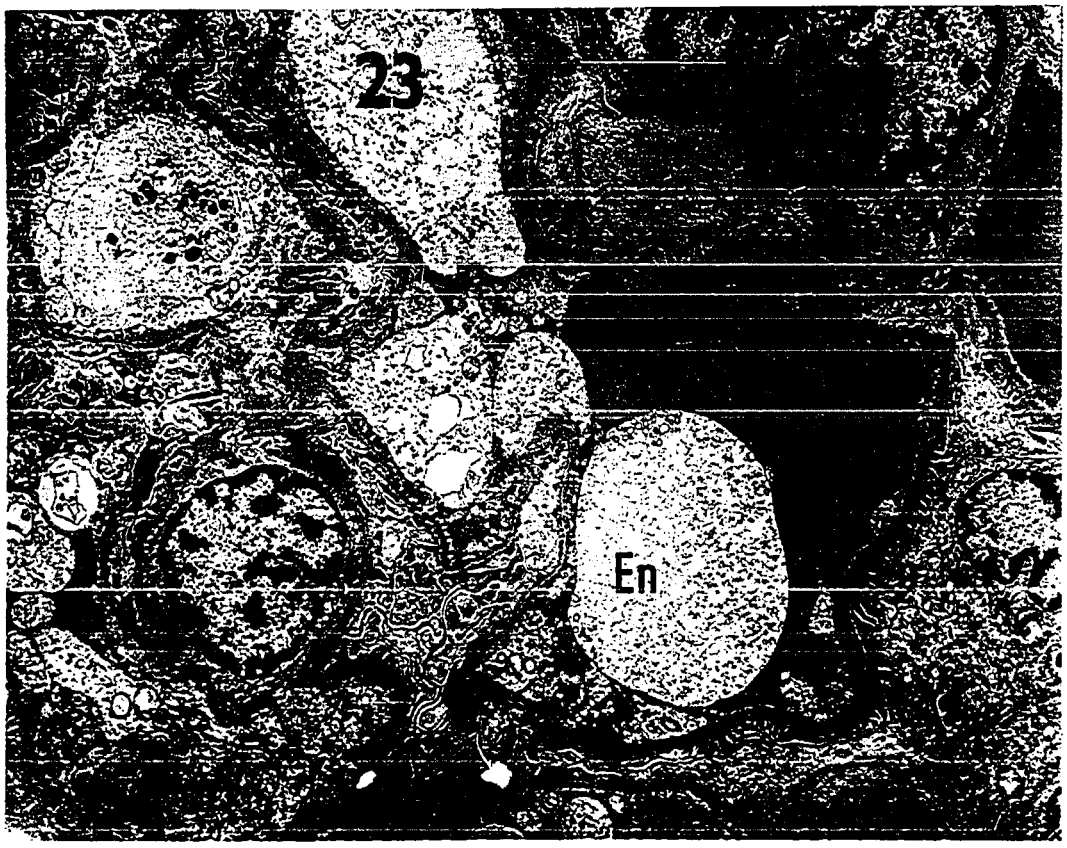
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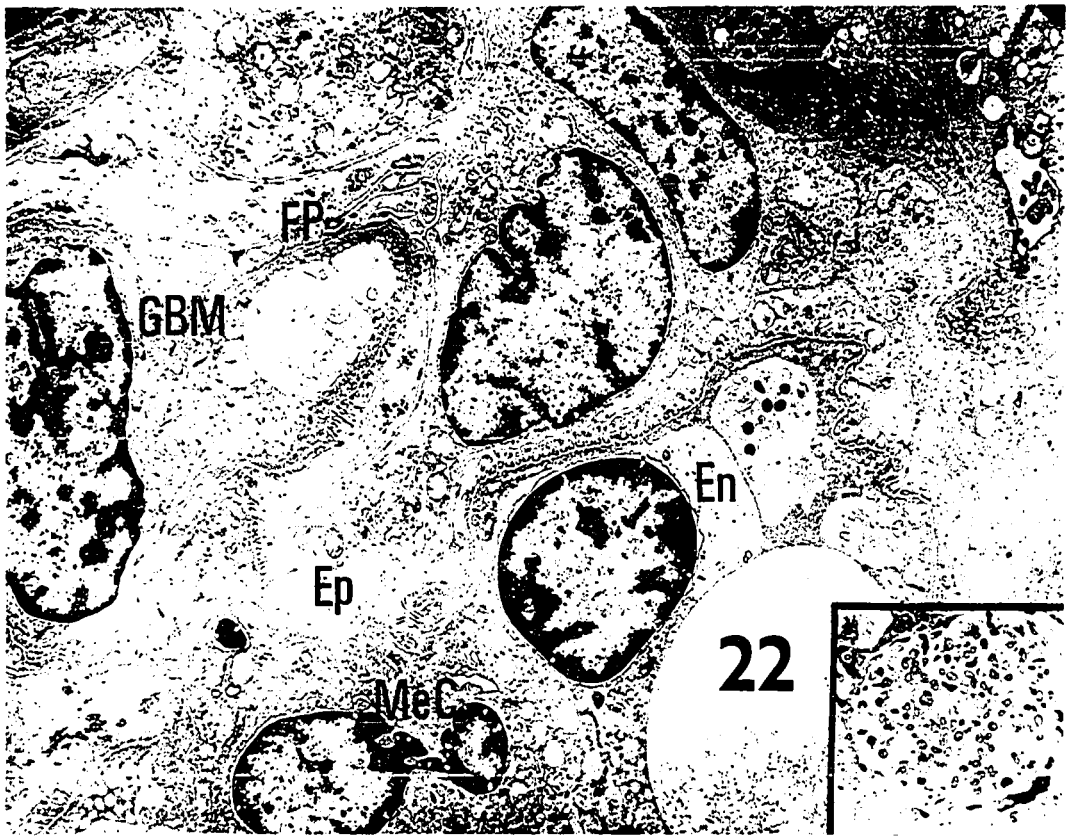
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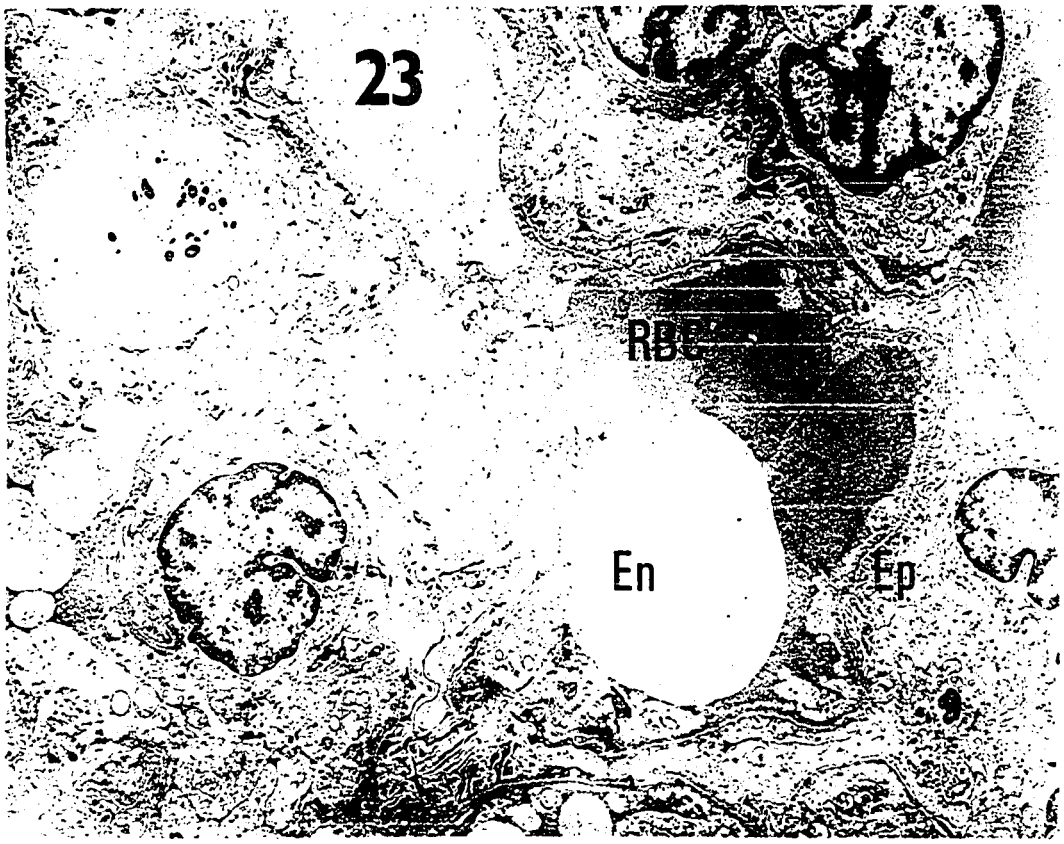
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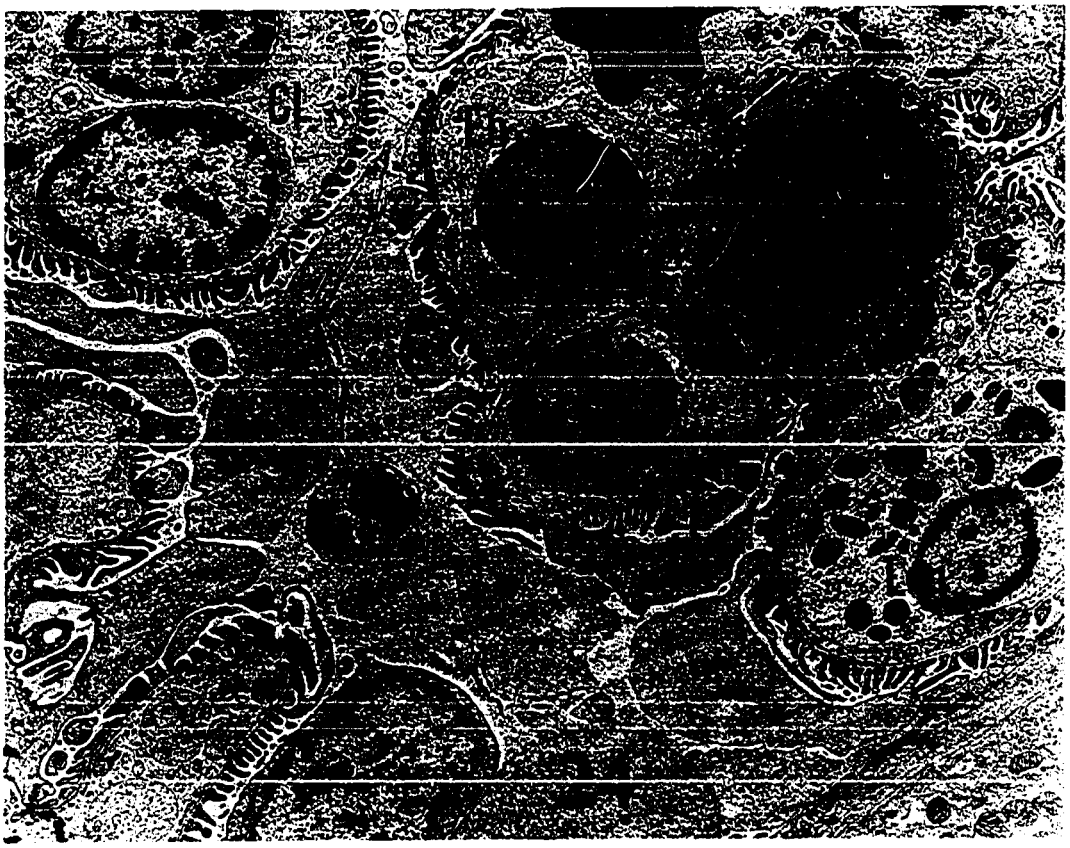


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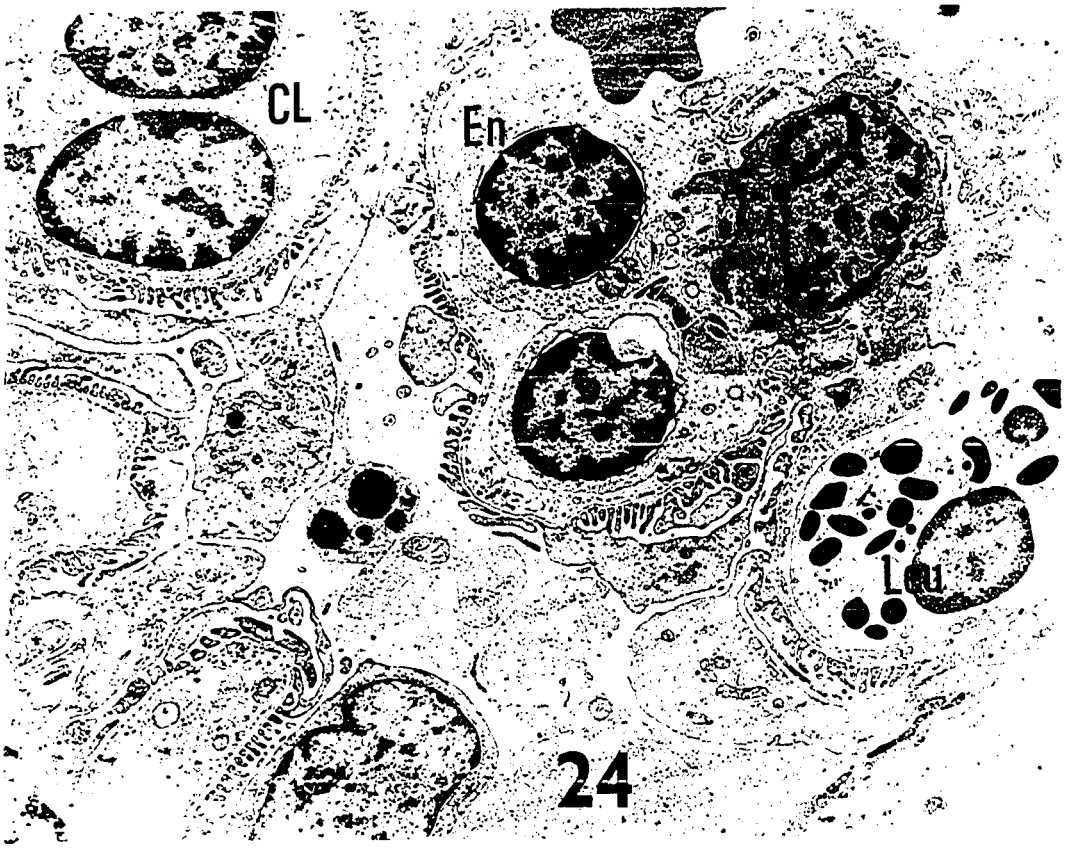
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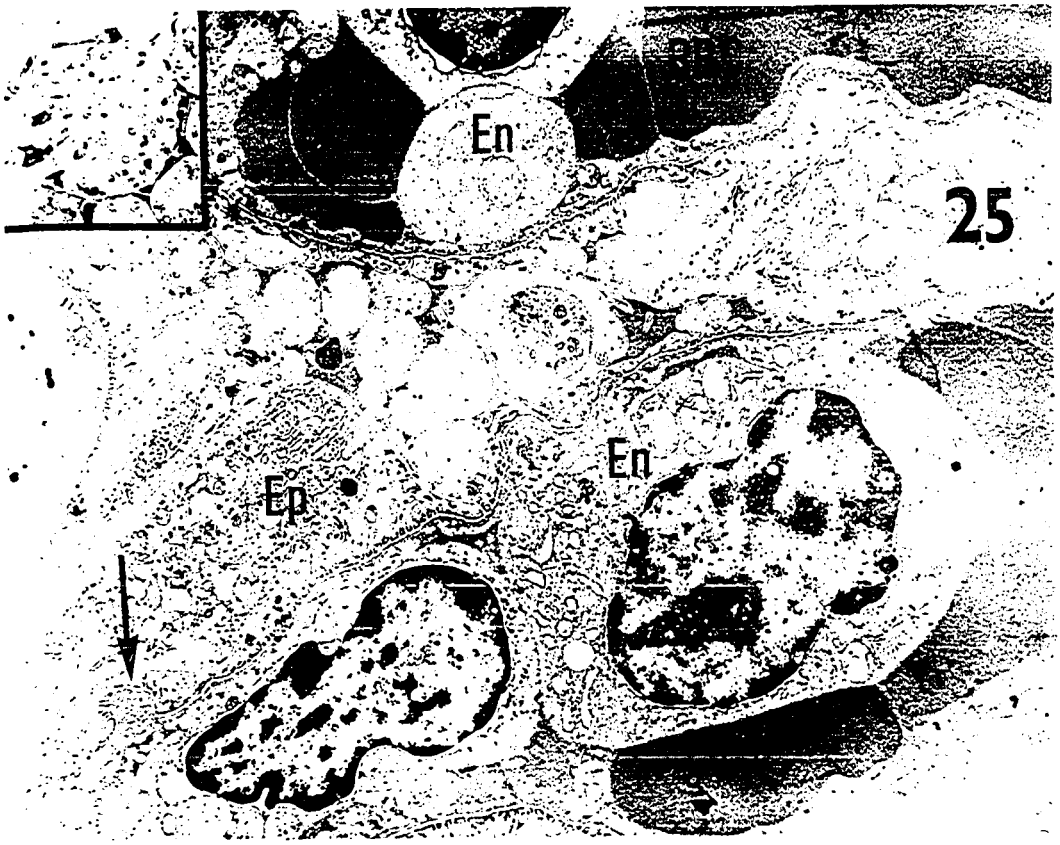
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B. *In Vitro* Tissue Culture Toxicity Studies Using Preparations of Nephrotoxin

Introduction - Primary chick embryo subcultures and the prepared cell cultures listed in Table 11 were used for the *in vitro* assay of possible direct, cellular toxic effects exerted by nephrotoxin associated preparations in contrast to the corresponding preparations obtained from type 6 streptococcal cultures.

When monolayers of cells were obtained in the case of the subcultured primary chick embryo and L cell cultures prepared in the laboratory, or when the commercially available cell cultures were received, the growth media over the monolayers were replaced by media containing the different preparations to be tested. The preparations used consisted of the Sephadex G-25 column separated fractions of the semi-purified extracts of type 6 and type 12 streptococcal broth cultures (see *Fig. 8*,p. 87). The column purified fractions, ranging from 1 to 4, were freeze-dried and reconstituted in a small amount of double distilled water to a concentration of 30 to 50 mg/ml. Maintenance medium No. 3 (Gibco, see Appendix) was used to dilute these fractions: Fraction 1 to a final concentration of 2 mg/ml, and Fractions 2, 3, and 4 to 1 mg/ml.

Results

The first changes, if any were seen in the cultured cells, were increased refractility and granularity. The cells began to round up, with subsequent necrosis and complete cell degeneration. Such changes

were seen with the chick embryo, L-929, and WI-38 cells. With the other cell cultures, no difference was observed between the cells which had been overlaid with nephrotoxin preparations and those with medium alone.

With the cell cultures in which changes were seen as compared to the controls, no difference was noted between those overlaid with type 12 and those with type 6 preparations. Furthermore, the very first changes were seen in those preparations which contained small amounts of ammonium sulfate, just before Fraction 2.

In conclusion, the subcultured chick embryo cells seemed to be most susceptible to the effects of both type 12 and type 6 preparations, followed by WI-38, a continuous line of human origin.

TABLE 11

Effect of Nephrotoxin Preparations on Tissue Culture

Cells	*Roller Tubes or Petri Plates*	*Observation Period**	*First Appearance of Tissue Damage (Both Type 6 and Type 12)*
Chick embryo	Tubes	3 days	12 hours
	Plates	3½ days	18 hours
	Tubes	2 days	20 hours
WI-38	Tubes	6½ days	48 hours
H Ep-2	Tubes	3 days	72 hours
L-929	Plates	5½ days	3½ days
Vero	Tubes	6 days	6 days
LLC-MK_2	Tubes	4 days	4 days

* The cells were observed until the controls were beginning to degenerate.

C. Chemical Characterisation of Nephrotoxin Associated Preparations

1. Dansylation in the Identification of N-*Terminal Amino Acids of Active Bands*

Introduction - Matheson and Reed (1959) had shown that the semi-purified nephrotoxin extract prepared by precipitation of the type 12 streptococcal culture filtrate dialysate had consisted mainly of the well-known, commonly occurring amino acids. Cheuk (1960) purified the semi-purified extract further by paper electrophoresis in 0.05 M sodium borate - 0.01 M NaCl buffer, pH 9.2, and obtained a fast migrating band, "Band I", which he had found to have toxicity in rabbits, causing hypertension and kidney lesions. This active band yielded 11 amino acids on acid hydrolysis. Micklea (1968) also obtained an active band which he concluded corresponded to Cheuk's Band I. In addition he discovered a Band I_1 which migrated even faster than Band I, but which was probably lost by Cheuk who routinely ran his electrophoretic separations for 6 hours instead of Micklea's 4 hours. In the present experiments, thin-layer electrophoretic separations were carried out in 0.01 M sodium borate - 0.003 M NaCl buffer, pH 9.2 (see Materials and Methods), which gave consistently reproducible separations of the semi-purified extracts. The two fastest migrating bands were collected and eluted, and the samples freeze-dried for use. Attempts were made to identify the N-terminal amino acids by Gray and Hartley's dansylation procedure as outlined in Materials and Methods.

Results

The di- and tripeptides, glycyl-D-asparagine, histidyl-histidine, DL-leucyl-glycine, and L-leucyl-glycyl-glycine, readily gave rise to the corresponding dansylated, N-terminal amino acids when the peptides were dansylated at room temperature for 3 hours under alkaline conditions, followed by acid hydrolysis.

The polyamino acids used, poly-DL-alanine (mw 1,700), poly-L-aspartic acid (mw 3,300), and poly-L-glutamic acid (mw 38,000), required the presence of 8 M urea and a longer period of dansylation, overnight at room temperature instead of just 3 hours.

Figs. 26 and 27 show the chromatographic patterns of the polyamino acids and Band 1 and Bands 1 + 2 of type 12 origin, fast migrating bands which are presumably active and which were obtained from electrophoretic separation of type 12 semi-purified extracts, and later subjected to Gray and Hartley's dansylation procedure in the presence of 8 M urea. *Fig. 26* shows the resulting products on cellulose thin-layer chromatography in 0.8% acetic acid - 0.4% pyridine buffer, pH 4.4. The samples, Bands 1 and Bands 1 + 2 of type 12 origin, all gave rise to fluorescent spots not detectable in either the "reagent control" or the "blank plate control" which was originally the eluate of a blank portion of the electrophoretic support. One prominent unknown spot was aligned approximately to the standard dansyl-leucine and dansyl-isoleucine. However, when the samples were run in a different ascending thin-layer chromatography system, as shown in *Fig. 27*, the spot was not detectable, and none was ligned with dansyl-leucine or dansyl-isoleucine. Similarly, when the same samples

were subjected to high voltage electrophoresis in the same pyridine-acetate buffer, pH 4.4, as above, no spots other than those present in the controls or as other reagent breakdown products as seen in "poly-aspartic acid", were seen (*Fig. 28*).

From these results it appears that no readily available N-terminal amino acids are present in the two fastest migrating bands obtained by electrophoretic separation of the type 12 semi-purified extract.

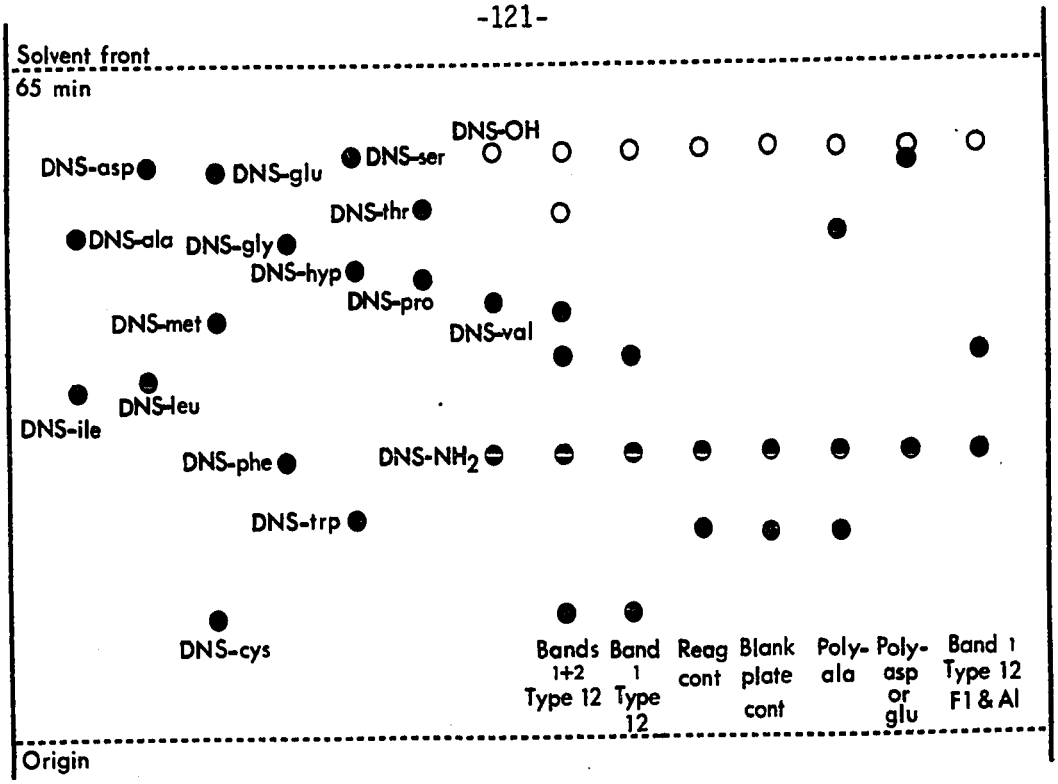

Fig. 26. Thin-layer chromatogram of hydrolysates of dansylated purified nephrotoxin and known polyamino acids along with standard dansyl amino acids. Band 1 *and* Bands 1+2 *were eluates from electrophoretically separated semi-purified extract.* Blank plate control *was eluate of blank area of electrophoretic support.* Reagent control *was reagents only subjected to same procedures. The MN 300 cellulose plates were developed in 0.8% acetic acid-0.4% pyridine.*

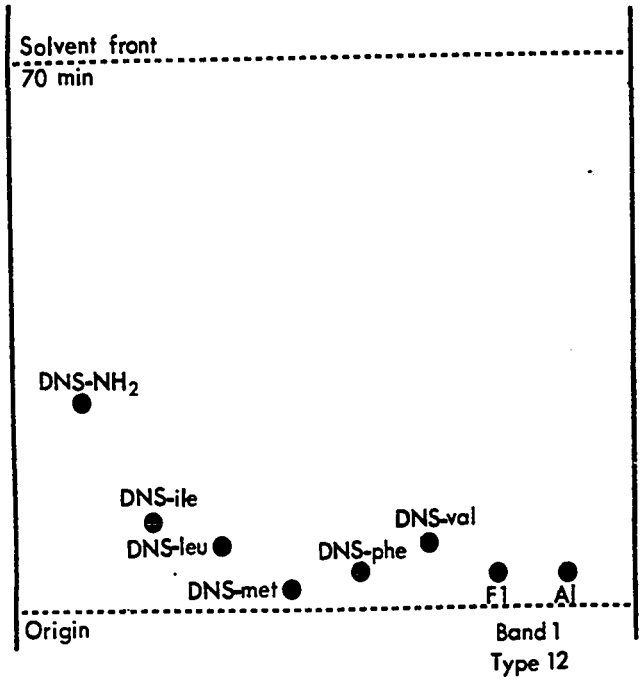

Fig. 27. Thin-layer chromatogram of dansylated products in an alternate system. The silica gel H:kieselgel G (5:1) layers were developed in petroleum ether: 2,4,6-trimethylpyridine: methyl ethyl ketone: acetic acid, 75:5:24:3.

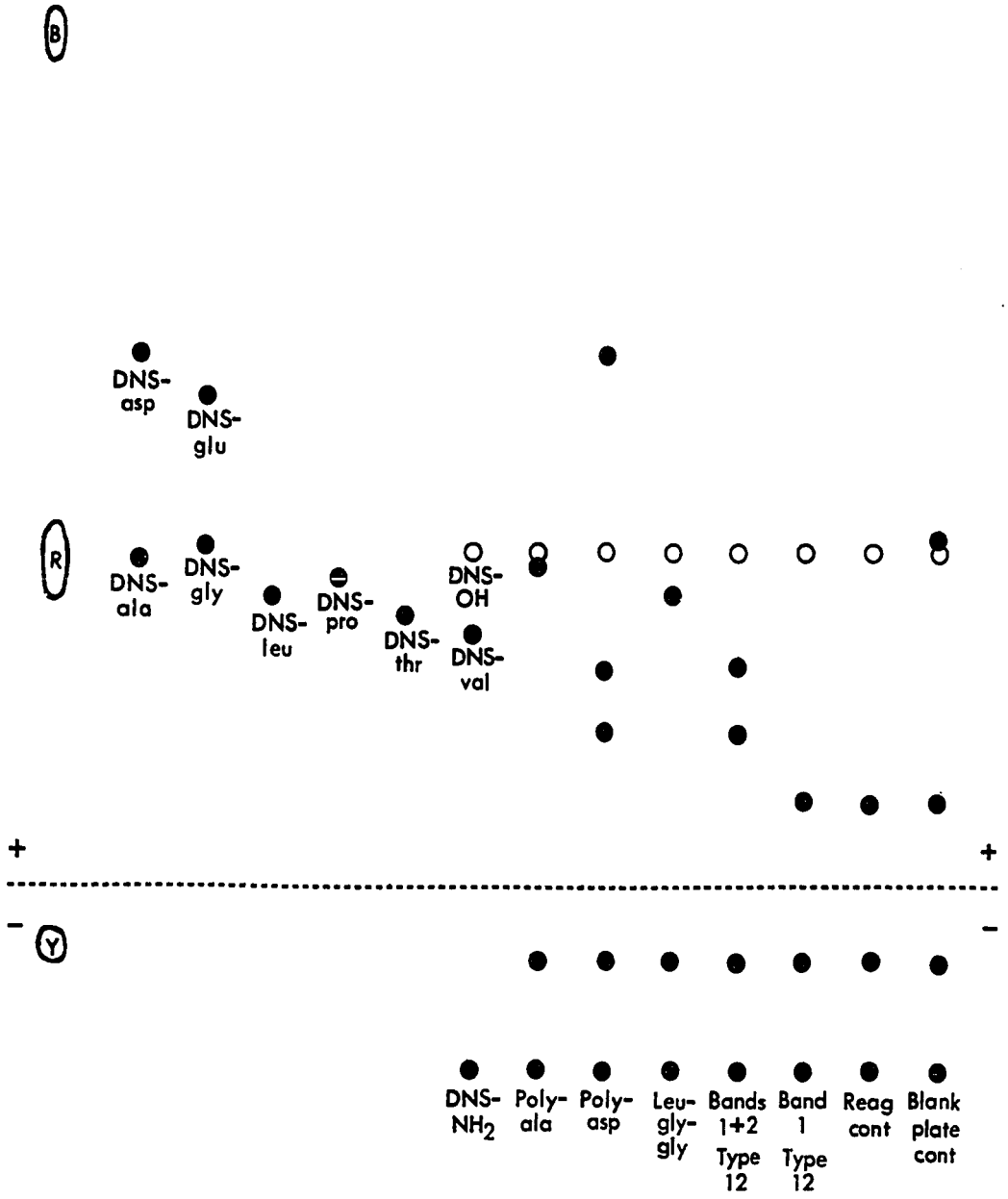

Fig. 28. High voltage electrophoretic separation of hydrolysates of dansylated purified nephrotoxin and known polyamino acids along with standard dansyl amino acids. Band 1 *and* Bands 1+2 *were eluates from electrophoretically separated semi-purified extract.* Blank plate control *was eluate of blank area of electrophoretic support.* Reagent control *was reagents only subjected to same procedures. Electrophoresis was performed in 0.8% acetic acid-0.4% pyridine, pH 4.4 buffer at 80 volts/cm for 2 hours.*

2. Amino Acid Analysis of the Semi-Purified Extracts

Introduction - As mentioned previously, the ammonium sulfate precipitated "semi-purified extract" of the type 12 streptococcal culture filtrate dialysate consists mainly of the well-known amino acid residues upon hydrolysis. Further purification of the semi-purified extract on paper electrophoresis in 0.05 M sodium borate - 0.01 M NaCl, pH 9.2 buffer revealed that this extract was extremely heterogeneous and consisted of a minimum of 8 or 9 distinct substances (Matheson and Reed, 1959). Cheuk (1960) studied the biological activity of each of the bands separated by electrophoresis, and found that a fast migrating "Band I" was active. Chemical characterisation of this Band I yielded a minimum of 11 amino acids. Micklea (1968) confirmed the biological activity of a band obtained by paper electrophoresis in the same buffer system corresponding to Band I. In addition, he found that another band, referred to as Band I_1, which migrated even faster than Band I, also was active, as well as a slow migrating band corresponding to Cheuk's Band A. He concluded that Band I_1 was lost from the paper electropherograms run by Cheuk who always carried out the run for 6 hours instead of the 4 hours of Micklea. In the interval before the present work commenced, the molarity of the electrophoresis buffer was decreased to give sharper delineation of the fast migrating bands. In the present work, it was found that a 3 hour electrophoretic separation on cellulose thin-layer plates in 0.01 M sodium borate - 0.003 M NaCl, pH 9.2 buffer gave better and more consistent separations of the fast migrating bands from the remaining substances in the semi-purified extracts. These three bands were all collected and eluted.

At the same time, further purification of the semi-purified extract was attempted using column chromatography by Fardy (1967), Friedlander (unpublished data), and in the present work. By using Sephadex G-15 and water as the eluant, Fardy was able to separate the semi-purified extract into two major fractions, Fraction I, the excluded fraction, and Fraction II, the fraction eluting before the ammonium sulfate. A better separation was achieved in the present work by using a Sephadex G-25 column with 0.02 M NaCl as eluant (see *Fig. 8*).

In the present experiments, all 4 fractions from the Sephadex G-25 column as well as the 3 fastest migrating bands eluted from electropherograms were subjected to acid hydrolysis (6.7 N HCl) for 18 hours and subsequent analysis by two-dimensional thin-layer chromatography, to see if there is any difference between the type 12 preparation as compared to type 6 and Todd-Hewitt preparations.

Results

Hydrolysis of the 3 fast migrating bands either together or separately only yielded very faint ninhydrin positive spots, which were unidentifiable either by thin-layer chromatography, or by use of the automatic amino acid analyser (Friedlander and Ng, unpublished data).

Photographs of the thin-layer chromatographic separations of the acid hydrolysates of the G-25 column fractions are shown in *Figs. 29 to 32*. The corresponding schematic drawings of the chromatograms are shown in *Fig. 33*. Some minor differences were seen among the hydrolysates of the fractions of semi-purified extracts from type 12 and type 6 broth cultures, and the growth medium alone. However, no outstanding differences nor consistent patterns of variation were seen.

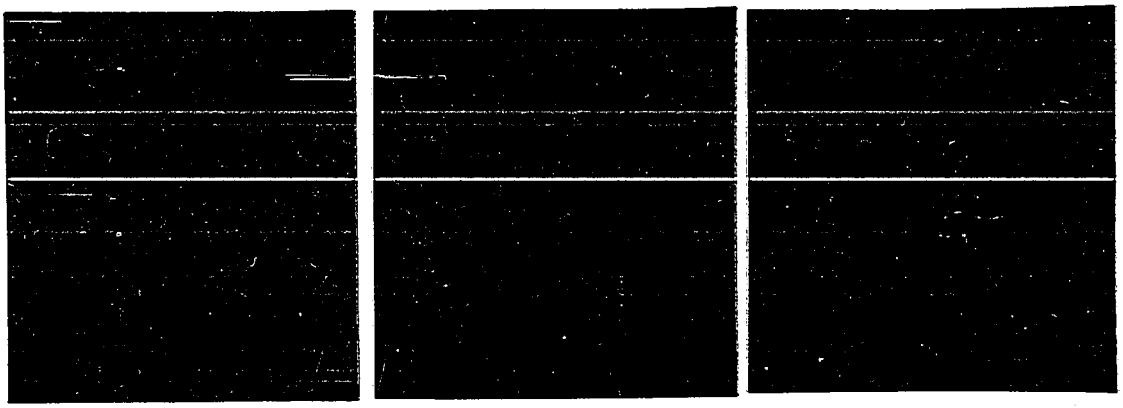

Fig. 29. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 1 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

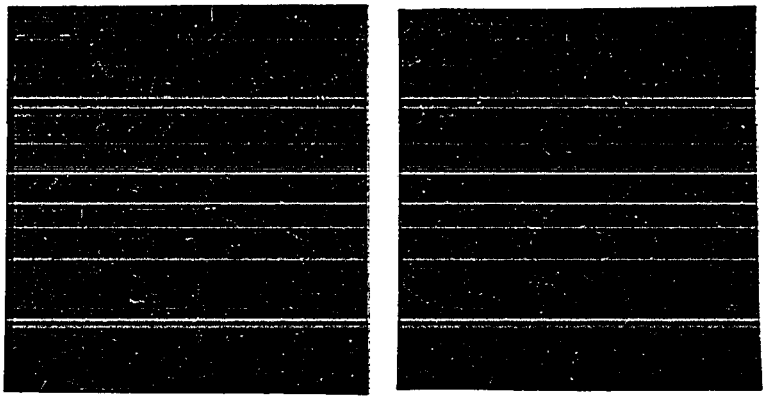

Fig. 30. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 1a which were obtained by gel filtration of type 12 and type 6 semi-purified extracts.

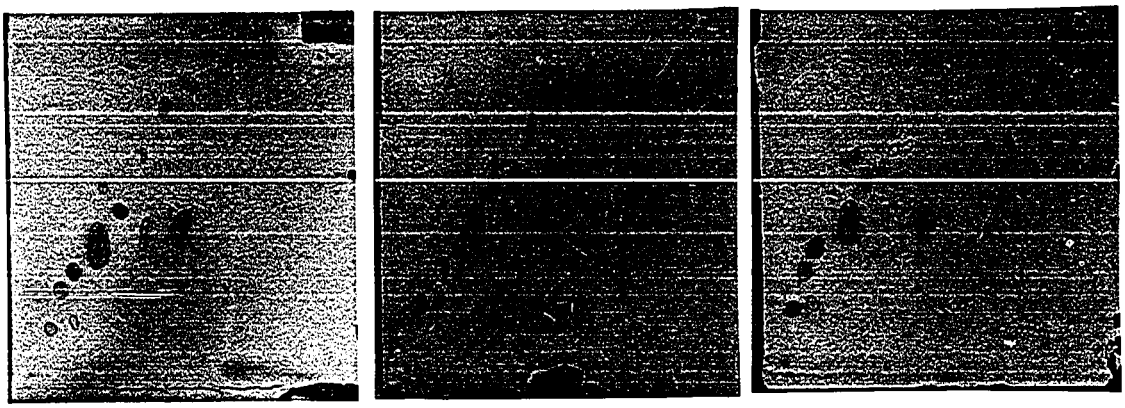

Fig. 29. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 1 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

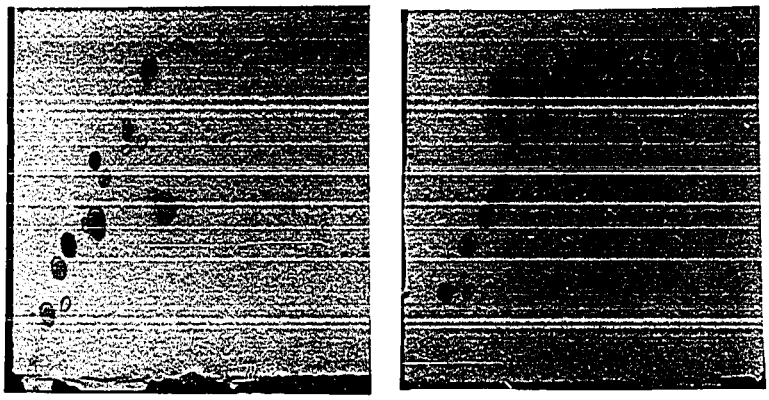

Fig. 30. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 1a which were obtained by gel filtration of type 12 and type 6 semi-purified extracts.

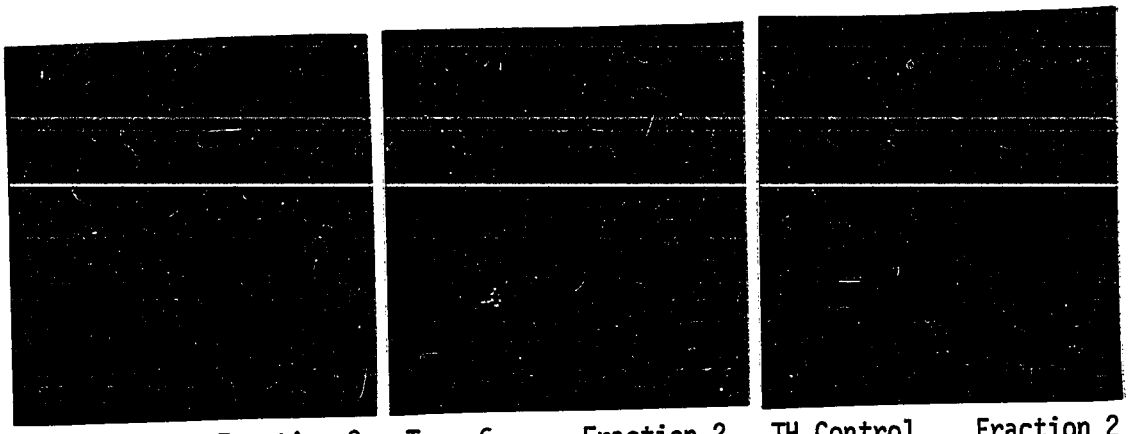

Fig. 31. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 2 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

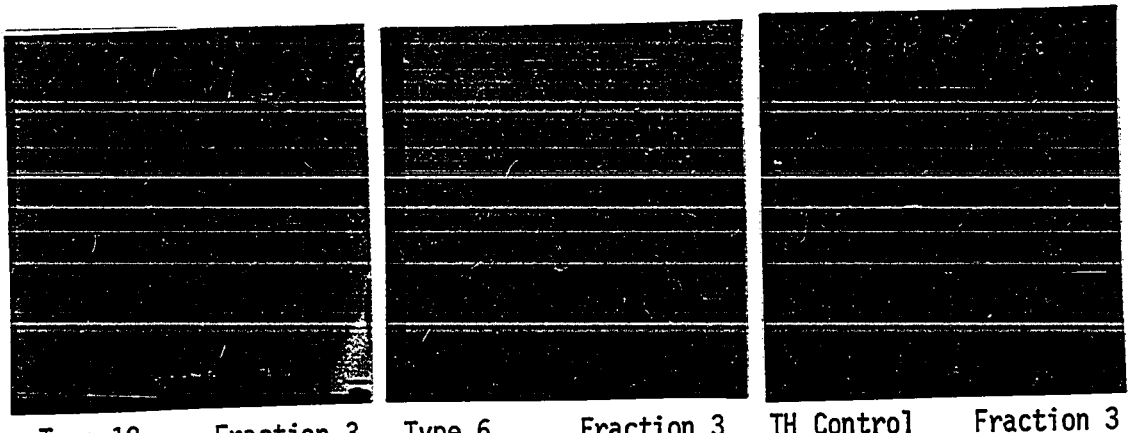

Fig. 32. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 3 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

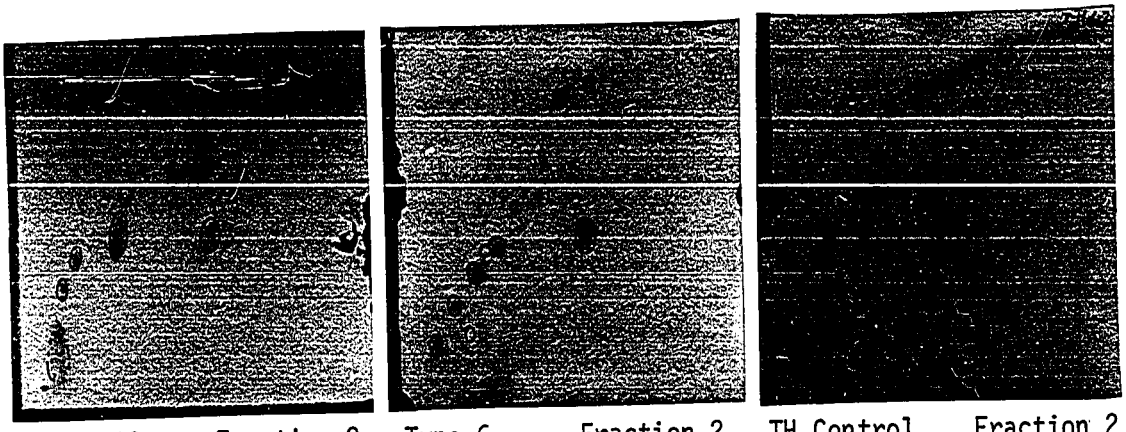

Fig. 31. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 2 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

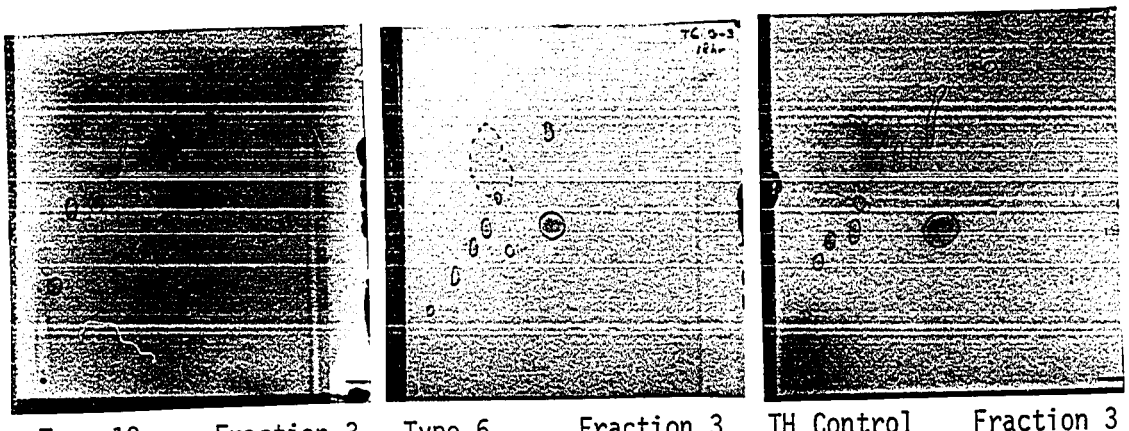

Fig. 32. Two-dimensional thin-layer chromatograms of acid hydrolysates of Fractions 3 which were obtained by gel filtration of type 12, type 6, and Todd-Hewitt (TH) semi-purified extracts.

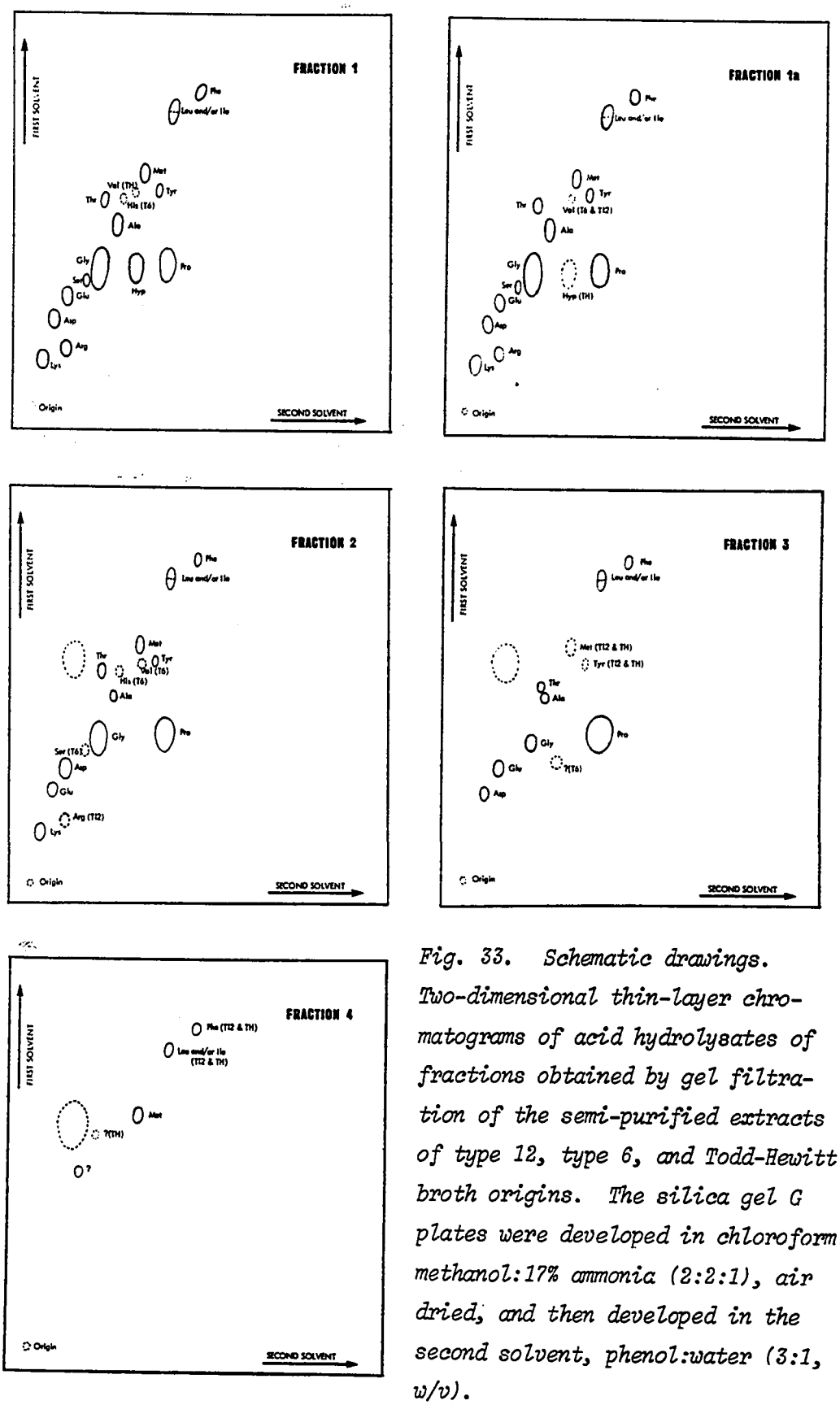

Fig. 33. Schematic drawings. Two-dimensional thin-layer chromatograms of acid hydrolysates of fractions obtained by gel filtration of the semi-purified extracts of type 12, type 6, and Todd-Hewitt broth origins. The silica gel G plates were developed in chloroform: methanol:17% ammonia (2:2:1), air dried, and then developed in the second solvent, phenol:water (3:1, w/v).

3. Gas-Liquid Chromatography

Introduction - The unknown samples, Bands 1, 2, and 3 which were the fastest migrating bands obtained from electrophoretic separation of the semi-purified extracts of both type 12 and type 6 broth cultures were examined by gas-liquid chromatography. Although the exact chemical nature of these samples were not known, their various staining reactions and fluorescent properties, to be discussed later, suggested that they might be related to amino acids, purines, or pyrimidines. Such substances possess available -H's as found in -OH, -COOH, or $-NH_2$ groups which can be replaced by trimethylsilyl groups using a silylating reagent (Pierce, 1968), thereby becoming sufficiently volatile for examination by gas-liquid chromatography. It was also essential that a method such as gas-liquid chromatography supplement paper chromatographic, ultraviolet, infrared and fluorescence spectral methods in the examination of the hydrolysates of Bands 1, 2, and 3, since as mentioned in the previous section, they only gave very faint ninhydrin positive staining.

Results

Bis-(trimethylsilyl)acetamide (BSA) was found to be the most valuable silylating reagent, giving rise to good recoveries and usually single peak derivatives. Somewhat lower recoveries were obtained when hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in a 2:1 ratio was used. The trimethylsilyldiethylamine (TMSDEA) gave rise to multiple peaks when it was used to silylate some of the standard compounds. A composite gas-liquid chromatogram showing the retention times of the

silylated derivatives of some of the standard compounds which were separated by the chromatographic column used is seen in *Fig. 34*.

In the mild acid hydrolysis, the unknown samples as well as the control were dissolved in 2 N HCl and heated for 1 hour at 100 C. This should have been sufficient to hydrolyse either small polysaccharides or nucleosides and nucleotides. *Fig. 35* shows the gas-liquid chromatogram of the silylated hydrolysate residues of Band 1 of type 12 origin. The remaining samples, Bands 2 and 3 of type 12 origin, Bands 1, 2, and 3 of type 6 origin, and an eluate of a blank portion of the electrophoretic support, gave rise to similar gas-liquid chromatograms. A careful study of these chromatograms in comparison to the retention times of standard compounds indicates that the peaks seen consist chiefly of sugar residues probably arising from impurities which still remained in the electrophoretic support after pre-washing of the cellulose support.

The more complete hydrolysis of the unknown samples and the control material was carried out in 6.7 N HCl at 105 C for 20 hours (see Materials and Methods). The gas-liquid chromatograms of the silylated residues of Bands 1, 2, and 3 of type 12 origin, Bands 1 and 2 of type 6 origin, and the control (eluate of a blank portion of the electrophoretic support) are shown in *Figs. 36 to 38*. It can be seen from these chromatograms that one prominent peak with a retention time of 11 minutes is present in the unknown samples which is absent in the control seen in *Fig. 38b*. This retention time was not identical to any of the standard compounds chromatographed (*Fig. 34*). In addition, the hydrolysate residues of the different bands had more peaks of shorter retention time than did

the control. No clearcut differences in the number of such peaks and their retention times could be seen among the different band eluates except one of quantity.

To summarise the gas-liquid chromatographic data, the mild acid hydrolysis did not reveal any differences between the eluted bands and the control material, whereas the stronger acid hydrolysis led to some noticeable differences. Presumably, the more complete hydrolysis released more monomeric residues which could be silylated and chromatographed with more ease. However, the exact identity of these substances found in the band eluates were not determined. The prominent broad peak with the retention time of 11 minutes was present in both the type 12 and type 6 band eluates whereas some differences were noted among the band eluates with regard to the substances with shorter retention times.

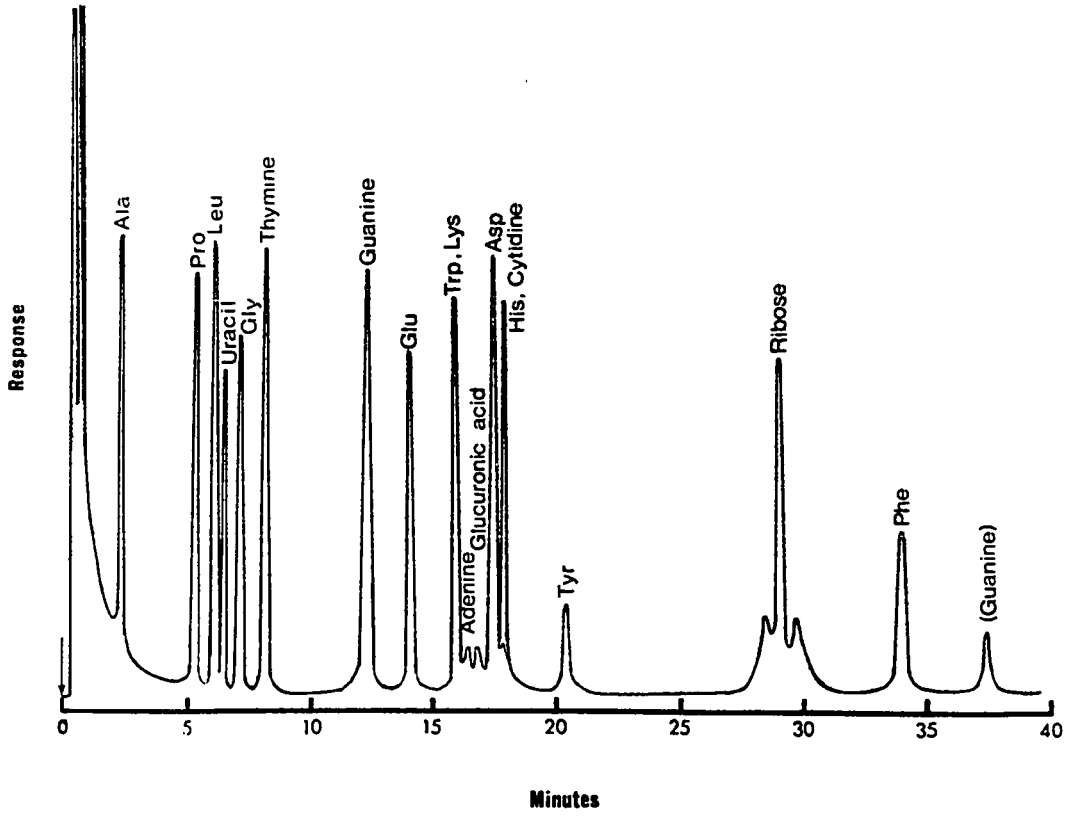

Fig. 34. Composite gas-liquid chromatogram of the silylated derivatives of some standard substances. Column: 6', 15% SE-52 on chromosorb W (AW,DMCS). Oven temperature: 160 C isothermal. N_2: 20 ml per minute.

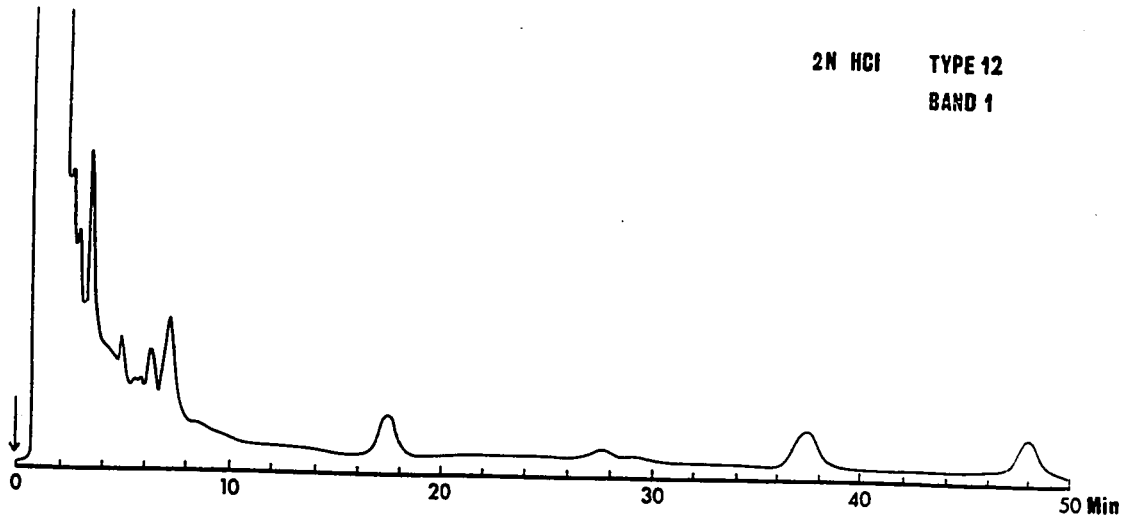

Fig. 35. Gas-liquid chromatogram of the silylated derivatives of Band 1 of type 12 origin which had undergone mild acid hydrolysis for 1 hour in 2N HCl at 100 C.

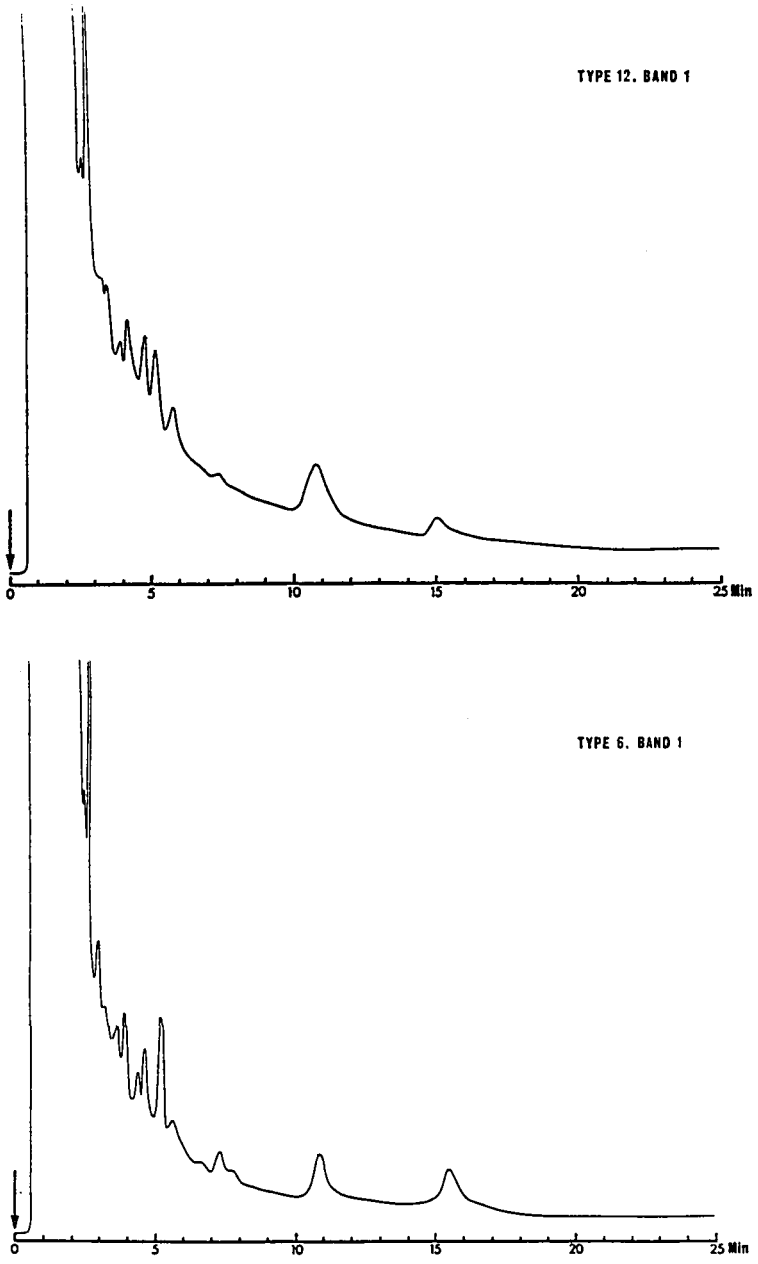

Fig. 36. Gas-liquid chromatograms of the silylated derivatives of Bands 1 of type 12 and type 6 origins which had undergone hydrolysis in 6.7N HCl for 20 hours at 105 C.

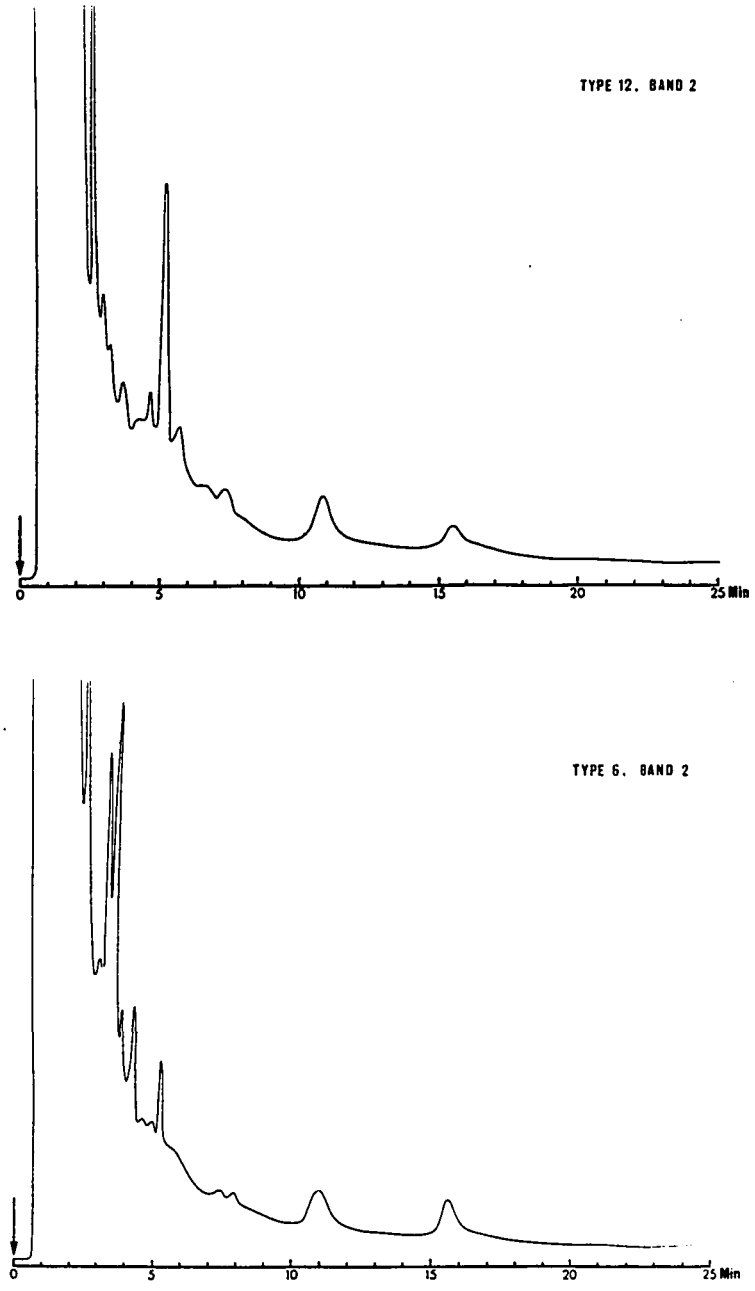

Fig. 37. Gas-liquid chromatograms of the silylated derivatives of Bands 2 of type 12 and type 6 origins which had undergone hydrolysis in 6.7N HCl for 20 hours at 105 C.

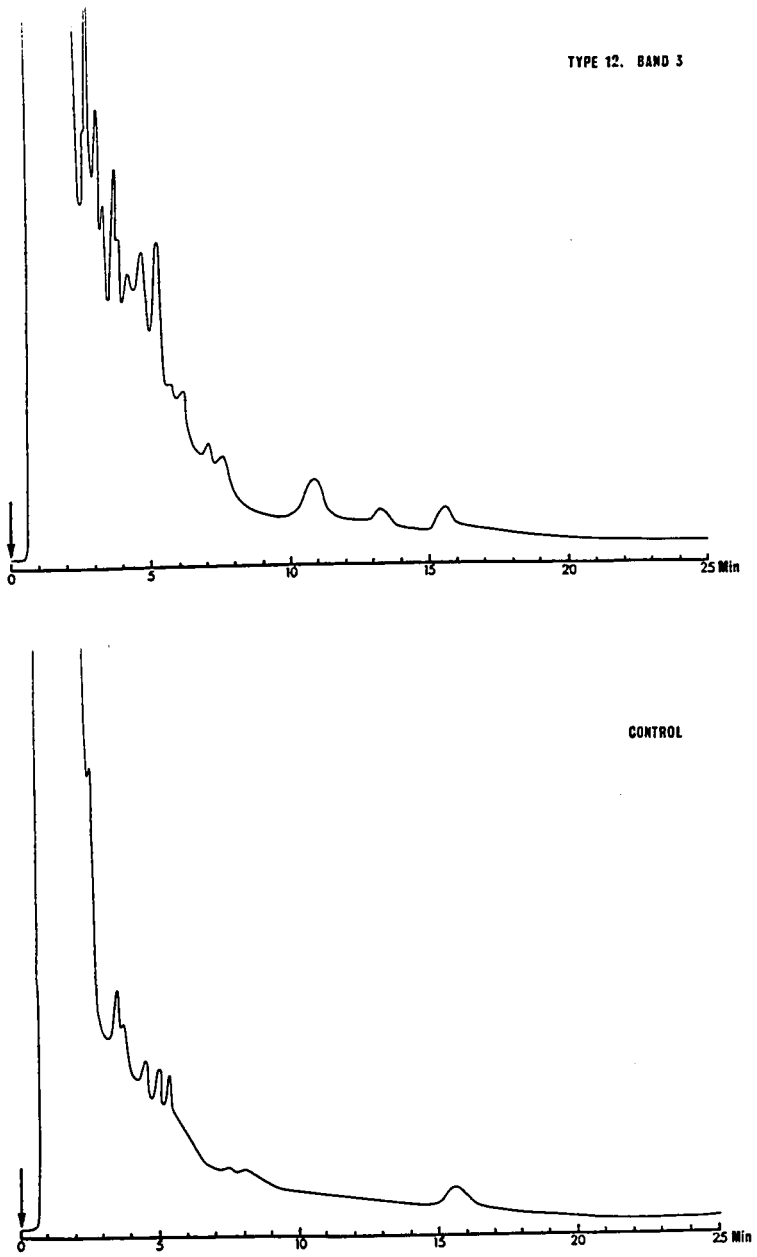

Fig. 38. Gas-liquid chromatograms of the silylated derivatives of Band 3 of type 12 origin and the control eluate of a blank portion of the electrophoretic support which had undergone hydrolysis in 6.7N HCl for 20 hours at 105 C.

4. Quantitative Analysis of Nephrotoxin Associated Preparations

Introduction - It has been difficult to quantitatively analyse nephrotoxin preparations due to two factors. One is the excessive amounts of salts encountered in the preparations. For instance, ammonium sulfate, which had been used for precipitation of the semi-purified extracts, was hard to remove completely, as was the sodium borate in the eluates of electrophoretically separated bands of the semi-purified extracts. In the latter case, the relatively small yield of purified nephrotoxin made the removal of salt a delicate procedure since nephrotoxin has been shown to have affinity for dextran gel and ion exchange resins used in attempted desalting and fractionation. Another factor is the lack of a suitable pure standard substance which can be used for comparison with the nephrotoxin preparations. In the quantitative analyses undertaken in these experiments, 4X crystalline bovine serum albumin (BSA) was used as a standard substance in protein assays by ultraviolet absorption, the biuret and the Lowry methods, all of which were described in Section III *4*. The semi-purified extracts of type 12, type 6, and Todd-Hewitt control origins were examined, as well as the Sephadex G-25 column separated fractions of type 12 and type 6 semi-purified extracts. In addition, the phosphorus and hexosamine contents were also determined.

The content of amino groups in the fast migrating bands eluted from electrophoretically separated semi-purified extracts of both type 12 and type 6 origins was determined by the modified ninhydrin reaction (Section III *4d*).

Results

The protein and phosphate contents of the semi-purified extracts are listed in Table 12. Hexosamine equivalent to less than 0.01% glucosamine of the proteinaceous material was obtained on a sample of type 12 semi-purified extract which was 5 times as concentrated as the samples in Table 12.

TABLE 12

Protein and Phosphate Contents of Semi-Purified Extracts

Origin of Semi-Purified Extract	*O.D.* $_{276\ nm}$ mg/ml	*Biuret* mg/ml	*Phosphorus* µg/ml *Total*	*C-M**	*Residue***	*Total*** (New Batch)*
Type 12	20.0	3.6	4.5	1.6	1.5	3.9
Type 6	20.0	4.4	6.5	3.2	0.6	4.9
Todd-Hewitt control	20.0	5.2	3.2	2.8	0.8	4.6

* C-M: P content in chloroform:methanol (2:1) extractable portion.

** Residue: P content in the residue following chloroform:methanol extraction.

*** Total P of new batch: The concentration of proteinaceous content had been adjusted to 20 mg/ml by absorption at 276 nm.

Table 13 shows the results of analyses of the Sephadex G-25 column separated fractions of the semi-purified extracts.

TABLE 13

Protein and Phosphate Contents of Sephadex G-25 Column Separated Fractions of Semi-Purified Extracts*

Fraction	*O.D.* $_{276\ nm}$ mg/ml Type 12	*Biuret* mg/ml Type 12	(Type 6)	*Lowry* mg/ml Type 12	(Type 6)	*Phosphorus* µg/ml Type 12
1	52.2	18.8	20.0	32.0	20.8	36.8
2	23.4	3.2	3.2	5.1	5.4	5.8
3	17.2	1.0	0.8	2.2	1.7	1.9
4	14.9	0.4	1.15	1.0	2.4	0

* Hexosamine values were all below the range of detection, or less than 0.05 µM/ml.

In the nitrogen determination of the eluates of electrophoretically separated semi-purified extracts, even the total eluate from approximately 10 thin-layer plates did not give rise to a detectable level of nitrogen which was more than the level detected in an equal amount of the eluate from blank portions of the plates.

To conclude, the phosphorus distribution in all the samples tested was from 0.01 to 0.05% of the proteinaceous material. Hexosamine content was less than 0.01%. The samples analysed seemed to contain residues which absorb at 276 nm but which do not react with the Lowry-Folin reagent to give rise to the color complex. The proportion of peptide bonds to the total material decreased from Fraction 1 to Fraction 4, as were the Lowry-Folin reagent reactive residues. It can be recalled that Fraction 4 upon electrophoresis gave rise to the 3 fast migrating active bands (*Fig. 8*).

5. Qualitative Spot Analysis of Nephrotoxin Associated Preparations

Introduction - As discussed previously, Fraction 4 derived from Sephadex G-25 column separation of the semi-purified extract contained in part the 3 fast migrating bands when it was subjected to electrophoresis. One or more of the bands could be active *in vivo*. Upon acid hydrolysis of Fraction 4, only faintly ninhydrin positive material could be seen. At the same time, further purification of this Fraction 4 on DEAE-Sephadex A-25 column revealed that two major peaks were obtained, the first of which contained the same fast migrating components upon electrophoresis (Friedlander, unpublished data). For the spot analysis, the Fractions 4 from the type 12 and type 6 semi-purified extracts and the first fraction from the DEAE column were hydrolysed and spotted for paper chromatographic separations. Attempts were also made in examining the hydrolysates of the eluates of the 3 fast migrating bands from those separated by electrophoresis of the semi-purified extract of type 12 origin. However, only the column separated samples gave rise to relatively salt-free hydrolysates which could easily be chromatographed.

Results

Ultraviolet illumination of the paper chromatograms revealed 2 major spots and 5 smaller ones from Fraction 4 of type 12 as well as from type 6 origin. None of these spots stained with aniline hydrogen phthalate (for reducing sugars). When milder acid hydrolysis was carried out in 2 N HCl at 100 C for 1 hour, no staining was seen either. Very faint staining was seen with the Elson-Morgan reagent for hexosamines.

Staining with the silver nitrate-bromophenol blue reagent (for purine and pyrimidine derivatives) revealed a prominent spot, the second major one from the origin, staining to a hue which is typical of a pyrimidine derivative. The first spot from the origin was probably due to Cl^-. Staining with quinine sulfate (for phosphate esters) and sodium fluoresceinate (for aromatic and heterocyclic compounds) also revealed the same spot. (*Fig. 39*).

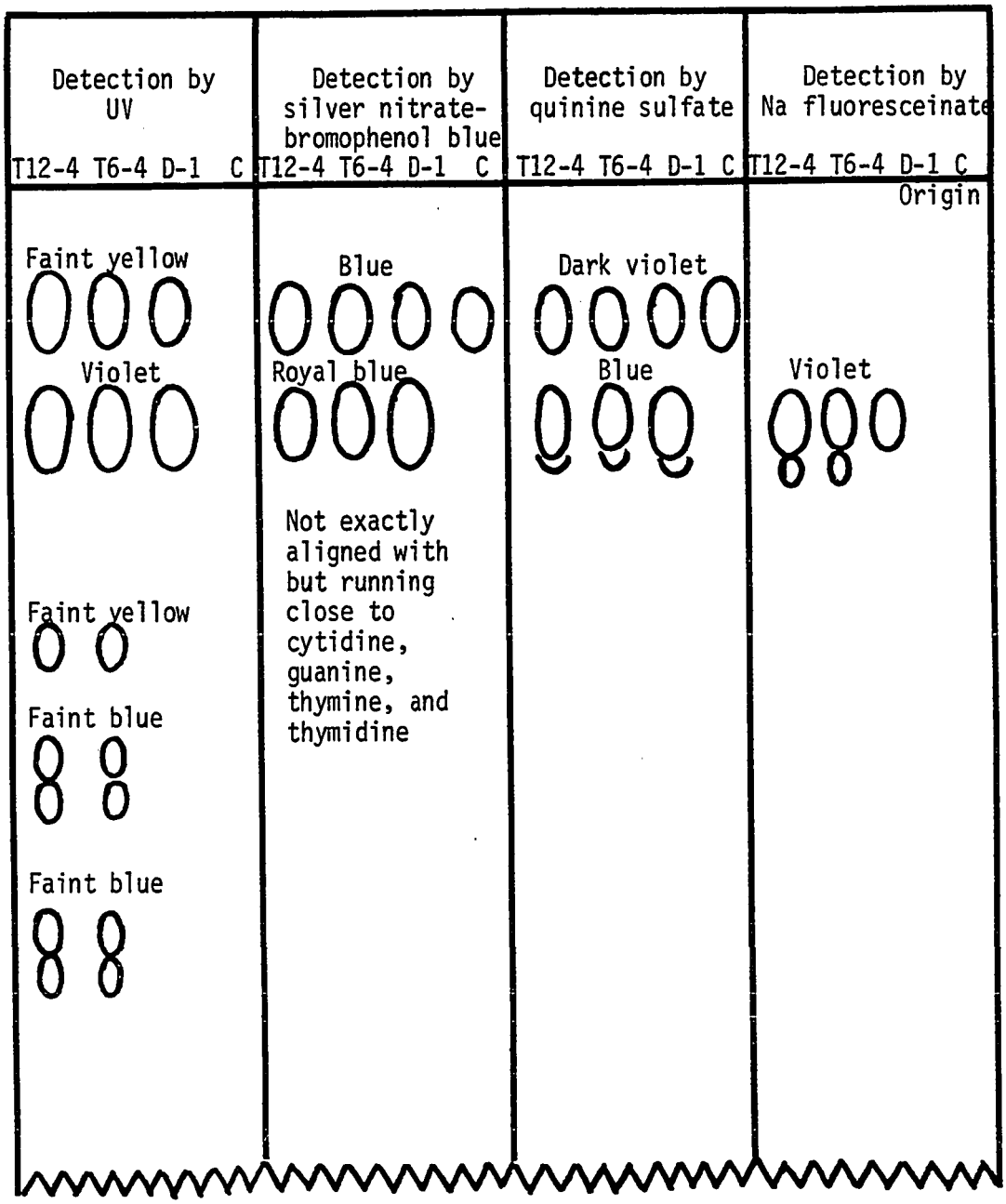

Fig. 39. Descending paper chromatography of acid hydrolysates of some purified nephrotoxin preparations in 1-butanol:acetic acid: water, 4:1:5. T12-4 = *Fraction 4 from G-25 column separated type 12 semi-purified extract.* T6-4 = *Fraction 4 from G-25 column separated type 6 semi-purified extract.* D-1 = *Fraction 1 from DEAE-Sephadex A-25 column separated Fraction 4 of type 12 origin from above.* C = *Concentrated eluate from unused DEAE-Sephadex A-25 column.*

6. Estimation of the Size of Nephrotoxin and Observations on Its Solubility Properties

a. Estimation of Size

i. Dialysis

One of the first steps in the purification of nephrotoxin involves dialysis using Visking cellophane tubing (1 1/8" inflated diameter), with the dialysate being active. According to this observation, the molecular weight of nephrotoxin has to be less than 12,000, the approximate exclusion limit of the cellophane pore size, providing nephrotoxin is globular.

ii. Ultrafiltration

Ultrafiltration using Diaflo membranes (Amicon Corp.) was attempted as a means of desalting the semi-purified nephrotoxin extract which had been prepared by ammonium sulfate precipitation from the dialysable portion of the culture filtrate. The membranes were not found to be a practical means for desalting the semi-purified extract since they became corroded after an initial period of use. However, some information as to the size range of substances from the extract was obtained.

The UM-10 membrane (10,000 mw exclusion limit) retained the major portion of the semi-purified extract. When this portion was separated by electrophoresis in the standard borate buffer system, the fast migrating bands were found to be missing. The portion of the extract which came through the UM-10 was completely retained by the UM-2 membrane (1,000 mw exclusion limit). This UM-2 retained portion upon electrophoresis gave rise to the fast migrating bands as well as some other bands.

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One of the first steps in the purification of nephrotoxin involves dialysis using Visking cellophane tubing (1 1/8" inflated diameter), with the dialysate being active. According to this observation, the molecular weight of nephrotoxin has to be less than 12,000, the approximate exclusion limit of the cellophane pore size, providing nephrotoxin is globular.

ii. Ultrafiltration

Ultrafiltration using Diaflo membranes (Amicon Corp.) was attempted as a means of desalting the semi-purified nephrotoxin extract which had been prepared by ammonium sulfate precipitation from the dialysable portion of the culture filtrate. The membranes were not found to be a practical means for desalting the semi-purified extract since they became corroded after an initial period of use. However, some information as to the size range of substances from the extract was obtained.

The UM-10 membrane (10,000 mw exclusion limit) retained the major portion of the semi-purified extract. When this portion was separated by electrophoresis in the standard borate buffer system, the fast migrating bands were found to be missing. The portion of the extract which came through the UM-10 was completely retained by the UM-2 membrane (1,000 mw exclusion limit). This UM-2 retained portion upon electrophoresis gave rise to the fast migrating bands as well as some other bands.

Therefore, according to the observations on ultrafiltration behavior of nephrotoxin, the size can be estimated as between 1,000 to 10,000 molecular weight.

iii. Gel filtration

The fractionation range of Sephadex G-25 cross-linked dextran gel is 1,000 to 5,000 mw. This gel was found to give better separation of the semi-purified extracts than either G-15 (Fardy, 1967) or Bio-Gel P-2 (personal observations) on which the sample eluted in a single peak. When the semi-purified extract was applied onto the G-25 column with 0.02 M NaCl as eluant, the portion which contained the fast migrating bands on electrophoresis was found in Fraction 4, the last peak obtained and one which was not excluded by the gel. The approximate size of the substances corresponding to the fast migrating bands can be said to be between 1,000 to 5,000 molecular weight. The substances in Fraction 4 also eluted following ammonium sulfate, which is indicative of interaction with the gel. This interaction could be due to either nonpolar adsorption due to the structure of substances being eluted (Katz and Burtis, 1969) or to electrostatic interaction of the substances with the gel under the weak electrolyte conditions used (Janson, 1967).

b. Solubility Properties

That nephrotoxin is soluble in water and insoluble in ether is well known (Matheson and Reed, 1959). It has also been found to be soluble in 0.8 N perchloric acid (pH 1.1) and 10% trichloroacetic acid (pH 1.35). It is precipitated by acidified tungstate (pH 2.4; 5 ml H_2O + 0.5 ml 0.6 N H_2SO_4 + 0.5 ml 10% sodium tungstate), and by cold acetone.

7. Comparison of the Electrophoretic Patterns of Nephrotoxin and Streptolysin S

Introduction - Streptolysin S (SLS) was suggested as being essential in the induction of renal tubular lesions in mice by the implantation of diffusion chambers containing live group A type 12 streptococci (Tan and Kaplan, 1962). Bernheimer (1967) estimated the molecular weight of purified SLS to be between 12,000 and 20,000. According to Koyama (1963), the molar ratio of polypeptide to oligonucleotide in such a preparation was 0.3. Therefore, the polypeptide moiety would be about 2,800. In the section just preceding, the molecular weight of the active substances found in nephrotoxin preparations was estimated to be between 1,000 and 10,000. Aside from this similarity in size, nephrotoxin has not yet been found to be antigenic either (Day, 1967), much like SLS (Bernheimer, 1949).

Electrophoretic separation of a SLS preparation, a gift from Dr. A.W. Bernheimer, was performed under the same conditions as those used for the further purification of nephrotoxin, on MN cellulose thin layers in 0.01 M sodium borate - 0.003 M NaCl, pH 9.2 buffer. The pattern which was obtained was compared to a corresponding separation of a type 12 semi-purified extract.

Results

The electrophoretic patterns as viewed under ultraviolet illumination are shown in *Fig. 40*. The violet colored fluorescent bands seen in the SLS preparation probably consisted of oligonucleotide components which

were not dominant in the nephrotoxin preparation. The component bands of the type 12 semi-purified extract were yellow except for a blue colored fluorescent band adjacent to the cathode. There were no bands in the SLS pattern which corresponded to the fast migrating bands in the nephrotoxin preparation. Electrophoretically then, SLS was dissimilar to nephrotoxin.

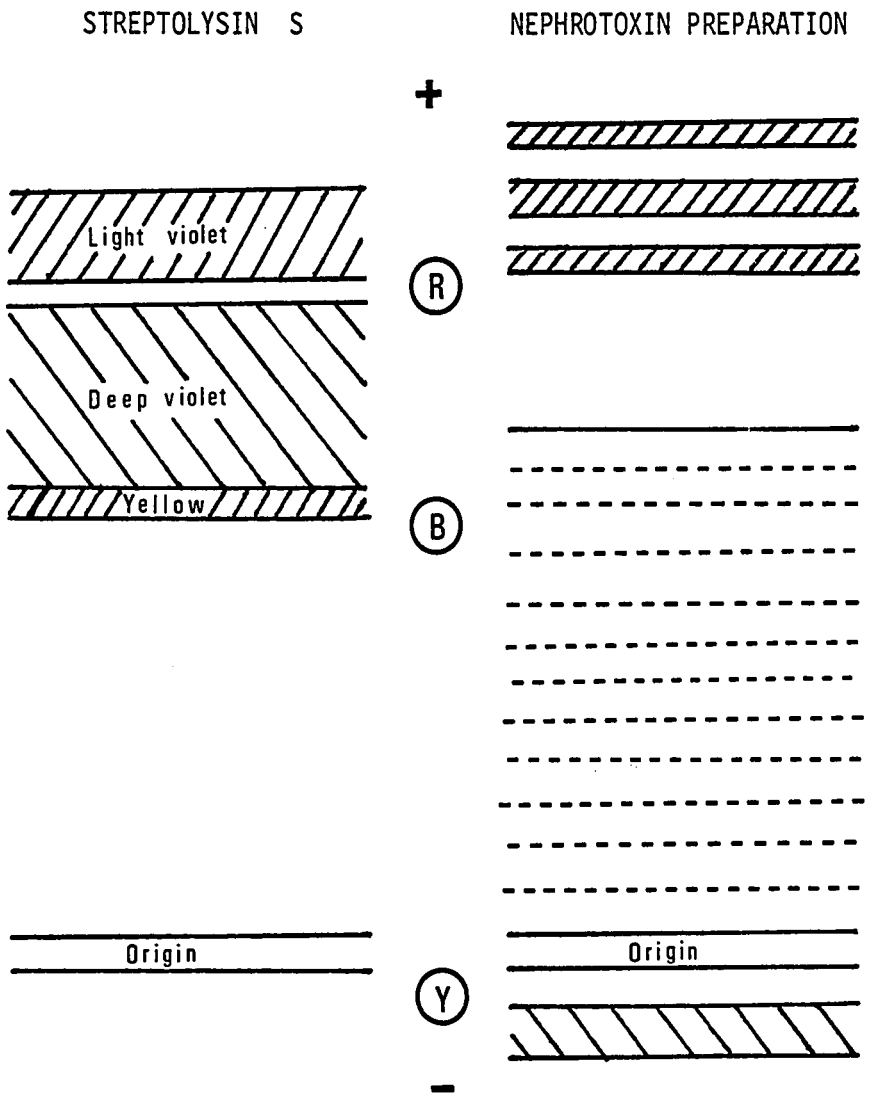

Fig. 40. Electrophoretic pattern of streptolysin S as compared to that of nephrotoxin preparation. This SLS preparation was known to consist of RNA and polypeptide components. The nephrotoxin preparation consisted of type 12 semi-purified extract.

8. Ultraviolet Spectra of the Component Fractions of the Semi-Purified Extracts and Purified Nephrotoxin

The ultraviolet spectra of the Sephadex G-25 fractions of both the type 12 and type 6 semi-purified extracts are shown in *Fig. 41*. Absorption maxima of the first 3 fractions are all 274 nm. The absorption maximum of the spectrum of Fraction 4 is 270 nm, with a shoulder at 280 nm, and another at 288 nm.

The 3 fast migrating bands eluted from electrophoretic separation of the semi-purified extracts had ultraviolet spectra similar to that of Fraction 2 above except that the range over 260 to 280 nm was flat. They also did not differ from the spectrum of the control, the eluate from a blank portion of the electrophoretic support. All showed high absorption below 280 nm. *Fig. 42* shows the ultraviolet spectrum of an eluate of the second fastest migrating band of a type 12 semi-purified extract which had been separated by electrophoresis and later desalted on Bio-Rex 70 column, a weak cation exchanger (see Section III *9*). The absorption maximum of this spectrum is 265 nm, and the peak is well defined as compared to the spectrum obtained without prior desalting.

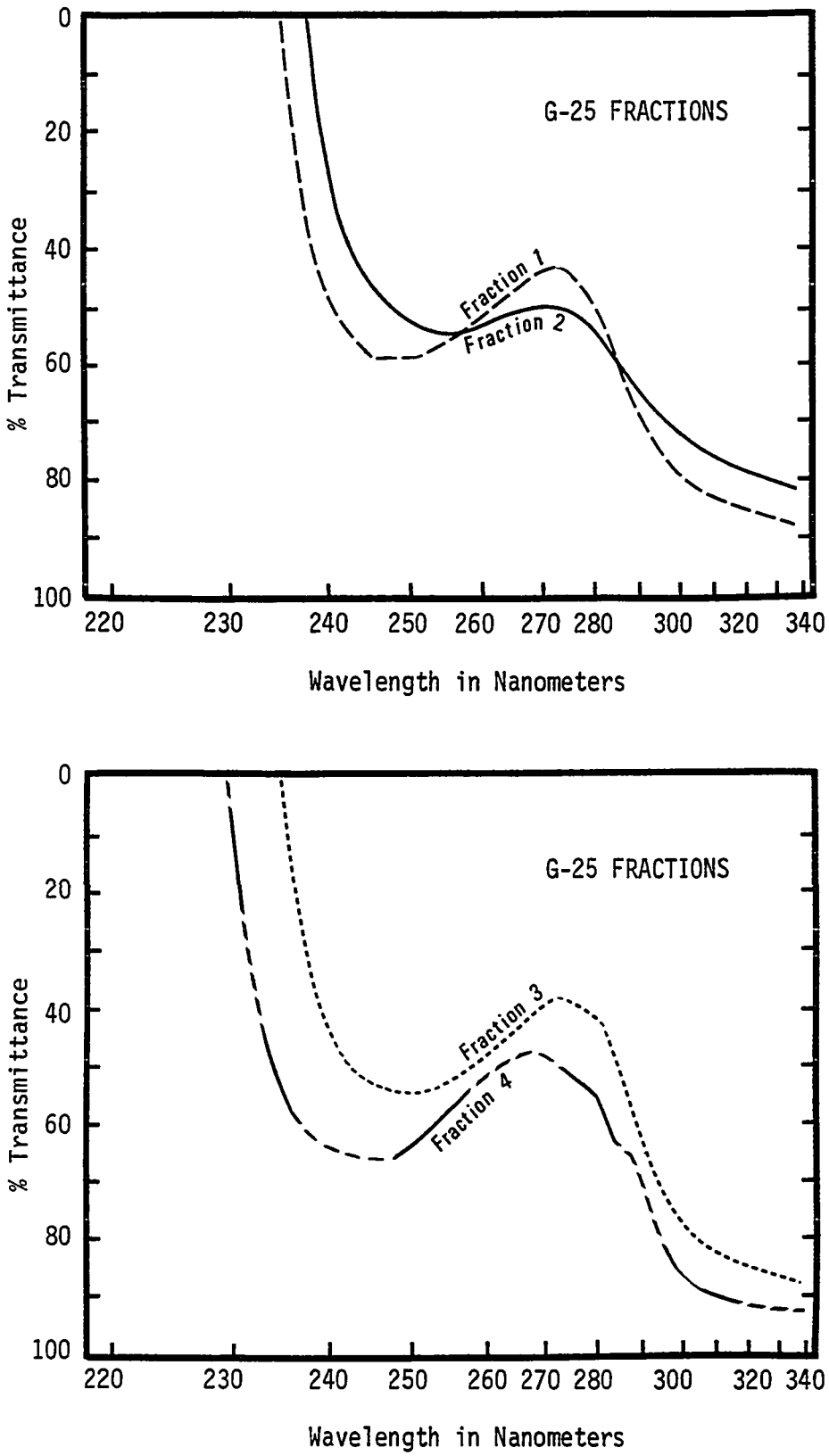

Fig. 41. Ultraviolet absorption spectra of Sephadex G-25 fractions of both type 12 and type 6 semi-purified extracts, pH 4.

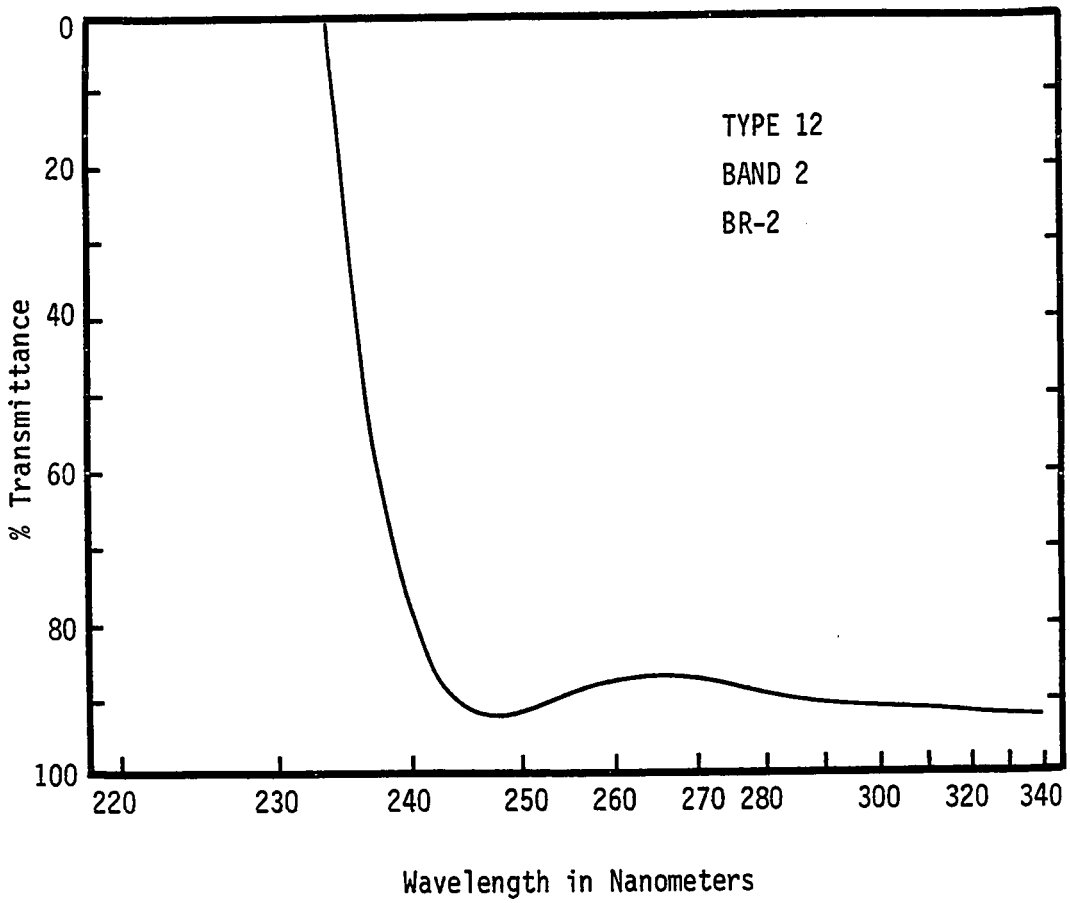

Fig. 42. Ultraviolet absorption spectrum of Band 2 of type 12 origin which had been desalted on Bio-Rex 70, pH 6.

9. Fluorescence Spectra of Further Purified Components of the Semi-Purified Extracts

The 3 fastest migrating bands eluted from the electrophoretic support on which the type 12 and type 6 semi-purified extracts were separated gave rise to identical fluorescence emission spectra, as seen in *Fig. 43*. The excitation maxima were all 330 nm, and the emission maxima 435 nm. The excitation maximum of an eluate of a blank portion of the electrophoretic support, the control, was 370 nm, with emission maximum also at that wavelength, indicating it to be due to Rayleigh and Tyndall light scatter. The spectra of an eluate of Bands 1, 2, and 3 combined of type 12 origin at 2 excitation wavelengths are seen in *Fig. 44*.

A similar fluorescence spectrum was seen with the first graction of the DEAE-Sephadex A-25 column, shown in *Fig. 45* at 3 different excitation wavelengths. The sample applied onto this column was Fraction 4 from a type 12 semi-purified extract as separated by a Sephadex G-25 column. The excitation maximum was shifted to 298 nm here although the emission maxima seen at all the different excitation wavelengths were 435 nm, which is similar to those obtained with the eluates of the fast migrating bands above. This first fraction from the DEAE A-25 column upon electrophoresis in the same system also gave rise to a fluorescent band corresponding to the position of the fast migrating bands, in addition to a blue fluorescent band near the origin. The second fraction of the DEAE-Sephadex A-25 column exhibited a fluorescence spectrum typical of proteinaceous material, with excitation and emission maxima at 275 nm and 375 nm respectively. However, the intensity of this fluorescence was very low compared to that of the first fraction.

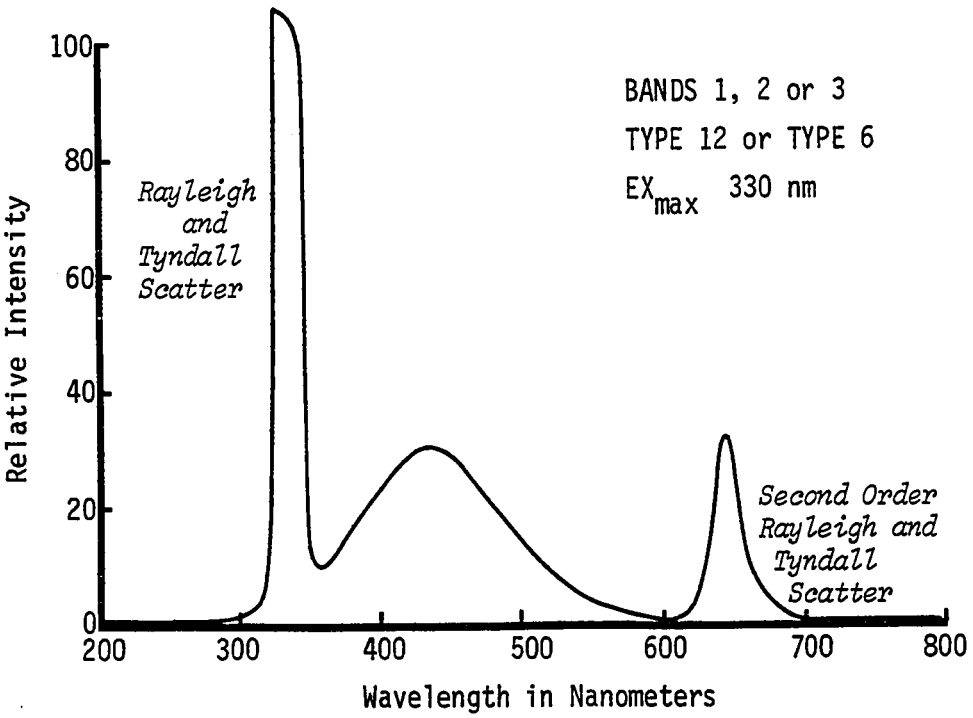

Fig. 43. The identical fluorescence spectra of eluates of Bands 1, 2 or 3 of type 12 or type 6 origin. Bands 3 exhibited relatively greater intensity. pH 4.

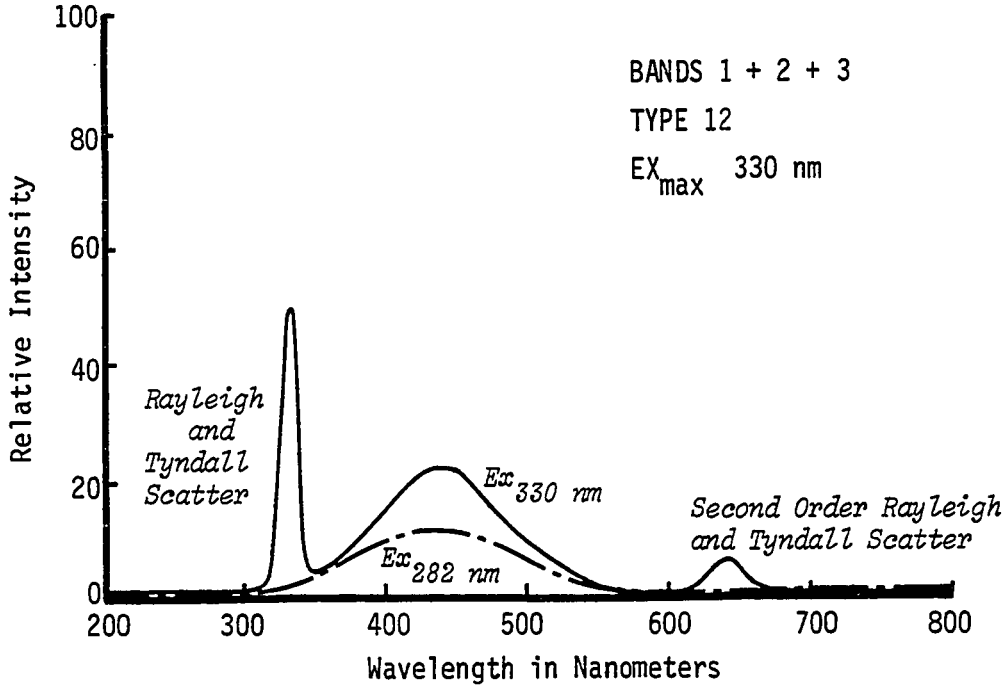

Fig. 44. Fluorescence spectra of combined eluate of Bands 1+2+3 of type 12 origin at two different excitation wavelengths, solvent 0.02 N NaOH.

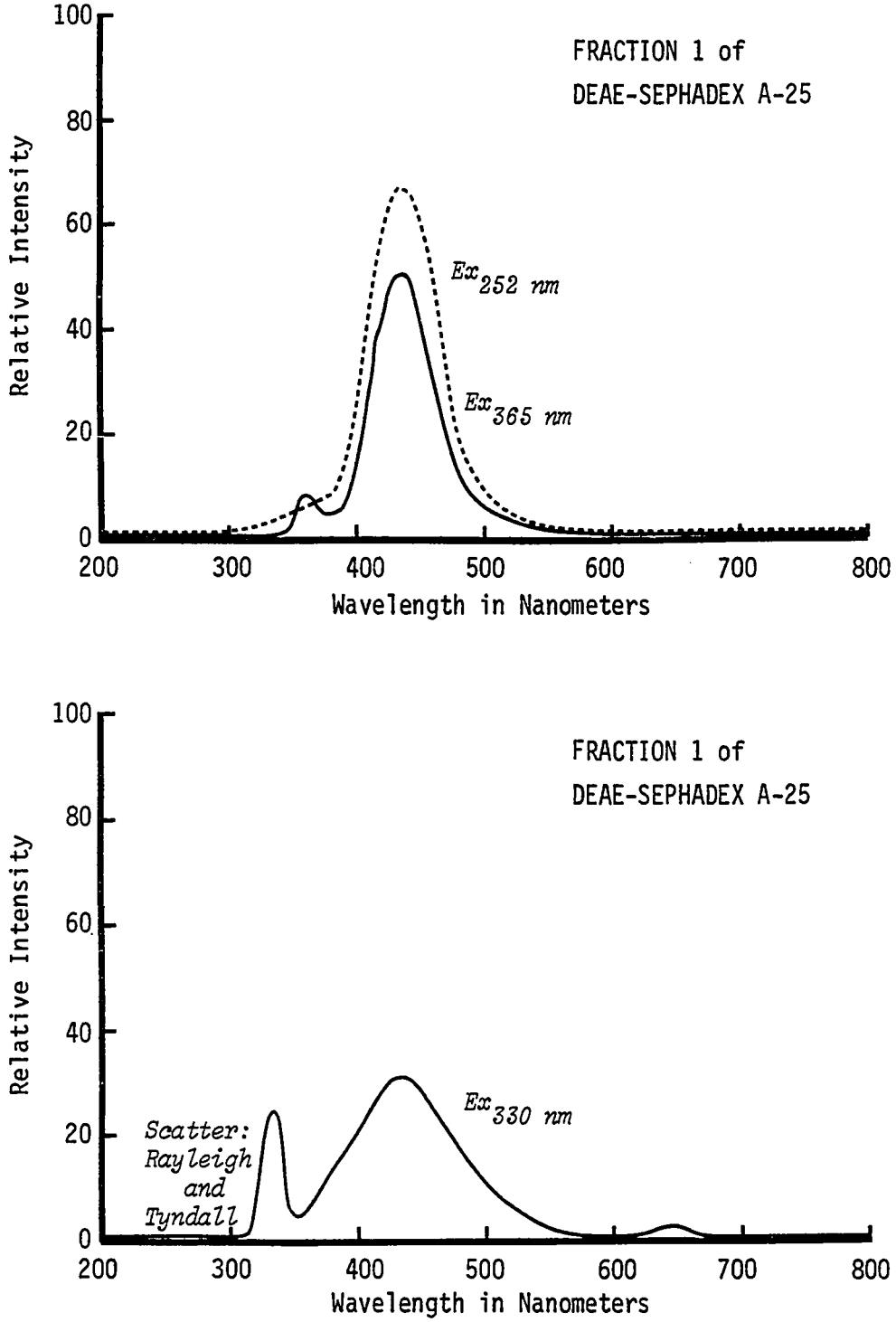

Fig. 45. Fluorescence spectra of the first fraction from DEAE-Sephadex A-25 column at 3 different excitation wavelengths. Excitation$_{max}$ = 298 nm, pH 4.2.

10. Infrared Spectra of the Active Bands

The samples which were subjected to examination on the infrared scanner included the eluates of the electrophoretically fast migrating bands of type 12 and type 6 origins, as well as a control, the eluate from a blank portion of the cellulose thin-layer electrophoresis support. All the samples were first desalted on the Bio-Rex column as described in Materials and Methods. The term BR-1 is used to designate the distilled water eluate of a sample which had been applied onto the Bio-Rex column, and BR-2 designates the material which did not elute with the distilled water, but which eluted with 1 N formic acid.

Fig. 46 shows the infrared spectrum of the distilled water eluate or BR-1 of the control material. Very similar spectra were seen with the distilled water eluates of the fast migrating bands of type 12 and type 6 origins. Therefore the BR-1 eluates did not provide any useful information.

Fig. 47a shows the spectrum of the formic acid or BR-2 eluate of the control material. *Fig. 47b* shows the spectrum of the BR-2 eluate of the 3 fast migrating bands combined of type 12 origin. In addition to the peaks which were also seen in the control, sharp peaks were seen in the 3.5 to 3.8 μm or 2630 to 2860 x 10 mm^{-1} region and the 12.5 to 13.5 μm or 740 to 800 x 10 mm^{-1} region. *Fig. 48* in turn shows the infrared spectra of BR-2 eluates of bands 2 of type 12 and type 6 origins. These are very similar to the spectrum seen in *Fig. 47b* except that the number and the exact location of the peaks differ. CH and NH stretching vibrations are known to absorb in the 3.5 to 3.8 μm or 2630 to 2860 x 10 mm^{-1} region.

As for the second region, 12.5 to 13.5 um, or 740 to 800 x 10 mm^{-1}, substituted aromatic and heterocyclic compounds such as furans, thiophenes and pyrazoles have characteristic absorptions in this region, the exact location of the peaks being dependent on the site of substitution. However, the other three major regions of broad absorption, which were also found in the control spectrum (*Fig. 47a*), do not exclude the presence of the following types of electron vibrational structures: 2.7 to 3.1 μm or 3200 to 3600 x 10 mm^{-1}, OH, NH, NH_2 and NH_3 stretching vibrations; 5.7 to 6.7 μm or 1500 to 1750 x 10 mm^{-1}, C=O, C=N, C=C and N=N stretching, asymmetrical N=O stretching, and NH_2 deformation vibrations; 7.1 to 7.8 μm or 1280 to 1400 x 10 mm^{-1}, OH bending, CH deformation, C---C (ring bonded) stretching, and N=O symmetrical stretching vibrations. The exact identity of the substances cannot be determined unless a known compound is scanned along with the unknown and provides the identical spectrum. (Randall *et al.*, 1949; Pouchert, 1970; Szymanski and Erickson, 1970.)

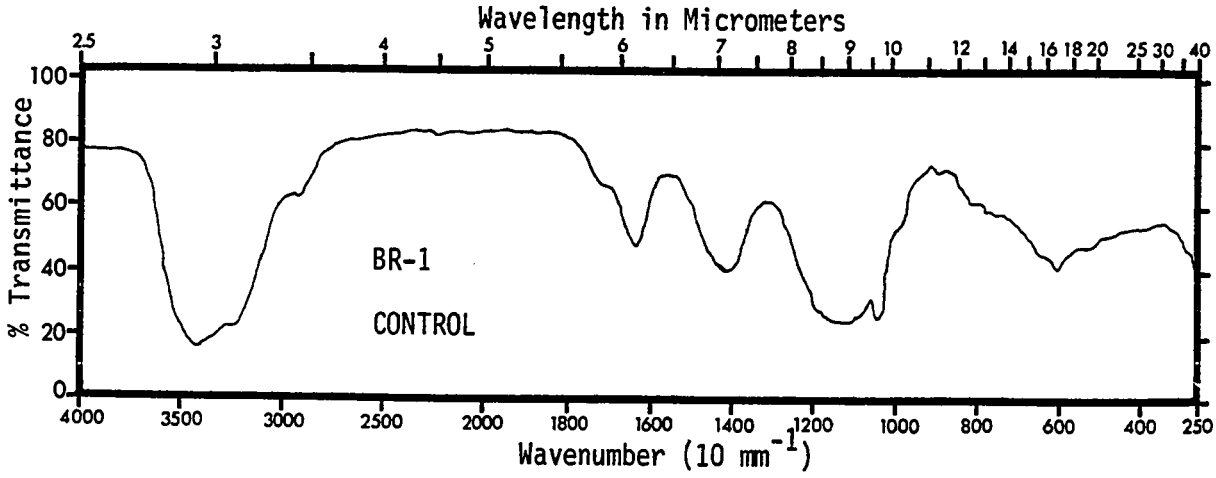

Fig. 46. Infrared spectrum of control material, the eluate from a blank portion of the cellulose electrophoretic support, which had been eluted with distilled water from a Bio-Rex column used for desalting.

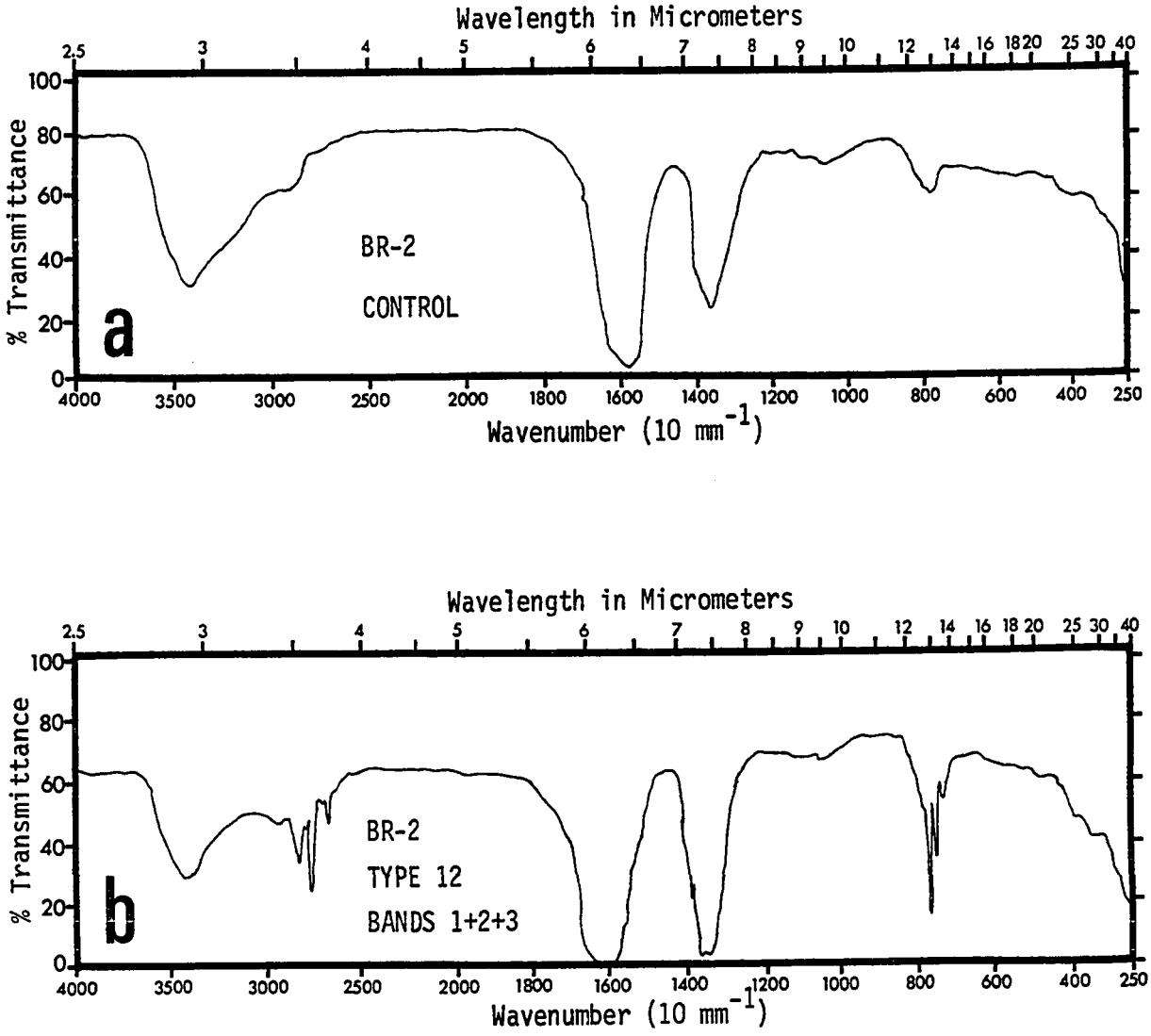

Fig. 47. a : *Infrared spectrum of control material, the eluate from a blank portion of the cellulose electrophoretic support, which had been eluted with 1 N formic acid from a Bio-Rex column used for desalting.* b : *Infrared spectrum of a purified nephrotoxin preparation, the 3 fast migrating bands combined, of type 12 origin, which had been eluted from the Bio-Rex column as in* a.

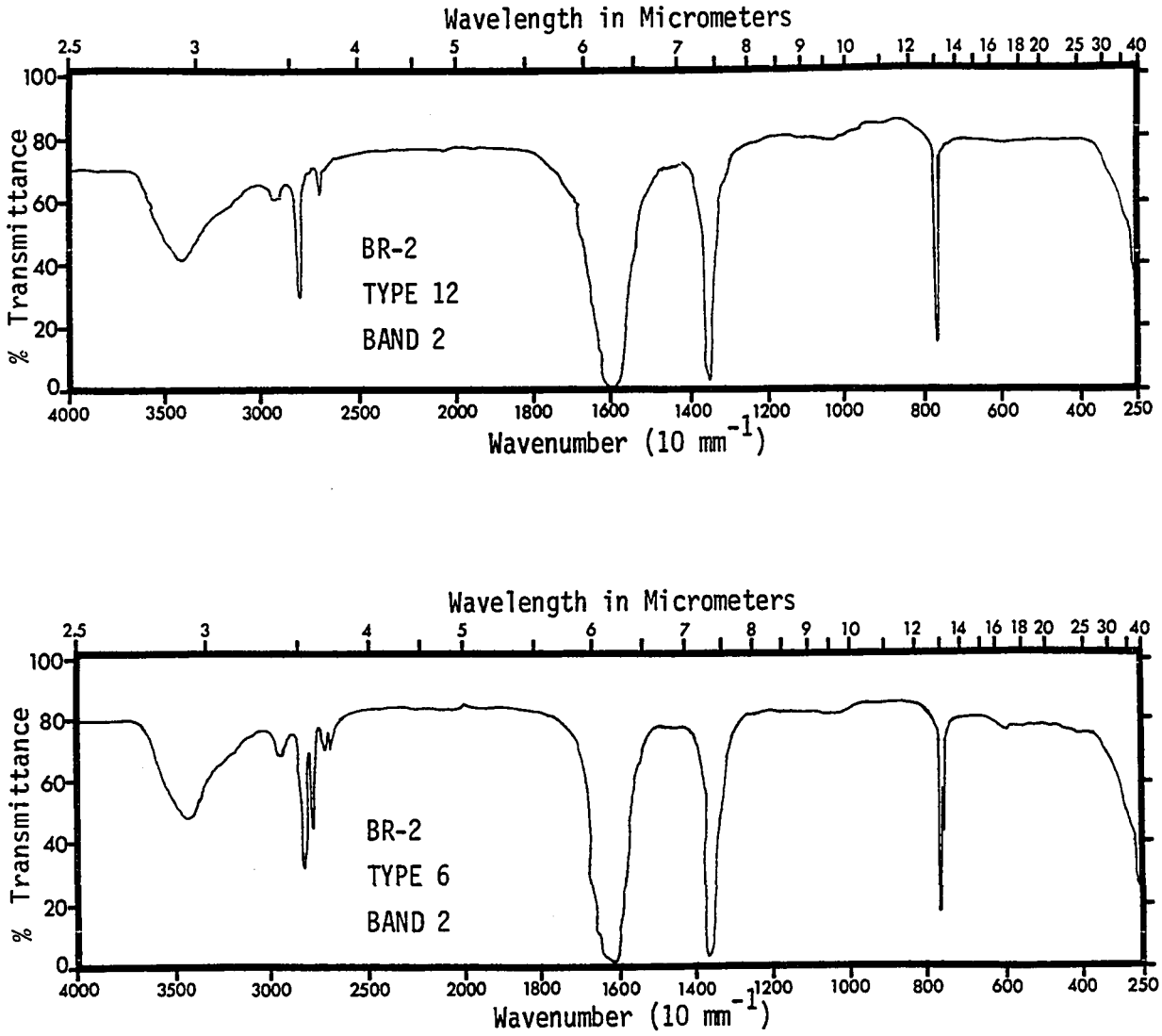

Fig. 48. Infrared spectra of the second fastest migrating bands of type 12 and type 6 origins, which had been eluted from a Bio-Rex column as stated in Fig. 47.

V. DISCUSSION

Reed and Matheson (1954*a*) first induced nephritis in rabbits by localised skin infection with a group A type 12 strain H-8 streptococcus or by the intravenous injection of its culture filtrate. The nephritogenicity was found to extend to all of the type 12 strains investigated at that time and subsequently (Holder, 1954; Franklin, 1964; Day, 1967; Fardy, 1967), but not to types 3, 6, 10, 18 or 25, nor to strains of *Streptococcus viridans*, *Staphylococcus aureus*, and *Escherichia coli*. On the average of 18 days post-infection, or 8 days post-injection, the nephritic rabbits exhibited almost uniform hypertension and urinary abnormalities. Proteinuria and hematuria, although usually not severe, were seen 17 to 20 days following the first treatment and continued until sacrifice at 35 days. Renal damage consisted chiefly of "degeneration and regeneration of the first convoluted tubules and glomerular congestion with the presence of numerous round cells in Bowman's capsule". In the experiments in which more than one series of treatment were given (Reed and Matheson, 1954*b*), either localised infection or intravenous filtrate each followed by another series of the first or the second treatment, the findings were similar. The renal lesions did not seem to be more severe than those seen after the single exposure to nephritogenic streptococci or their culture filtrate.

Later the filtrate was dialysed and the nephrotoxic activity was located in the dialysate (Matheson and Reed, 1959). Through further purification, the precipitate obtained between 65 to 75% of saturation with ammonium sulfate was found to give rise to hypertension, proteinuria

equivalent to 0.03% albumin, microscopic hematuria, and renal changes in both glomeruli and tubules. The portions of the dialysate which were precipitated between 60 to 65% and 70 to 75% of satruation with ammonium sulfate both induced only renal changes without hypertension. A combination of the above three precipitates, or the material precipitating between 60 to 75% of saturation with ammonium sulfate, was named "nephrotoxin" and used extensively in subsequent animal experiments. Eighteen mg of this precipitate could induce hypertension and proteinuria equivalent to a maxiumum of 0.15% albumin, and hematuria in rabbits. Similar results were obtained when the dosage was increased to 96 mg. The nephrotoxin was also found to be active in other animal species. Rhesus monkeys were found to respond with renal lesions which were more typical of those seen in man (Reed and Matheson, 1960), and this was attributed to the genetic proximity of the primate to man. Cats and dogs also exhibited renal changes although these were not noticeably different than those seen in rabbits (Reed and Matheson, unpublished data). Mice did not respond to the nephrotoxin (Matheson, 1957; Franklin *et al.*, 1969). For obvious reasons, blood pressure measurements were not performed on the animals other than the rabbits. Urinalyses were also not performed on animals other than rabbits.

The *in vivo* method of assay of nephrotoxin which has just been discussed has been the only assay method relied upon. Among the animals used, rabbits were used most often, chiefly for practical reasons. In the course of the present work, the rabbits model has been critically assessed for its resemblance to the human disease, since many more details

concerning the manifestation of acute glomerulonephritis in man have become well established in recent years, although there has been no concurrent clearcut establishment of its pathogenesis. It becomes obvious then that it is valuable to have an animal model in which the inciting agent of the disease, the nephritogenic streptococci, and its manifestations are similar to those in man.

In the light microscopic examination of the kidneys of the rabbits which had been given intravenous nephrotoxin preparations, the glomerular changes were usually not diffuse, as compared to what has been seen in human biopsies. Even in the monkeys, the extent of glomerular involvement of those diagnosed as having nephritis was estimated to be between 25 to 40% (Reed, 1959). The proportion of rabbits receiving nephrotoxin containing preparations which displayed renal changes in the present study has been lower than what has been seen in the nephrotoxin treated rabbits in the past. However this proportion is still greater than the approximately 12% susceptibility to disease found by Becker and Murphy (1968) by light microscopic examination of their rabbits which had been multiply infected with different strains of nephritogenic streptococci.

Hypertension has been a constant finding in rabbits which had been injected with preparations of nephrotoxin. Therefore, it was disturbing to find that some control animals also exhibited different degrees of hypertension. This finding is difficult to explain since at least in man, hypertension of renal origin is mainly of arterio-glomerular nature and not associated with tubular-interstitial changes which are seen with pyelonephritis (Kassirer, 1971), cases of which have been noted with

the present work, as they had been in the past. Another factor which may be important is the inherent differences in blood pressures which have been found among different strains of inbred rabbits (Fox *et al.*, 1969). The New Zealand white rabbits which have been used for testing nephrotoxin preparations are not inbred.

Day (1967) had found some indication that host gamma globulin deposits were seen preferentially in the kidney glomeruli of rabbits treated with type 12 nephrotoxin preparations as compared to those treated with type 6 control preparations. Such a finding would imply that an immune complex mediated process might be taking place in the kidneys of rabbits which had been treated with nephrotoxin. In the course of the present work, this finding was not verified. In contrast, some incidences of greater glomerular deposition of host gamma globulin, as evidenced by the brighter immunofluorescent staining, were seen in the control groups. It is not surprising to find the occurrence of such unexplained host gamma globulin deposition in the kidneys since immunologically mediated processes can have many origins. For instance, it has been well-documented that immune complexes localise in the kidneys of animals with chronic viral infections such as the Aleutian disease of mink, chronic lymphocytic choriomeningitis in mice, and hog cholera (Cheville *et al.*, 1970; Pan *et al.*, 1970; Oldstone and Dixon, 1971). "Spontaneous" glomerular deposits of IgG and complement have been noted in guinea pigs (Steblay and Rudofsky. 1971), so it is conceivable that this may also occur in rabbits although it has not been reported before.

The electron microscopic findings were interesting in that the three rabbits with numerous "humps" in the kidney glomeruli were among those treated with the type 12 nephrotoxic preparation. The "hump", a deposit which may or may not be electron dense, and which may appear as a protrusion of the basement membrane, has been suggested to consist of immune complexes. One difference between our electron microscopic observations and those made in man is that none of these humps were electron dense. Again, as with light microscopy, cellular proliferation and edema were not confined to the animals which were given type 12 preparation only, but were also seen in those given the type 6 control preparation or saline only. Noticeably absent were fibrin deposits which can be detected by their periodic structure in the electron micrographs and which is associated with exudative processes.

The technique for measuring blood pressure using the medial artery of the rabbit ear, and the histological diagnosis based on microscopic observations, especially by light microscopy, are ofttimes very subjective, and the more objective criteria based on urinalyses and serum profiles of components such as urea and complement are valuable adjuncts.

Urinary findings have been consistently negative in the present series of experiments except for the occasional proteinuria equivalent to 0.01% albumin. In the earliest experiments both the qualitative method for detection of proteinuria using Robert's reagent (one volume nitric acid to five volumes of saturated magnesium sulfate; Matheson, 1953 and Holder, 1954), and the quantitative method using sulfosalicylic acid (Kingsbury *et al.*, 1926) were used (Agnew, 1955; Matheson, 1957). Although the maximum

amount of proteinuria obtained was usually no more than 0.05% albumin, positives were frequent. Only occasional hematuria was seen. Since that time the incidence of proteinuria appeared to decrease. Cheuk (1960) those who followed him observed virtually no urinary abnormalities. Although it is not essential that urinary abnormalities accompany cases which are diagnosed as acute glomerulonephritis (Cohen and Levitt, 1963; Berman and Vogelsang, 1963; Kandall *et al.*, 1969), the presence of such abnormalities is certainly a good objective indicator of renal damage. Strain attenuation has been blamed for the decreased incidence of urinary abnormalities in the nephrotoxin treated animal model. When a few freshly isolated strains of type 12 streptococci became available, nephrotoxin was prepared from their broth cultures. The Castonguay and Witcher strains (Franklin, 1964) as well as the Inglis strain (Day, 1967; Fardy, 1967) did not give rise to a higher incidence of urinary abnormalities.

Concurrent with the finding of urinary abnormalities in human acute glomerulonephritis, the inefficient glomerular filtration process can also lead to increased blood urea nitrogen levels (Bradley *et al.*, 1950; Earle *et al.*, 1951). However Fardy (1967) and Elliott and Williamson (1967) could not detect such an increase, the latter working independently with nephrotoxin treated rats. Another independent index of renal damage is the increase in serum lactate dehydrogenase (LDH) which can be due to either increased leakage of the enzyme from damaged cells or to decreased clearance of endogenous LDH due to permeability changes, as were seen in mice which were infected with lactic dehydrogenase virus (Notkins, 1965). Elliott and Williamson (1967) pursued

this line of reasoning and were able to obtain a significant increase in serum LDH in rats which had been treated with nephrotoxin. However, preceding this rise was a marked decrease which could not be explained easily.

Another puzzling aspect of AGN in man is the decrease of serum complement which is now believed to be due to the probable inactivation of complement component C'3 through the release of an inhibitor or inactivator (Soothill, 1967; Pickering *et al.*, 1968; Spitzer *et al.*, 1969). Previously it had been speculated that immune complex formation may be vital for this decrease but it has been shown that a large amount of antigen reinjected into a pre-immunised rabbit caused only a very transient decrease of hemolytic activity of complement (Stavitsky *et al.*, 1949). Conceivably, continued depression of complement due to immune complex formation alone would require a massive amount of immune reactants, which is possible but not likely. Whether the release of a complement inactivator is associated with the nephritic process is not yet known. In one of the experiments performed in the course of the present work, serum complement levels of a group of nephrotoxin treated rabbits were titrated and compared to a group which were treated with the corresponding type 6 control preparation. Some depression of the hemolytic activity of complement was seen in both groups, thereby indicating that any change in serum complement levels cannot be attributed to a nephritic process solely. In addition, no significant amount of complement inactivating activity was detected in either group. Until more is known about the process which leads to complement depression in human AGN, the significance

of our findings cannot be fully appreciated.

As one can see, the bioassay system for nephrotoxin is very complex. No attempt can be made to use it for the accurate quantitation of nephrotoxin, since due to unknown factors, some rabbits will not respond to it at all. For those which respond, the severity of the renal disease has not been shown to be dose-dependent. It is hoped that by assessing the response of a large number of experimental animals, one can somewhat compensate for the individual variation.

Franklin *et al.* (1969) were not successful in attempting to use primary kidney monolayers derived from rabbits to titrate nephrotoxin. In the course of the present work, a restricted survey of some cell cultures and cell lines from different origins for susceptibility to nephrotoxin was carried out, since it is known that a given toxin can affect different cell cultures to different extents, depending on factors such as the status of the cell culture, whether primary or continuous, the organ of cell origin, and the species of origin, with the last not necessarily following the evolutionary scale (Solotorovsky and Johnson, 1970). The semi-purified nephrotoxin was added to the maintenance medium of monolayers of cell cultures to detect any cytotoxic effects. In general, the established cell lines were very resistant to 1 to 2 mg/ml concentrations of nephrotoxin and its corresponding type 6 counterpart. In particular, the African green monkey kidney (Vero) and the Rhesus monkey kidney (LLC-MK_2) cell cultures were not affected at all. The primary chick embryo cultures showed some damage at 12 to 20 hours in response to both type 12 and type 6 preparations. Obviously, whatever substance was

cytotoxic was not restricted to the nephrotoxin alone, but was also found in the type 6 preparation, be it a substance affecting the membrane chiefly, as was seen with SLS (Ginsburg and Grossowicz, 1960), or be it a substance interfering with metabolism, as was seen with diphtheria toxin (Goor and Pappenheimer, 1967; Goor *et al.*, 1967). Other examples of active bacterial products include the peptide antibiotics. Very minute amounts of the cyclic decapeptide gramicidin S can induce the lysis of erythrocytes (Hotchkiss, 1944). Actinomycin D, a polypeptide consisting of two cyclic pentapeptides connected via a three-ring heterocyclic chromophore, can interact with guanine containing DNA (Kirk, 1960). The cyclododecadepsipeptide valinomycin can uncouple oxidative phosphorylation in rat liver mitochondria (McMurray and Begg, 1959). Perhaps a more specialised system, such as one which can detect changes in selective permeability, will be found to be more useful in the study of nephrotoxin.

Another aspect of the studies which were pursued in the course of this work involved the further purification and characterisation of the components of the semi-purified nephrotoxin, the "60 to 75%" precipitate. Purification was performed by both gel filtration on Sephadex G-25 and by electrophoresis. When the further purified components were tested in rabbits, neither the G-25 fractions nor the fast migrating bands from electrophoresis which had been previously shown to be active (Friedlander, unpublished data) gave rise to a higher incidence or more severe renal lesions than the semi-purified nephrotoxin alone. Of the 4 fractions obtained by further purification of the semi-purified nephrotoxin on the G-25 column, the first and last were found to induce more extensive renal

changes than the second and third fractions as well as some hypertension. At the same time, the last fraction was shown by electrophoresis to contain the 3 fast migrating bands, two of which were shown to have activity by Friedlander, as stated before. Gel filtration was then used extensively to obtain greater amounts of purified nephrotoxin preparations, so that their structure could be studied and correlated with function.

Simultaneous electrophoretic separation of the semi-purified nephrotoxin was also performed to collect the active components. According to Friedlander, the single fast migrating band which was obtained by Cheuk and found by him to be active could be separated into 2 distinct bands when the molarity of the electrophoresis buffer was decreased. Some confusion existed in this area since Micklea (1969) felt that in fact the fast migrating bands obtained by electrophoretic separation of the nephrotoxin in the borate buffer of reduced ionic strength and decreased length of time of electrophoresis were actually lost by Cheuk in his electrophoretic separations. However, activity of these 2 fast migrating bands were detected by Friedlander, with the first band giving rise to hypertension in 3 out of 4 rabbits, but inducing no renal damage, and the second band giving rise to renal damage only. In the course of the present work, the same 2 fast migrating bands as well as the band immediately following were collected for chemical characterisation.

Several tentative conclusions can be drawn from the various data obtained by the study of the chemical nature of the nephrotoxic components. First of all, the 3 fast migrating bands from electrophoresis were very similar to each other and to their counterparts of type 6 origin, with

only minor variations, as detected by fluorescence and infrared spectroscopy and by gas-liquid chromatography. There is well known precedence to support the suggestion that these 3 bands may be heterogeneous molecular forms of the same substance. For instance, Baird-Parker and Joseph (1964) found that staphylococcal enterotoxin B could be dissociated into two major immunologically related components by starch gel electrophoresis. On their part, Poulik and Poulik (1958) found that diphtheria toxin could be separated into several toxic components by electrophoresis. Many other such examples were related by Alouf and Raynaud (1970). Although no obvious differences could be found between the nephrotoxin preparations and their type 6 counterparts in the analyses performed, it is still possible that the differences in toxicity may be due to very minor structural dissimilarities which could not be detected by the methods used. The 3 fast migrating bands which were detected by their yellow fluorescence under ultraviolet illumination following electrophoresis of the semi-purified extracts also appeared to consist of substances which had complexed with borate since re-electrophoresis of any one of them would reveal the presence of a blue-violet fluorescent band near the origin in addition to the yellow fluorescent fast migrating band. The ability of borate to form complexes with biological substances has been reported before, such as with bovine serum albumin (Cann, 1968), and can take place whenever adjacent -OH groups are available, or when -OH groups occur with sufficient proximity, as in ortho-dihydroxybenzene (Böeseken, 1949). The blue-violet fluorescent substance might then repre-

sent the dissociated form of purified nephrotoxin.

A second general statement which can be made concerning the chemical composition of the nephrotoxin is that the active components of the semi-purified extract of nephrotoxin may not be a linear polypeptide consisting of the usual protein amino acid residues. From the quantitative analytical data, the semi-purified nephrotoxin appeared to consist chiefly of polypeptide material. Less than 0.01% hexosamine was found. The content of reducing sugars had been found to be less that trace amounts (Fardy, 1967). The phosphorus content was from 0.01 to 0.05% of the polypeptide material. Amino acid mapping of the acid hydrolysates of the first 3 Sephadex G-25 fractions indicated that the usual amino acids were found. The composition of Fraction 1 almost agreed entirely with Fardy's automatic amino acid analyser data (Fardy *et al.*, 1969) of the composition of the heterogeneous Sephadex G-15 column excluded fraction which had been shown to have nephrotoxicity. Similar composition was found in the corresponding hydrolysates of type 6 and Todd-Hewitt broth origins. However, the last G-25 fraction, or Fraction 4, which electrophoretically contained the 3 fast migrating bands among others, on amino acid mapping gave rise to 5 to 6 very faint ninhydrin positive spots, only 3 of which could be tentatively identified. Repeated attempts at mapping of the hydrolysates of the 3 fast migrating bands obtained by electrophoresis gave rise to very faint ninhydrin positive spots which could not be identified. In addition, the fluorescence spectra of the 3 fast migrating bands indicate the presence of an aromatic or heterocyclic structure which does not resemble that of any of the well known amino

acids. Proteins usually have excitation maxima of between 270 to 300 nm and fluorescence emission maxima at 313 nm and 350 nm due to primarily the tyrosine and tryptophan residues (Konev, 1967). Our substances were excited maximally at 330 nm and emitted maximally at 435 nm. Gas-liquid chromatography of the acid hydrolysates of the 3 fast migrating bands revealed a major peak in addition to various peaks of shorter retention times, none of which appeared to correspond to any of the usual amino acids. Since acid hydrolysis destroys the indole nucleus of the tryptophan ring, additional tests for tryptophan residues which might have been present in the fast migrating bands were performed. Neither oxidation by N-bromosuccinimide (Spande and Witkop, 1967) nor the colorimetric reaction with the glyoxyllic acid reagent (Hamilton, 1960) revealed any tryptophan. At the same time, the last G-25 fraction and one of its components obtained by further purification on DEAE-Sephadex A-25 column were hydrolysed and examined by paper chromatography. Again very faint ninhydrin positive spots were seen. A prominent spot was found to absorb ultraviolet light strongly, being dark when viewed under an ultraviolet source. This spot stained a violet color with Wood's reagent (Wood, 1955), typifying the pyrimidine type of ring rather than the purine type. The maximum absorbance of the last G-25 fraction in the ultraviolet was at 271 nm, and that of the second electrophoretic band of type 12 origin was at 265 nm. The infrared data suggest the presence of substituted aromatic or heterocyclic compounds. One may propose that a polypeptide moiety occurs in close association with a nucleotide derivative in purified nephrotoxin, thus paralleling the studies on streptolysin S. However, the very low

phosphate content and the lack of reducing sugar residues cannot account for the phosphate and ribose or deoxyribose residues one would expect to find in a nucleotide. It is also possible that the aromatic or heterocyclic residues are similar to ones which have been found in certain characterised peptides of bacterial origin (Kopple, 1966). Further work is necessary in order to establish the exact identity of the residues so that the functional significance of the purified nephrotoxic material can be ascribed. Furthermore, providing sufficient quantities of purified nephrotoxin can be obtained, it would be desirable to examine the effects of purified nephrotoxic components on larger groups of animals, so that their activity can be firmly established.

VI. SUMMARY AND CLAIM TO ORIGINALITY

1. The total hemolytic activity of serum complement in rabbits was not affected by group A type 12 streptococcal nephrotoxin as compared to treatment by the corresponding group A type 6 control preparation. No inhibitors of complement activity were found.
2. Renal changes were seen in the rabbits which were injected with Fractions 1 and 4 of the Sephadex G-25 column separated semi-purified nephrotoxin.
3. The deposition of host gamma globulin in kidneys of rabbits was not restricted to those receiving nephrotoxin containing preparations.
4. Electron microscopy of kidney glomeruli revealed a greater incidence of proliferative glomerulonephritis among animals which received nephrotoxin containing preparations as compared to the type 6 and saline controls.
5. Chick embryo, WI-38, H Ep-2 and L-929 cell cultures grown in monolayers were damaged equally by type 12 nephrotoxic and type 6 control preparations as compared to the uninoculated controls, whereas Vero and LLC-MK_2 cell cultures were not affected.
6. No readily available N-terminal amino acid was found in purified nephrotoxin by use of the dansyl chloride end group analytical procedure.
7. Although the major portion of the semi-purified extract of nephrotoxin consisted of polypeptide material, the active components did not contain amino acids recognisable on two-dimensional thin-layer chromatography.

8. Gas-liquid chromatography of the purified nephrotoxin revealed the presence of a prominent residue which was found in the type 6 preparation as well. However, differences were noted between the preparations in the residues which eluted with shorter retention times.
9. The semi-purified extract of nephrotoxin and its component Sephadex G-25 fractions contained less than 0.01% hexosamines and 0.01 to 0.05% phosphorus.
10. Examination of paper chromatograms of hydrolysates of active nephro-components revealed a prominent residue which absorbed ultraviolet light and reacted to staining procedures in a similar fashion as aromatic or heterocyclic compounds.
11. The active components of nephrotoxin were not excluded by Sephadex G-25 gel which has a fractionation range of 1,000 to 5,000.
12. The nephrotoxin is soluble in 0.8 N perchloric acid and 10% trichloro-acetic acid but is precipitated by acetone.
13. The physical behavior of nephrotoxin on electrophoresis did not resemble that of streptolysin S.
14. Purified nephrotoxin absorbs maximally in the ultraviolet at 265 nm.
15. Purified nephrotoxin fluoresces maximally at 435 nm and is excited maximally by ultraviolet illumination of 330 nm.
16. Infrared studies of purified nephrotoxin revealed that it possesses residues with the group frequencies of substituted aromatic or heterocyclic compounds.

APPENDIX

A. Buffers and Electrolytes for Electrophoresis

1. Borate buffer, 0.01 M, pH 9.2

0.01 M sodium tetraborate ($Na_2B_4O_7.10\ H_2O$)	3.81 g per liter
0.003 M NaCl	0.18 g per liter

2. Phosphate buffered saline, 0.01 M, pH 7.2

   a. 0.1 M monobasic phosphate: 6.8 g KH_2PO_4 or 6.9 g NaH_2PO_4 per 500 ml.

   b. 0.1 M dibasic phosphate: 7.1 g Na_2HPO_4 per 500 ml.

   Mix 50 ml of *a* with 91.5 ml of *b*. Adjust to pH 7.2 and make up to 1415 ml with distilled water. Add NaCl to a final concentration of 0.8% (by adding 11.32 g).

3. Sorensen's phosphate buffer, 0.1 M, pH 7.4

   a. 0.2 M monobasic phosphate: 31.2 g $NaH_2PO_4.2\ H_2O$ per liter

   b. 0.2 M dibasic phosphate: 28.39 g Na_2HPO_4 or 71.7 g $Na_2HPO_4.12\ H_2O$ per liter

   Add 19 ml of *a* to 81 ml of *b* and dilute to 200 ml.

4. Carbonate-bicarbonate buffer, 0.5 M, pH 10

0.5 M Na_2CO_3	1 volume
0.5 M $NaHCO_3$	3 volumes

5. Barbital buffer, 0.05 M, pH 8.6 (used for immunoelectrophoresis)

Sodium barbital	8.76 g per liter
Barbital	1.38 g per liter
Calcium lactate	0.38 g per liter

6. Barbital buffer, Γ 0.05, pH 8.2 (used for cellulose acetate electrophoresis)

Sodium barbital	10.0 g per liter
1 N HCl	15.8 ml per liter

B. Bacteriological Media

1. Homemade McGill Todd-Hewitt broth

Add 1 liter of distilled water to 450 g of fresh minced beef heart trimmed of fat. Stir and skim off the fat particles which rise to the surface. Place the misture at 4 to 8 C overnight. Next day heat to 85 C and maintain this temperature for 30 minutes. Filter through coarse filter paper. To each liter of filtrate add 20 g of neopeptone (Difco) and adjust to pH 7.0 with 5 N NaOH.

To each liter add the following:

NaCl	2 g
$NaHCO_3$	2 g
$Na_2HPO_4.12\ H_2O$	1 g
Glucose	2 g

Boil for 15 min and filter. Dispense and sterilise in the autoclave at 121 C for 15 to 25 min. The final pH should be 8.0±0.1.

2. Difco Todd-Hewitt broth

To rehydrate the medium, dissolve 30 g of the dehydrated product in 1 liter distilled water. Ingredients per liter are:

Beef heart (infusion from)	500 g
Neopeptone (Difco)	20 g
Bacto-dextrose	2 g
NaCl	2 g
Na_2HPO_4	0.4 g
Na_2CO_3	2.5 g

Sterilise in the autoclave at 121 C for 15 to 25 min. The final pH should be 8.0±0.1.

3. Sheep blood agar plates

a. Heart infusion agar: Add 1 liter of distilled water to 450 g of minced beef heart trimmed of fat. Heat at 75 to 80 C for 1½ hours. Siphon off the meat infusion. To 20% of the infusion add 1.1% agar (BDH) and 1% proteose peptone (Difco). Heat at 121 C for 2 hours. To the molten agar add the remainder of the infusion and 1% stock salt solution (25% NaCl, 2% KCl, 1% $CaCl_2$). Adjust the pH to 8.4. Precipitate the phosphates by heating the contents for 30 min at 100 C and filter. Adjust the final pH to 7.2 with 1 N HCl. Sterilise in the autoclave at 121 C for 20 min.

b. Addition of sheep blood (5%): To 500 ml of the molten heart infusion agar at 50 to 53 C add 25 ml sterile defribrinated sheep blood. Dispense 20 ml for each plate. Incubate the plates at 37 C for 24 hours to check for sterility.

C. Tissue Culture Media and Reagents

Sterilise the media and reagents below by membrane filtration (0.22 μm, Millipore).

1. Maintenance medium

Maintenance medium No. 3 (Gibco; Taylor-Robinson *et al.*, 1963) consists of the following ingredients:

Minimal essential medium (Eagle) with Earle's salts, 49%.

Medium 199, 49%.

Gamma globulin-free calf serum (heat inactivated), 2%.

L-glutamine, 2 mM.

To 500 ml of the above medium add 5 ml of the following sterile antibiotic mixture solution: 90 ml distilled water containing 1,000,000 units penicillin and 500 mg streptomycin + 10 ml distilled water containing 5 mg fungizone.

2. Growth medium

Add calf serum to the above medium to bring the final serum concentration up to 10%.

3. Trypsin, 0.25% in 0.01 M phosphate buffered saline

Dissolve the following ingredients in 1500 ml distilled water:

0.68 g KH_2PO_4

1.42 g Na_2HPO_4

3.75 g trypsin

15 ml 0.2% phenol red

D. Histological Methods

1. Sternheimer-Malbin stain for urine sediment (Sternheimer and Malbin, 1951)

Solution *a*:	Crystal violet	3 g
	Ethanol	20 ml
	Ammonium oxalate	0.8 g
	Distilled water to	80 ml
Solution *b*:	Safranine O	0.25 g
	Ethanol	10 ml
	Distilled water to	100 ml

Solutions *a* and *b* are stable indefinitely.

Make a working solution by adding 3 parts of *a* to 97 parts of *b*. To stain the urine sediment, add an equal amount of the working solution.

2. Light microscopy

a. Dehydration and paraffin infiltration of tissue

Solution	*Duration*
(1) 10% formalin in saline	1 hour
(2) 80% methylated ethanol	1 hour
(3) 90% methylated ethanol	1 hour
(4) 100% ethanol	2 hours
(5) 100% ethanol	2 hours

(6) 100% ethanol	3 hours
(7) Xylol	1 hour
(8) Xylol	2 hours
(9) 10% cedarwood oil in chloroform	2 hours
(10) First paraffin, 56 C	2 hours
(11) Second paraffin, 56 C	2 hours
(12) Third paraffin, 56 C	2 hours

b. Hematoxylin-eosin stain

Prepare the Harris hematoxylin reagent as follows:

(1) Dissolve 5 g hematoxylin (Chroma-Gesellschaft hematoxylin purris, Otto Watzka & Co.) in 50 ml ethanol.

(2) Dissolve 100 g alum, $Al_2(SO_4)_3.K_2SO_4.24\ H_2O$ in 1000 ml distilled water over heat.

(3) Add the alum solution to the hematoxylin solution. Boil for 5 min and add 2.5 g HgO.

(4) Boil the whole mixture for 5 min, cool to room temperature and filter before use.

(5) Add 3 ml glacial acetic acid to 95 ml of Harris hematoxylin at the time of use to increase its nuclear staining properties.

Bring the sections on slides "to water" as follows:

(1) 2.5 min in xylol (2X).

(2) 0.5 min in ethanol (2X).

(3) 0.5 min in 80% ethanol.

(4) 0.5 min in 70% ethanol.

(5) 0.5 min in 50% ethanol.

(6) 1.0 min in tap water.

The staining procedure is as follows:

(1) Stain 5 min in Harris hematoxylin.

(2) Remove excess stain in running tap water.

(3) Immerse briefly in acid water (1% HCl) until salmon pink.

(4) Immerse briefly in alkaline water (1% $NaHCO_3$) until blue.

(5) Counterstain 1 min in aqueous eosin Y (0.5%).

(6) Rinse briefly in water.

Dehydration and mounting:

(1) 0.5 min in 50% ethanol.

(2) 0.5 min in absolute ethanol (2X).

(3) 0.5 min in absolute ethanol-xylol (1:1).

(4) 0.5 min in xylol (2X).

(5) Mount in Permount (Fisher).

c. Periodic acid-Schiff stain

Prepare Schiff's reagent:

(1) Dissolve 1 g basic fuchsin (BDH standard) in 200 ml hot distilled water and bring the solution to boil.

(2) Cool to 50 C and add 20 ml 1 N HCl.

(3) Cool further and add 1 g potassium metabisulfite ($K_2S_2O_5$).

(4) Store 24 hours at 4 to 8 C.

(5) Add 1 g decolorising charcoal, shake the mixture and filter after 10 min. The reagent should be colorless.

Prepare sulfite rinses:

To 6 ml 10% sodium or potassium metabisulfite add 5 ml 1 N HCl and 89 ml distilled water. Change solutions daily.

Harris hematoxylin:

Prepare the reagent in the same way as before for the hematoxylin-eosin stain.

The staining procedure is as follows:

(1) Bring the sections "to water" as before.

(2) Oxidise 10 min in periodic acid solution (1% in distilled water).

(3) Rinse 5 min in tap water.

(4) Rinse thoroughly in distilled water.

(5) Place 10 min in Schiff's reagent.

(6) Rinse in 3 changes of sulfite solutions, 2 min each.

(7) Rinse briefly in tap water.

(8) Stain 2 min in Harris hematoxylin.

(9) Immerse briefly in acid water (1% HCl) until salmon pink.

(10) Immerse briefly in alkaline water (1% $NaHCO_3$) until blue.

(11) Rinse briefly in tap water.

Dehydration and mounting:

The procedure is identical to that used before.

d. Alcian blue-periodic acid-Schiff stain

Prepare the alcian blue stain by making a solution of 0.1% alcian blue 8GX (Allied Chemical) in 3% acetic acid.

Schiff's reagent and the sulfite rinses are the same as those used in the periodic acid-Schiff stain.

The staining procedure is as follows:

(1) Bring the sections "to water" as before.

(2) Stain 30 min in alcian blue.

(3) Decolorise 5 min in 3% acetic acid.

(4) Rinse 5 min in water.

(5) Oxidise 10 min in 1% periodic acid.

(6) Rinse 5 min in tap water.

(7) Rinse thoroughly in distilled water.

(8) Place 10 min in Schiff's reagent.

(9) Rinse in 3 changes of sulfite solutions, 2 minutes each.

(10) Rinse briefly in tap water.

Dehydration and mounting:

The procedure is identical to that described previously.

3. Fluorescence microscopy

Glycine buffered glycerol for mounting coverslips (Roitt and Doniach, 1966)

Add the following ingredients and make up to 1 liter with distilled water:

Glycine	14.0 g
NaOH	0.7 g
NaCl	17.0 g
Sodium azide	1.0 g

4. Electron microscopy

a. Osmic acid fixative for post-fixation

(1) 0.28 M veronal acetate buffer

Sodium barbital	2.88 g
Sodium acetate (anhydrous)	1.15 g
Add distilled water up to	100 ml

(2) Shortly before use add approximately 10 ml of 0.1 N HCl to 10 ml of (1) to adjust the pH to 7.5. Bring the total volume up to 45 ml with distilled water.

(3) To solution (2) add 1 g osmium tetroxide (Polysciences, Inc.) and 2.25 g sucrose. Adjust the final volume to 50 ml with distilled water.

b. Dehydration of fixed tissue

(1) 20% ethanol	20 min
(2) 40% ethanol	20 min
(3) 60% ethanol	20 min
(4) 95% ethanol	20 min
(5) 100% ethanol	30 min
(6) 100% ethanol	30 min
(7) Propylene oxide	10 min
(8) Propylene oxide	10 min

c. Infiltration and embedding

The soft modification of the Spurr low-viscosity embedding medium (Polysciences, Inc.) consists of:

Vinylcyclohexene dioxide	10 g
Diglycidyl ether of polypropyleneglycol	7 g
Nonenyl succinic anhydride	26 g
Dimethylaminoethanol	0.4 g

Procedure:

(1) Spurr resin:propylene oxide (1:1)	40 min
(2) Add equal volume of Spurr resin to (1)	30 min
(3) Replace with fresh Spurr resin	1 hour
(4) Replace again with fresh Spurr resin	overnight
(5) Cure in dried Beem capsules (Ladd Research Industries, Inc.) at 70 C	8 hours

d. Lead citrate stain (Reynolds, 1963)

In a 50 ml volumetric flask place 1.33 g lead nitrate, 1.76 g sodium citrate and 30 ml distilled water. Shake vigorously for 1 min. Allow to stand 30 min to allow full formation of lead citrate. Add 1 ml of 1 N NaOH (carbonate-free). Dilute to 50 ml with distilled. The final pH should be 12 and the solution should be clear. Store in glass or polyethylene bottles for up to 6 months.

E. Staining Reagents for Thin-Layer and Paper Chromatograms

1. Moffat-Lytle polychromatic stain for amino acids (Moffat and Lytle, 1959)

a. Mix the following together:

50 ml 0.2% ninhydrin in ethanol

10 ml glacial acetic acid

2 ml 2,4,6-trimethylpyridine

b. Make a 1% $Cu(NO_3)_2.3\ H_2O$ solution in ethanol.

To make the working reagent solution, mix 50 parts of *a* to 3 parts of *b* just before use. Spray or dip the dried chromatogram through the reagent. Observe the amino acids which appear at room temperature and those which appear after heating at 110 C for 5 min.

2. Aniline hydrogen phthalate for reducing sugars (Partridge, 1949)

Dissolve 1 g aniline hydrogen phthalate in 95 ml 1-butanol + 5 ml distilled water. Heat the dipped paper chromatogram at 110 C for 5 min. Reducing sugars will appear pink to brown.

3. Elson-Morgan reagent for hexosamines (modified from Elson and Morgan, 1933)

a. Dip the dried chromatogram through a mixture of 1 volume alcoholic KOH solution (5 g KOH in 20 ml H_2O + 80 ml ethanol) and 10 volumes 1% acetylacetone in ethanol. Heat to 110 C for 5 min.

b. Dip the chromatogram from through a mixture of 1 volume 10% *p*-dimethylaminobenzaldehyde in concentrated HCl and 9 volumes

ethanol. Dry with hair drier for 1 to 2 min.

c. Reheat to 80 C for a few minutes. Hexosamines appear red and reducing sugars blue-green.

4. Quinine sulfate for phosphate esters (Rorem, 1959)

Immerse the dried chromatogram in a solution of 0.5% quinine sulfate.2 H_2O in ethanol. Dry at room temperature for a few minutes and view under ultraviolet light. Phosphate esters appear as light areas against a grey-blue fluorescent background. Purine and pyrimidines as well as inorganic salts are also detected.

5. Silver nitrate-bromophenol blue for purines and pyrimidines (Wood, 1955)

Immerse the dried chromatogram in a mixture of 1 volume bromophenol blue (0.4% in acetone) and 1 volume $AgNO_3$ (2% in water). Hang in air for 10 min. Wash off excess stain in distilled water for 5 min. Rinse in fresh distilled water. Dry in air. Purines appear blue, pyrimidines, royal blue, and anions violet to grey.

6. Sodium fluoresceinate for aromatic and heterocyclic compounds (Waldi, 1965)

Dissolve 25 mg sodium fluorescein in 100 ml 50% methanol. Immerse the dried chromatogram, dry in air, and view under ultraviolet light.

F. Quantitative Chemical Assay Procedures

1. Biuret method (Gornall *et al.*, 1949)

Biuret reagent:

Cupric sulfate.5 H_2O	1.5 g
Sodium potassium tartrate ($NaKC_4H_4O_6.4\ H_2O$)	6.0 g
Potassium iodide	2.0 g
10% NaOH	300 ml

Make up to 2 liters with distilled water.

Procedure:

Prepare standard solutions of bovine serum albumin ranging from 1 to 10 mg per ml.

To 1 ml sample or standard solution add 3 ml biuret reagent. Mix well and allow to stand at room temperature for 30 min. Read at 540 nm.

2. Lowry method (Wu, 1922; Lowry *et al.*, 1951)

Reagents:

a. 2% Na_2CO_3 in 0.1 N NaOH.

b. 0.5% $CuSO_4.5\ H_2O$ in 1% sodium citrate.

c. 2 ml *b* + 100 ml *a*.

d. Folin reagent: Dilute Fisher Folin reagent 1:1 with water.

Procedure:

Prepare standard solutions of bovine serum albumin ranging from 0.05 to 0.5 mg per ml.

Add 2 ml of reagent *c* to 0.4 ml sample or standard solution. Allow to stand at room temperature for 10 min.

Add 0.2 ml Folin reagent and allow to stand at room temperature for 30 min.

Read at 750 nm.

3. Hexosamine determination (Elson and Morgan, 1933; Davidson, 1966)

Reagents (to be made fresh daily):

a. Dissolve 2,4-pentanedione (acetylacetone) in 98 ml of 1 N Na_2CO_3.

b. Dissolve 677.5 mg of *p*-dimethylaminobenzaldehyde in 25 ml ethanol:concentrated HCl, 1:1.

Procedure:

Prepare standard solutions of glucosamine HCl in concentrations of 0.05 to 0.1 μM per ml.

To 1 ml sample or standard solution add 1 ml reagent *a*. Close tubes with Teflon-lined caps. Place in a 90 C bath for 45 min. Cool in running water.

Add 4 ml 95% ethanol. Mix. Add 1 ml reagent *b*. Allow to stand at room temperature for 1 hour.

Read at 540 nm.

4. Nitrogen determination (Jacobs, 1959)

Digestion:

Digest samples and standards ranging from 50 to 500 μg

of bovine serum albumin (0.5 to 5.0 μM of NH_3) in 0.5 ml H_2SO_4 and 3 to 5 mg of a mixture of $CuSO_4$:K_2SO_4:HgO:Se, 5:15:5:1 in a Kjeldahl flask for 45 min. If the digests are not cleared, cool flasks and add 0.2 ml H_2O_2 to each. Continue heating for 2 hours.

Reagents:

Sodium citrate buffer, 0.4 M, pH 5.0:

Sodium citrate, 0.4 M	58.8 g in 500 ml
Citric acid, 0.4 M	42.0 g in 500 ml

Add enough of the second solution to the first to bring the pH to 5.0.

Ninhydrin reagent:

2 g indanetrione hydrate (ninhydrin)

50 ml methyl "Cellosolve" (ethylene glycol monomethyl ether)

25 ml 4.0 M sodium acetate buffer, pH 5.5 (544 g sodium acetate.3 H_2O + 100 ml acetic acid per liter)

25 ml distilled water

0.08 g stannous chloride ($SnCl_2$.2 H_2O)

Procedure:

Dissolve the digested samples in 25 ml of the citrate buffer. Dilute 0.5 ml of this to 2 ml with buffer. Add 2 ml of the ninhydrin reagent. Heat in a waterbath at 100 C for 30 min.

Cool and dilute with 6 ml ethanol:water, 1:1 (v/v). Read at 570 nm.

5. Phosphate determination (Meun and Smith, 1968)

Reagents:

Ammonium molybdate.4 H_2O, 0.28% in water.

7.2 N H_2SO_4: Dilute 200 ml concentrated H_2SO_4 to 1 liter.

Aminonaphtholsulfonic acid: Dissolve 50 mg sodium sulfite, 3 g sodium bisulfite, and 50 mg aminonaphtholsulfonic acid and make up to 50 ml with water. This will store 1 to 2 weeks only.

Procedure:

Prepare standard phosphate solution: To make a solution of 80 µg P per ml, dissolve 0.351 g KH_2PO_4 in 1 liter water. Add 10 ml of 10 N H_2SO_4 before making up to volume. This stock solution can keep in the cold indefinitely.

Place 1 ml samples and standard solutions (1 to 6 µg per ml) in Kjeldahl flasks. Add 1.5 ml 7.2 N H_2SO_4 and 2 boiling chips. Digest until constant boiling sulfuric acid is obtained, when white fumes are seen. Reduce heat and digest 30 min longer. If digests have not cleared, add 1 to 2 drops 30% H_2O_2 and digest further until clear.

Cool flasks and add 4.5 ml of the molybdate reagent, followed by 0.5 ml of the aminonaphtholsulfonic acid reagent. Heat in boiling waterbath for 10 min. Cool in running water for 3 min. Read at 830 nm.

BIBLIOGRAPHY

AGNEW, R. 1955. Observations on the pathogenesis of experimental nephritis due to type 12 streptococci. M.Sc. Thesis, Dalhousie University, Halifax.

ALOUF, J.E. and Raynaud, M. 1970. Isolation and purification of bacterial toxic proteins. *In* Microbial Toxins, Vol. I. Bacterial Protein Toxins, pp. 119-182. Editors: S.J. Ajl, S. Kadis and T.C. Montie. Academic Press, New York and London.

ANDRES, G.A., Accinni, L., Hsu, K.C., Zabriskie, J.B. and Seegal, B.C. 1966. Electron microscopic studies of human glomerulonephritis with ferritin conjugated antibody. J. Exp. Med. *123*: 399-412.

ANDRES, G.A., Hsu, K.C. and Accinni, L. 1964. Immunoferritin studies of the pathogenesis of human acute glomerulonephritis. J. Cell Biol. *23*: 5A-6A, abst.

ANDRES, G.A., Seegal, B.C., Hsu, K.C., Rothenberg, M.S. and Chapeau, M.L. 1963. Electron microscopic studies of experimental nephritis with ferritin-conjugated antibody. Localization of antigen-antibody complexes in rabbit glomeruli following repeated injections of bovine serum albumin. J. Exp. Med. *117*: 691-704.

ANTHONY, B.F., Kaplan, E.L., Chapman, S.S., Quie, P.G. and Wannamaker, L.W. 1967. Epidemic acute nephritis with reappearance of type 49 streptococcus. Lancet ii: 787-789.

ANTHONY, B.F., Perlman, L.V. and Wannamaker, L.W. 1967. Skin infections and acute nephritis in American Indian children. Pediatrics *39*: 263-279.

ARNOTT, M.S. and Ward, D.N. 1967. Separation of dansyl amino acids in a single analysis. Anal. Biochem. *21*: 50-56.

BAIRD-PARKER, A.C. and Joseph, R.L. 1964. Fractionation of staphylococcal enterotoxin. Nature *202*: 570-571.

BASSETT, D.C.J. 1970. *Hippelates* flies and streptococcal skin infection in Trinidad. Trans. Roy. Soc. Trop. Med. Hyg. *64*: 138-147.

BATES, R.C., Jennings, R.B. and Earle, D.P. 1957. Acute nephritis unrelated to group A hemolytic streptococcus infection: Report of ten cases. Am. J. Med. *23*: 510-528.

BECKER, C.G. 1967. Enhancing effect of type specific antistreptococcal antibodies on emergence of streptococci rich in M-protein. Proc. Soc. Exp. Biol. Med. *124*: 331-335.

BECKER, C.G. and Murphy, G.E. 1968. The experimental induction of glomerulonephritis like that in man by infection with group A streptococci. J. Exp. Med. *127*: 1-24.

BELL, E.T. 1936. The early stages of glomerulonephritis. Am. J. Pathol. *12*: 801-824.

BELL, E.T., Clawson, B.J. and Hartzell, T.B. 1925. Experimental glomerulonephritis. Am. J. Pathol. *1*: 247-258.

BELL, E.T. and Hartzell, T.B. 1922. The etiology and development of glomerulonephritis. Arch. Internal Med. *29*: 768-820.

BENDIXEN, G. 1968. Organ-specific inhibition of the in vitro migration of leukocytes in human glomerulonephritis. Acta Med. Scand. *184*: 99-103.

BERMAN, L.B. and Vogelsang, P. 1963. Poststreptococcal glomerulonephritis without proteinuria. New Engl. J. Med. *268*: 1275-1277.

BERNHEIMER, A.W. 1960. Recent studies on SLO and streptococcal DPNase. Quart. Rev. Pediat. *15*: 237.

BERNHEIMER, A.W. 1967. Physical behavior of SLS. J. Bacteriol. *93*: 2024-2025.

BERNHEIMER, A.W. and Cantoni, G.L. 1945. The cardiotoxic action of preparations containing the oxygen-labile hemolysin of *Streptococcus pyogenes*. I. Increased sensitivity of the isolated frog's heart to repeated application of the toxin. J. Exp. Med. *81*: 295-306.

BERNHEIMER, A.W. and Schwartz, L.L. 1960. Leucocidal agents of haemolytic streptococci. J. Pathol. Bacteriol. *79*: 37-46.

BERNSTEIN, S.H. and Stillerman, M. 1960. A study of the association of group A streptococci with acute glomerulonephritis. Ann. Internal Med. *52*: 1026-1034.

BIRKOFFER, L. and Ritter, A. 1960. Silylierung von Aminosäuren. Chem. Ber. *93*: 424-427.

BLACKMAN, S.S. and Rake, G. 1932. Acute pneumococcal nephritis. Bull. Johns Hopkins Hosp. *51*: 217-233.

BLATT, W.F., Feinberg, M.A., Hoppenberg, H. and Saravis, C.A. 1965. Protein solutions: Concentration by a rapid method. Science *150*: 224-226.

BLOOMFIELD, A.L. 1919. The relations of spontaneous nephritis of rabbits to experimental lesions. Bull. Johns Hopkins Hosp. *30*: 121-125.

BÖESEKEN, J. 1949. The use of boric acid for the determination of the configuration of carbohydrates. Advan. Carbohydrate Chem. *4*: 189-210.

BOHLE, A. and Herfarth, C. 1958. Zur Frage eines intercapillaren Bindezewebes im Glomerulus der Niere des Menschen. Arch. Pathol. Anat. Physiol. *331*: 573-590.

BRADLEY, S.E., Bradley, G.P., Tyson, C.J., Curry, J.J. and Blake, W.D. 1950. Renal function in renal diseases. Am. J. Med. *9*: 766-798.

BRIGHT, R. 1827. Reports of medical cases, selected with a view of illustrating the symptoms and cure of diseases by a reference to morbid anatomy. Vol. I, pp. 1-88. Longman, Rees, Orme, Brown and Green, London.

CANN, J.R. 1968. Recent advances in the theory and practice of electrophoresis. Immunochemistry *5*: 107-134.

CARY, W.E. 1924. Bacteriology of urine in nephritis and experimental bacterial nephritis. J. Infect. Diseases *34*: 599-607.

CAVELTI, P.A. and Cavelti, E.S. 1945. Studies on the pathogenesis of glomerulonephritis. III. Clinical and pathological aspects of the experimental glomerulonephritis produced in rats by means of autoantibodies to kidney. Arch. Pathol. *40*: 163-172.

CHANNING, A.A., Kasuga, T., Horowitz, R.E., Dubois, E.L. and Demopoulos, H.B. 1965. An ultrastructural study of spontaneous lupus nephritis in the NZB/BL-NZW mouse. Am. J. Pathol. *47*: 677-694.

CHEUK, S.F. 1960. Nutritional and other studies of nephrotoxin-producing streptococci. Ph.D. Thesis, McGill University, Montreal.

CHEVILLE, N.F., Mengeling, W.L. and Zinober, M.R. 1970. Ultrastructural and immunofluorescent studies of glomerulonephritis in chronic hog cholera. Lab. Invest. *22*: 458-467.

CLARK, H.F. and Shepard, C.C. 1963. A dialysis technique for preparing fluorescent antibody. Virology *20*: 642-644.

CLASENER, H.A.L., Ensering, H.L. and Hijmans, W. 1970. Persistence in mice of the L-phase of three streptococcal strains adapted to physiological osmotic conditions. J. Gen. Microbiol. *62*: 195-202.

CLAUSEN, J. 1969. Immunochemical techniques for the identification and estimation of macromolecules. *In* Laboratory Techniques in Biochemistry and Molecular Biology, pp. 399-572. Editors: T.S. Work and E. Work. North-Holland Publishing Company.

COCHRANE, C.G. 1971. Mechanisms involved in the deposition of immune complexes in tissues. J. Exp. Med. *134*: 75s-89s.

COHEN, G.J. 1955. Acute nephritis complicating an illness resembling infectious mononucleosis; a case report. Clin. Proc. Child. Hosp. *11*: 38-42.

COHEN, J.A. and Levitt, M.F. 1963. Acute glomerulonephritis with few urinary abnormalities: Report of two cases proved by renal biopsy. New Engl. J. Med. *268*: 749-753.

COOK, E.S., Kreke, W.C., Barnes, E.B. and Motzel, W. 1954. Infra-red and ultra-violet absorption spectra of proteins in the solid state. Nature *174*: 1144-1145.

DAVIDSON, E.A. 1966. Analysis of sugars found in mucopolysaccharides. *In* Methods in Enzymology, Vol VIII, pp. 52-60. Editors: E.F. Neufeld and V. Ginsburg. Academic Press, New York and London.

DAY, N. 1967. Streptococcal glomerulonephritis: Immunological studies. Ph.D. Thesis, McGill University, Montreal.

DERRICK, C.W., Reeves, M.S. and Dillon, H.C. 1970. Complement in overt and asymptomatic nephritis after skin infection. J. Clin. Invest. *49*: 1178-1187.

DILLON, H.C. and Reeves, M.S. 1969. Streptococcal antibody titers in skin infections and acute glomerulonephritis. Pediat. Res. *3*: 362, abst.

DILLON, H.C., Reeves, M.S. and Maxted, W.R. 1968. Acute glomerulonephritis following skin infection due to streptococci of M-type 2. Lancet i: 543-545.

DINH, B.L. and Brassard, A. 1967. Induction of experimental glomerulonephritis in the rat by homologous major urinary protein. Clin. Exp. Immunol. *2*: 633-644.

DIXON, F.J. 1965. Antigen-antibody complexes and autoimmunity. Ann. N.Y. Acad. Sci. *124*: 162-166.

DIXON, F.J., Feldman, J.D. and Vazquez, J.J. 1961. Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. J. Exp. Med. *113*: 899-920.

DIXON, F.J., Vazquez, J.J., Weigle, W.O. and Cochrane, C.G. 1958. Pathogenesis of serum sickness. Arch. Pathol. *65*: 18-27.

DRUMMOND, K.N. and Chiu, J.M.T. 1970. Alterations in glomerular sialoprotein (GSP) during acute poststreptococcal glomerulonephritis (AGN). Abst. Am. Soc. Nephrology Fourth Annual Meeting, Washington, p. 22.

DUVAL, C.W. and Hibbard, R.J. 1926. Experimental glomerulonephritis induced in rabbits with the endotoxic principle of streptococcus scarletinae. J. Exp. Med. *44*: 567-580.

EARLE, D.P. 1965. Hypertension in parenchymal renal disease. Progr. Cardiovas. Dis. *8*: 195-209.

EARLE, D.P., Farber, S.J., Alexander, J.D. and Pellegrino, E.D. 1951. Renal function and electrolyte metabolism in acute glomerulonephritis. J. Clin. Invest. *30*: 421-433.

EDGINTON, T.S., Glassock, R.J. and Dixon, F.J. 1967. Autologous immune complex pathogenesis of experimental allergic glomerulonephritis. Science *155*: 1432-1434.

EDINGTON, G.M. and Mainwaring, A.R. 1966. Nephropathies in West Africa. *In* Int. Acad. of Pathology Monograph No. 6, The Kidney, pp. 488-502. Editors: F.K. Mostofi and D.E. Smith. The Williams and Wilkins Company, Baltimore.

EISEN, A.H., Eidinger, D., Rose, B. and Richter, M. 1964. Prolonged exposure to nephritogenic beta-hemolytic streptococci in intraperitoneal diffusion chambers. Proc. Soc. Exp. Biol. Med. *115*: 367-369.

ELDER, T.R., Barry, J.L., Folcik, P.M. and Knights, E.M. 1962. Simplified test for hematuria. Ann. Intern. Med. *56*: 957-959.

ELLIOTT, S.C. and Williamson, C.K. 1967. Lactic dehydrogenase studies in animals infected with type 12 streptococcal nephrotoxin. Can. J. Microbiol. *13*: 1027-1032.

ELLIS, A.W.M. 1942. Natural history of Bright's disease: Clinical, histological and experimental observations. Lancet i: 1-7, 34-36, and 72-76.

ELSON, L.A. and Morgan, W.T.J. 1933. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem J. *27*: 1824-1828.

FABER, H.K. and Murray, V. 1917. Studies in glomerulonephritis. III. An attempt to produce glomerulonephritis by repeated injections of bacteria. J. Exp. Med. *26*: 707-719.

FAHMY, A.R., Niederwieser, A., Pataki, G. and Brenner, M. 1961. Dünnschicht-Chromatographie von Aminosäuren auf Kieselgel G. 2 Mitteilung. Eine Schnellmethode. Helv. Chim. Acta *44*: 2022-2026.

FARDY, P.W. 1967. Further characterization of a streptococcal nephritogenic agent. Ph. D. Thesis, McGill University, Montreal.

FARDY, P.W., Matheson, B.H. and Reed, R.W. 1969. Experimental nephritis due to type specific streptococci. VI. Further characterization of type 12 nephrotoxin. Can. J. Microbiol. *15*: 555-561.

FARQUHAR, M.G., Vernier, R.L. and Good, R.A. 1957. An electron microscopic study of the glomerulus in nephrosis, glomerulonephritis, and lupus erythematosus. J. Exp. Med. *106*: 649-660.

FELDMAN, J.D. 1963. Pathogenesis of ultrastructural glomerular changes induced by immunologic means. *In* Immunopathology, Third Int. Symposium, pp. 263-281. Editors: P. Grabar and P.A. Miescher. Schwabe, Basel.

FELDMAN, J.D., Hammer, D. and Dixon, F.J. 1963. Experimental glomerulonephritis. III. Pathogenesis of glomerular ultrastructural lesions in nephrotoxic serum nephritis. Lab. Invest. *12*: 748-763.

FERRIERI, P., Dajani, A.S., Chapman, S., Jensen, J.B. and Wannamaker, L.W. 1970. Appearance of nephritis associated with type 57 streptococcal impetigo in North America. New Engl. J. Med. *283*: 832-836.

FISCHEL, E.E. and Gajdusek, D.C. 1952. Serum complement in acute glomerulonephritis and other renal diseases. Am. J. Med. *12*: 190-196.

FISH, A.J., Herdman, R.C., Michael, A.F., Pickering, R.J. and Good, R.A. 1970. Epidemic acute glomerulonephritis associated with type 49 streptococcal pyoderma. II. Correlative study of light, immunofluorescent and electron microscopic findings. Am. J. Med. *48*: 28-39.

FISH, A.J., Michael, A.F., Vernier, R.L. and Good, R.A. 1966. Acute serum sickness nephritis in the rabbit. An immune deposit disease. Am. J. Pathol. *49*: 997-1022.

FLEMING, J. 1949. An epidemic of acute nephritis. Lancet i: 763-766.

FOX, R.R., Schlager, G. and Laird, C.W. 1969. Blood pressure in thirteen strains of rabbits. J.Heredity *60*: 312-314.

FRANKLIN, M.A. 1964. The biological detection of streptococcal nephrotoxin. Ph.D. Thesis, McGill University, Montreal.

FRANKLIN, M., Matheson, B.H. and Reed, R.W. 1969. Experimental nephritis due to type specific streptococci. V. Attempted use of tissue cultures and mice for the assay of nephrotoxic streptococcal polypeptides. Can. J. Microbiol.*15*: 543-548.

FREEDMAN, P. and Markowitz, A.S. 1962. Gamma globulin and complement in the diseased kidney. J. Clin. Invest. *41*: 328-334.

FREEDMAN, P., Peters, J.H. and Kark, R.M. 1960. The localization of γ-globulin in the diseased kidney. Arch. Intern. Med. *105*:524-535.

FREIMER, E.H., Krause, R.M. and McCarty, M. 1959. Studies of L-forms and protoplasts of group A streptococci. I. Isolation, growth and bacteriological characteristics. J. Exp. Med. *110*: 853-874.

GEHRKE, C.W. and Leimer, K. 1970. Trimethylsilylation of amino acids.

Effects of solvents on derivation using bis(trimethylsilyl)-trifluoroacetamide. J. Chromatog. *53*: 201-208.

GEORGE, J.T.A., McDonald, J.C., Payne, D.J.H. and Slade, D.A. 1958. Nephritis in North Yorkshire. Brit. Med. J. *2*: 1381-1382.

GERMUTH, F.G. 1953. A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. J. Exp. Med. *97*: 257-282.

GILLES, H.M. and Hendrickse, R.G. 1963. Nephrosis in Nigerian children. Role of *Plasmodium malariae* and effect of antimalarial treatment. Brit. Med. J. *2*: 27-31.

GINSBURG, J. and Grossowicz, N. 1960. Effect of streptococcal hemolysins on Ehrlich ascites tumor cells. J. Pathol. Bacteriol. *80*: 111-119.

GINSBURG, J., Zeiri, N., Silberstein, Z., Bentwitch, Z. and Lavi, S. 1966. Effects of streptolysin S in rabbits. *In* Current Research on Group A Streptococcus, pp. 190-191. Editor: R. Caravano. Excerpta Medica Foundation, Amsterdam.

GOLDSMITH, H.J., Cowan, M.A. and Gooder, E. 1958. Familial outbreak of acute glomerulonephritis due to Griffith type 1 streptococcus. Lancet ii: 674-675.

GOLDSTEIN, J. and Trung, P.H. 1966. Study of the pathogenic action of group A streptococcal group-polysaccharide, and of sonicated extracts of the group A streptococcal cell wall. *In* Current Research on Group A Streptococcus, pp. 165-170. Editor: R. Caravano. Excerpta Medica Foundation, Amsterdam.

GONZAGA, A.J. and Rammelkamp, C.H. 1962. Diphosphopyridine nucleotidase and acute glomerulonephritis. Arch. Internal Med. *110*: 615-618.

GOODER, H. and Maxted, W. R. 1958. Protoplasts of group A beta-haemolytic streptococci. Nature *182*: 808-809.

GOOR, R.S. and Pappenheimer, A.M. 1967. Studies on the mode of action of diphtheria toxin. III. Site of toxin action in cell-free extracts. J. Exp. Med. *126*: 899-912.

GOOR, R.S., Pappenheimer, A.M. and Ames, E. 1967. Studies on the mode of action of diphtheria toxin. V. Inhibition of peptide bond formation by toxin and NAD in cell-free systems and its reversal by nicotinamide. J. Exp. Med. *126*: 923-939.

GORNALL, A.G., Bardawill, C.J. and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. *177*: 751-766.

GOTOFF, S.P., Isaacs, E.W., Muehreke, R.C. and Smith, R.D. 1969. Serum $beta_{1c}$ globulin in glomerulonephritis and systemic lupus erythematosus. Ann. Internal Med. *71*: 327-333.

GOTZE, O. and Muller-Eberhard, H.J. 1971. The C3-activator system: An alternate pathway of complement activation. J. Exp. Med. *134*: 90s-108s.

GRANT, R.T. and Rothschild, P. 1934. A device for estimating blood pressure in a rabbit. J. Physiol. *81*: 265-269.

GRAY, W.R. 1967. Dansyl chloride procedure. *In* Methods in Enzymology, Vol. XI. Enzyme Structure, pp. 139-151. Editor: C.H.W. Hirs. Academic Press, New York and London.

GRAY, W.R. and Hartley, B.S. 1963. A fluorescent end-group reagent for proteins and peptides. Biochem. J. *89*: 59p.

HALBERT, S.P. 1966. Pathogenic significance of streptococcal extracellular products. *In* Current Research on Group A Streptococcus, pp. 173-187. Editor: R. Caravano. Excerpta Medica Foundation, Amsterdam.

HALBERT, S.P., Dahler, E., Keatinge, S.L. and Bircher, R. 1965. Studies on the role of potassium ions in the lethal toxicity of streptolysin O. *In* Recent Advances in Pharmacology of Toxins, p. 149. Pergamon Press, Oxford.

HAMILTON, L.D.G. 1960. The estimation of side chain groups in the intact protein. *In* A Laboratory Manual of Analytical Methods of Protein Chemistry, Vol. 2. The Composition, Structure and Reactivity of Proteins, pp. 59-100. Editors: P. Alexander and R.J. Block. Pergamon Press, Oxford.

HAYFLICK, L. and Moorhead, P.S. 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*: 585-621.

HELMHOLZ, H.F. 1932. The experimental production of glomerulonephritis in the rabbit. Arch. Pathol. *13*: 592-604.

HEPTINSTALL, R.H. 1966. Pathology of the Kidney. Little, Brown and Company, Boston.

HERBUT, P.A. 1944. A diffuse glomerulonephritis following revaccination for smallpox. Am. J. Pathol. *20*: 1011-1023.

HERDSON, P.B., Jennings, R.B. and Earle, D.P. 1966. Glomerular fine structure in post-streptococcal acute glomerulonephritis. Arch. Pathol. *81*: 117-128.

HEYMANN, W., Hackel, D.B., Harwood, S., Wilson, S.G.F. and Hunter, J.L.P. 1959. Production of nephrotoic syndrome in rats by Freund's

adjuvants and rat kidney suspensions. Proc. Soc. Exp. Biol. Med. *100*: 660-664.

HEYMER, B., Bültmann, B. and Haferkamp, O. Toxicity of streptococcal mucopeptides *in vivo* and *in vitro*. J. Immunol. *106*: 858-861.

HICKS, J.D. and Burnet, F.M. 1966. Renal lesions in the "auto-immune" mouse strains NZB and F1 NZB x NZW. J. Pathol. Bacteriol. *91*: 467-477.

HILL, L.L., Guerra, S. and Rosenberg, H. 1965. Acute glomerulonephritis (AGN) secondary to pneumococcal infection. J. Pediat. *67*: 904, abst.

HINKLE, N.H., Partin, J. and West, C.D. 1960. Nephrepathy in mice after exposure to group A, type 12 streptococci. J. Lab. Clin. Med. *56*: 265-276.

HIRATA, A.A. and Terasaki, P.I. 1970. Cross-reactions between streptococcal M proteins and human transplantation antigens. Science *168*: 1095-1096.

HOLDER, M.A. 1954. The nature of the nephrotoxic substance of Lancefield group A, hemolytic streptococci. M.Sc. Thesis, Dalhousie University, Halifax.

HOLM, S.E., Braun, D. and Jönsson, J. 1968. Antigenic factors common to human kidney and nephritogenic and non-nephritogenic streptococcal strains. Intern. Arch. Allergy Appl. Immunol. *33*: 127-130.

HOLM, S.E., Jönsson, J. and Zettergren, L. 1967. Experimental streptococcal nephritis in rabbits. Acta Pathol. Microbiol. Scand. *69*: 417-430.

HOTCHKISS, R.D. 1944. Gramicidin, tyrocidine, and tyrothricin. Advan. Enzymol. *4*: 153-199.

HULL, R.N., Cherry, W.R. and Tritch, O.J. 1962. Growth characteristics of monkey kidney cell strains LLC-MK_1, LLC-MK_2, and LLC-MK_2 (NCTC-3196) and their utility in virus research. J. Exp. Med. *115*: 903-917.

HUMPHREY, J.H. 1948. The pathogenesis of glomerulonephritis: A re-investigation of the auto-immune hypothesis. J. Pathol. Bacteriol. *60*: 211-218.

ISHIZAKA, K., Ishizaka, T. and Campbell, D.H. 1959. Biologic activity of soluble antigen-antibody complexes. III. Various antigen-antibody systems and the probable role of complement. J. Immunol. *83*: 105-115.

ISHIZAKA, K., Ishizaka, T. and Sugahara, T. 1961. Biological activity of aggregated γ-globulin. III. Production of Arthus-like reactions. J. Immunol. *86*: 220-227.

JACOBS, S. 1959. Determination of nitrogen in proteins by means of indanetrione hydrate. Nature *183*: 262.

JANSON, J.-C. 1967. Adsorption phenomena on Sephadex. J. Chromatog. *18*: 12-20.

JENSEN, M.M. 1967. Viruses and kidney disease. Am. J. Med. *43*: 897-911.

JOHNSON, J.C., Baskin, R.C., Beachey, E.H. and Stollerman, G.H. 1968. Virulence of skin strains of nephritogenic group A streptococci: New M protein serotypes. J. Immunol. *101*: 187-191.

JONES, D.B. 1953. Glomerulonephritis. Am. J. Pathol. *29*: 33-43.

KABAT, E.A. and Mayer, M.M. 1961. Experimental Immunochemistry, 2nd Edition, pp. 133-240. Charles C. Thomas, Springfield, Illinois.

KAGAN, G.J. 1962. L forms of β-haemolytic streptococcus and their pathogenic role. Intern. (8) Congr. Microbiol., Abst., p. 125.

KANDALL, S., Edelmann, C.M. and Bernstein, J. 1969. Acute poststreptococcal glomerulonephritis: A case with minimal urinary abnormalities. Am. J. Dis. Child. *118*: 426-430.

KANTOR, F.S. 1961. Renal glomerular lesions in mice following injection of streptococcal M protein. Yale J. Biol. Med. *34*: 70-71.

KANTOR, F.S. 1964. Glomerular lesions induced by intravenous injection of streptococcal M protein. J. Clin. Invest. *43*: 1251-1252.

KANTOR, F.S. and Cole, R.M. 1959. A fibrinogen precipitating factor (FPF) of group A streptococci. Proc. Soc. Exp. Biol. Med. *102*: 146-150.

KAPLAN, E.L., Anthony, B.F., Chapman, S.S. and Wannamaker, L.W. 1970*a*. Epidemic acute glomerulonephritis associated with type 49 streptococcal pyoderma. 1. Clinical and laboratory findings. Am. J. Med. *48*: 9-27.

KAPLAN, E.L., Anthony, B.F., Chapman, S.S., Ayoub, E.M. and Wannamaker, L.W. 1970*b*. The influence of the site of infection on the immune response to group A streptococci. J. Clin. Invest. *49*: 1405-1414.

KAPLAN, M.H. 1958. Localization of streptococcal antigens in tissues. I. Histologic distribution and persistence of M protein, types 1, 5, 12, and 19 in the tissues of the mouse. J. Exp. Med. *107*: 341-352.

KARK, R.M. 1967. Renal biopsy and diagnosis. Ann. Rev. Med. *18*: 269-298.

KASSIRER, J.P. 1971. Current concepts: Clinical evaluation of kidney function - glomerular function. New Engl. J. Med. *285*: 385-389.

KATZ, S. and Burtis, C.A. 1969. Relationship between chemical structure and elution position. J. Chromatog. *40*: 270-282.

KELLY, D.K. and Winn, J.F. 1958. Renal lesions produced by group A type 12 streptococci. Science *127*: 1337.

KENDALL, F.E. 1937. Studies on serum proteins. I. Identification of a single serum globulin by immunological means. Its distribution in the sera of normal individuals and of patients with cirrhosis of the liver and with chronic glomerulonephritis. J. Clin. Invest. *16*: 921-931.

KIBUKAMUSOKE, J.W. and Hutt, M.S.R. 1967. Histological features of the nephrotic syndrome associated with quartan malaria. J. Clin. Pathol. *20*: 117-123.

KIBUKAMUSOKE, J.W., Hutt, M.S.R. and Wilks, N.E. 1967. The nephrotic syndrome in Uganda and its association with quartan malaria. Quart. J. Med. *36*: 393-408.

KIM, Y.B. and Watson, D.W. 1970. A purified group A streptococcal pyrogenic exotoxin. J. Exp. Med. *131*: 611-628.

KIMMELSTIEL, P., Kim, O.J. and Beres, J. 1962. Studies on renal biopsy specimens, with the aid of the elctron microscope. II. Glomerulonephritis and glomerulonephrosis. Am. J. Clin. Pathol. *38*: 280-296.

KINGSBURY, F.B., Clark, C.P., Williams, G. and Post, A. 1926. The rapid determination of albumin in urine. J. Lab. Clin. Med. *11*: 981-989.

KIRK, J.M. 1960. The mode of action of actinomycin D. Biochim. Biophys. Acta *42*: 167-169.

KJEMS, E. 1960. Studies on streptococcal bacteriophages. 4. The occurrence of lysogenic strains among group A hemolytic streptococci. Acta Pathol Microbiol. Scand. *49*: 199-204.

KLEBE, J., Finkbeiner, H. and White, D. 1966. Silylations with bis-(trimethylsilyl)acetamide, a highly reactive silyl donor. J. Am Chem. Soc. *88*: 3390-3395.

KLEINMAN, H. 1954. Epidemic acute glomerulonephritis at Red Lake. Minnesota Med. *37*: 479-483.

KLEMPERER, M.R., Gotoff, S.P., Alper, C.A., Levin, A.S. and Rosen, F.S. 1965. Estimation of the serum $beta_{1c}$ globulin concentration: Its relation to serum hemolytic complement titer. Pediatrics *35*: 765-769.

KLODNITSKAYA, N.S. 1962. Isolation of L-forms of streptococcus from the penicillin-treated scarlet fever patients, and the experience of obtaining oohemocultures (*in Russian*). Zh. Mikrobiol. (Mosk.) *33*: 31-35.

KODAMA, T., Miyamoto, Y., Kotake, I. and Ochiai, S. 1958. Acute nephritis in Japan. Yokohama Med. Bull. *9*: 105-118.

KOFFLER, D. and Paronetto, F. 1965. Immunofluorescent localization of immunoglobulins, complement and fibrinogen in human diseases. II. Acute, subacute, and chronic glomerulonephritis. J. Clin. Invest. *44*: 1665-1671.

KONEV, S.V. 1967. Fluorescence and phosphorescence of proteins and nucleic acids. Translation editor: S. Udenfriend. Plenum Press, New York.

KOPPLE, K.D. 1966. Peptides and Amino Acids. W.A. Benjamin, Inc., New York.

KOYAMA, J. 1963. Biochemical studies on streptolysin S'. II. Properties of a polypeptide component and its role in the toxin activity. J. Biochem. *54*: 146-151.

KRAMER, N.C., Watt, M.F., Howe, J.H. and Parrish, A.E. 1961. Circulating antihuman kidney antibodies in human renal disease. Am. J. Med. *30*: 39-45.

KRASNER, R.I., Young, G. and Heitmann, P. 1964. The presence of a virulence factor in M protein extracts of group A streptococci. I. The enhancement of virulence of homologous streptococci by these extracts. J. Infect. Diseases *114*: 401-411.

KRAUSE, R.M. 1957. Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell. J. Exp. Med. *106*: 365-383.

KUTTNER, A.G. 1966. Production of bacteriocines by group A streptococci with special reference to the nephritogenic types. J. Exp. Med. *124*: 279-291.

LACHMANN, P.J., Müller-Eberhard, H.J., Kunkel, H.G. and Paronetto, F. 1962. The localization of *in vivo* bound complement in tissue sections. J. Exp. Med. *115*: 63-82.

LANCEFIELD, R.C. 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. *89*: 307-313.

LANGE, C.F. 1969. Chemistry of cross-reactive fragments of streptococcal cell membrane and human glomerular basement membrane. Trans. Proc. *1*: 959-963.

LANGE, C.F. and Markowitz, A.S. 1969. Chemistry of possible transplantation antigens. Trans. Proc. *1*: 502-505.

LANGE, K., Gold, M.M.A., Weiner, D. and Simon, V. 1949. Autoantibodies in human glomerulonephritis. J. Clin. Invest. *28*: 50-55.

LANGE, K., Wachstein, M., Treser, G. and McPherson, S.E. 1965. The possible role of autoantibodies in chronic human and experimental glomerulonephritis. Ann. N.Y. Acad. Sci. *124*: 329-331.

LANGE, K., Wasserman, E. and Slobody, L.B. 1960. The significance of serum complement levels for the diagnosis and prognosis of acute and subacute glomerulonephritis and lupus erythematosus disseminatus. Ann. Internal Med. *53*: 636-646.

LANGERHANS, T. 1879. Ueber die Veränderungen der Glomeruli bei der Nephritis nebst einigen Bemerkungen über die Entstehung der Fibrincylinder. Archiv. Path. Anat. *76*: 85-118.

LASCH, E.E., Frankel, V., Vardy, P.A., Bergner-Rabinowitz, S., Ofek, J. and Rabinowitz, K. 1971. Epidemic glomerulonephritis in Israel. J. Infect. Diseases *124*: 141-147.

LAU, H.S. and Scherago, M. 1967. Leukocytic hypersensitivity in experimental group A streptococcal infections. J. Bacteriol. *94*: 1722-1727.

LE COUNT, E.R. and Jackson, L. 1914. The renal changes in rabbits inoculated with streptococci. J. Infect. Diseases XV: 389-408.

LERNER, R.A. and Dixon, F.J. 1968. The induction of acute glomerulonephritis in rabbits with soluble antigens isolated from normal homologous and autologous urine. J. Immunol. *100*: 1277-1287.

LINDBERG, L.H., Raffel, S. and Vosti, K.L. 1964. Streptococcal glomerulonephritis in rats. Fed. Proc. *23*: 509, abst.

LINDBERG, L.H. and Vosti, K.L. 1969. Elution of glomerular bound antibodies in experimental streptococcal glomerulonephritis. Science *166*: 1032-1033.

LINDBERG, L.H., Vosti, K.L. and Raffel, S. 1967. Experimental streptococcal glomerulonephritis in rats. J. Immunol. *98*: 1231-1240.

LINDEMANN, W. 1900. Sur le mode d'action de certains poisons rénaux. Ann. Inst. Pasteur *14*: 49-59.

LIU, C.T. and McCrory, W.W. 1958. Autoantibodies in human glomerulonephritis and nephrotic syndrome. J. Immunol. *81*: 492-498.

LONGCOPE, W.T., O'Brien, D.P., McGuire, J., Hansen, O.C. and Denny, E.R. 1928. Relationship of acute infections to glomerular nephritis. J. Clin. Invest. *5*: 7-30.

LOWRY, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*: 265-275.

LUKENS, F.O.W. and Longcope, W.T. 1931. Experimental acute glomerulitis. J. Exp. Med. *53*: 511-526.

LYTTLE, J.D., Seegal, D., Loeb, E.N. and Jost, E.L. 1938. The serum antistreptolysin titer in acute glomerulonephritis. J. Clin. Invest. *17*: 631-639.

MAKI, S. 1968. Studies on the nephritogenicity of hemolytic streptococcus. Med. J. Osaka Univ. *19*: 175-227.

MARKOWITZ, A.S. 1969. Streptococcal-related glomerulonephritis in the Rhesus monkey. Trans. Proc. *1*: 985-991.

MARKOWITZ, A.S., Armstrong, S.H. and Kushner, D.S. 1960. Immunological relationships between the rat glomerulus and nephritogenic streptococci. Nature *187*: 1095-1097.

MARKOWITZ, A.S., Clasen, R., Nidus, B.D. and Ainis, H. 1967. Streptococcal related glomerulonephritis. II. Glomerulonephritis in rhesus monkeys immunologically induced both actively and passively with a soluble fraction from human glomeruli. J. Immunol. *98*: 161-170.

MARKOWITZ, A.S. and Lange, C.F. 1962. Streptococcus-related glomerulonephritis. J. Lab. Clin. Med. *60*: 1001-1002.

MARKOWITZ, A.S. and Lange, C.F. 1964. Streptococcal related glomerulonephritis. I. Isolation, immunochemistry and comparative chemistry of soluble fractions from type 12 nephritogenic streptococci and human glomeruli. J. Immunol. *92*: 565-575.

MASUGI, M. 1934. Über die experimentelle Glomerulonephritis durch das spezifische Antinierensern. Beitr. Path. Anat. *92*: 429-466.

MATHESON, B.H. 1953. An investigation of the nephrotoxic action of a strain of Lancefield group A, Griffith type 12 streptococcus. M.Sc. Thesis, Dalhousie University, Halifax.

MATHESON, B.H. 1957. A study of the nephritogenic substance produced by type 12 streptococci. Ph.D. Thesis, McGill University, Montreal.

MATHESON, B.H. and Reed, R.W. 1959. Experimental nephritis due to type specific streptococci. III. Biological, chemical and physical studies on type 12 nephritogenic substance. J. Infect. Diseases *104*: 213-232.

MAXTED, W.R. 1964. Streptococcal bacteriophages. *In* The Streptococcus,

Rheumatic Fever and Glomerulonephritis, pp. 25-44. Editor: J.W. Uhr. Williams and Wilkins Co., Baltimore.

MAXTED, W.R., Fraser, C. and Parker, M.T. 1967. *Streptococcus pyogenes*, type 49; a nephritogenic streptococcus with a wide geographical distribution. Lancet i: 641-644.

MAXTED, W.R. and Potter, E.V. 1967. The presence of type 12 M-protein antigen in group G streptococci. J. Gen. Microbiol. *49*: 119-125.

MAYER, M.M., Eaton, B.B. and Heidelberger, M. 1945. Spectrophotometric standardization of complement for fixation tests. J. Immunol. *53*: 31-35.

McCLUSKEY, R.T., Benacerraf, B., Potter, J.L. and Miller, F. 1960. The pathologic effect of intravenously administered soluble antigen-antibody complexes. I. Passive serum sickness in mice. J. Exp. Med. *111*: 181-194.

McGIVEN, A.R. and Lynraven, G.S. 1968. Glomerular lesions in NZB/NZW mice: Electron microsopic study of development. Arch. Pathol. *85*: 250-261.

McGREGOR, L. 1929. The finer histology of the normal glomerulus. Am. J. Pathol. *5*: 545-557.

McMURRAY, W. and Begg, R.W. 1959. Effect of valinomycin on oxidative phosphorylation. Arch. Biochem. Biophys. *84*: 546-548.

McPHAUL, J.J. and Dixon, F.J. 1969. Basement membrane antigens in serum and urine. Trans. Proc. *1*: 964-967.

MELLORS, R.C. and Ortega, L.G. 1956. Analytical pathology. III. New observations on the pathogenesis of glomerulonephritis, lipid nephrosis, periarteritis nodosa and secondary amyloidosis in man.

Am. J. Pathol. *32*: 455-499.

MESROB, B. and Holeyšovský, V. 1966. Differentiation of dimethyl-aminonaphthalenesulphonic derivatives (DNS) of valine, leucine and isoleucine by chromatography on a thin layer of silica gel. J. Chromatog. *21*: 135-137.

MEUN, D.H.C. and Smith, K.C. 1968. A micro phosphate method. Anal. Biochem. *26*: 364-368.

MICHAEL, A.F., Drummond, K.N., Good, R.A. and Vernier, R.L. 1964. Immunoglobulins: Clarification of their significance in renal disease and demonstration of response to immunosuppressive therapy. J. Clin. Invest. *43*: 1291, abst.

MICHAEL, A.F., Drummond, K.N., Good, R.A. and Vernier, R.L. 1966. Acute poststreptococcal glomerulonephritis: Immune deposit disease. J. Clin. Invest. *45*: 237-248.

MICKLEA, G.D. 1968. Further studies on streptococcal nephrotoxin. Ph.D. Thesis, McGill University, Montreal.

MINOKOWITZ, S., Weuk, R., Friedman, E., Yuceoglu, A.M. and Berkovich, S. 1968. Acute glomerulonephritis associated with varicella infection. Am. J. Med. *44*: 489-492.

MIYAMOTO, Y., Kotake, I., Ochiai, S. and Kodama, T. 1960. Experimental nephritis produced in rabbits by inoculation of group A hemolytic streptococci isolated from epidemic nephritis patients. A focal infection experiment in the paranasal sinuses. Jap. J. Microbiol. *4*: 105-114.

MOFFAT, E.D. and Lytle, R. 1959. Polychromatic technique for the identification of amino acids on paper chromatograms. Anal. Chem. *31*: 926-928.

MOORE, E.A., Sabachewsky, L. and Toolan, H.W. 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Res. *15*: 598-602.

MORTIMER, E.A. 1965. Production of L-forms of group A streptococci in mice. Proc. Soc. Exp. Biol. Med. *119*: 159-163.

MOVAT, H.Z., Steiner, J.W. and Huhn, D. 1962. The fine structure of the glomerulus in acute glomerulonephritis. Lab. Invest. *11*: 117-135.

NEUSTEIN, H.B. and Davis, W. 1965. Acute glomerulonephritis. A light and electron microscopy study of 8 serial biopsies. Am. J. Clin. Pathol. *44*: 613-626.

NG, W. 1969. Studies on streptococcal nephrotoxin and glomerulonephritis. M.Sc. Thesis, McGill University.

NOTKINS, A.L. 1965. Lactic dehydrogenase virus. Bacteriol. Rev. *29*: 143-160.

OKUDA, R., Kaplan, M., Cuppage, F. and Heymann, W. 1965. Deposition of autologous gamma globulin in kidneys of rats with nephrotic renal disease of various etiologies. J. Lab. Clin. Med. *66*: 204-215.

OLDSTONE, M.B.A. and Dixon, F.J. 1971. Immune complex disease in chronic viral infections. J. Immunol. *134*: 32s-40s.

OPHÜLS, W. 1917. The etiology and development of nephritis. J. Am. Med. Ass. *69*: 1223-1227.

ORT, M., Salinas, M.L., Metcoff, J. and Pirani, C.L. 1969. Some clinical and morphological features of the acute phase of acute glomerulonephritis in children. Acta Univ. Carolinae Medica *15*: 207-225.

OVERTURF, G.D. and Mortimer, E.A. 1970. Studies of the relationship between the production of bacteriocines by group A streptococci and acute glomerulonephritis. J. Exp. Med. *132*: 694-701.

PACÁKOVÁ, V., Miller, V. and Černohorský, I.J. 1971. Gas-liquid chromatography of some pyrimidine derivative. Anal. Biochem. *42*: 549-554.

PAN, I.C., Tsai, K.S. and Karstad, L. 1970. Glomerulonephritis in Aleutian disease of mink: Histological and immunofluorescence studies. J. Pathol. *101*: 119-127.

PARTRIDGE, S.M. 1949. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature *164*: 443.

PECK, J.L. and Thomas, L. 1948. Failure to produce lesions or autoantibodies in rabbits by injecting tissue extracts, streptococci and adjuvants. Proc. Soc. Exp. Biol. Med. *69*: 451-453.

PICKERING, R.J., Gewurz, H. and Good, R.A. 1968. Complement inactivation by serum from patients with acute and hypocomplementemic chronic glomerulonephritis. J. Lab. Clin. Med. *72*: 298-307.

PIEL, C.F., Dong, L., Modern F.W.S., Goodman, J.R. and Moore, R. 1955. The glomerulus in experimental renal disease in rats as observed by light and electron microscopy. J. Exp. Med. *102*: 573-580.

PIERCE, A.E. 1968. Silylation of Organic Compounds. Pierce Chemical Company, Rockford, Illinois.

PITAL, A. and Janowitz, S.L. 1963. Enhancement of staining intensity in the fluorescent antibody reaction. J. Bacteriol. *86*: 888-889.

PLEYDELL, M.J. and Hall-Turner, W.J.A. 1958. An outbreak of nephritis in Northamptonshire. Brit. Med. J. *2*: 1382-1383.

POON-KING, T., Mohammed, I., Cox, R., Potter, E.V., Simon, N.M., Siegel, A.C. and Earle, D.P. Recurrent epidemic nephritis in South Trinidad. New Engl. J. Med. *277*: 728-733.

POTTER, E.V., Moran, A.F., Poon-King, T. and Earle, D.P. 1968. Characteristics of beta hemolytic streptococci associated with acute glomerulonephritis in Trinidad, West Indies. J. Lab. Clin. Med. *71*: 126-137.

POTTER, E.V., Ortiz, J.S., Sharrett, A.R., Burt, E.G., Bray, J.P., Finklea, J.F., Poon-King, T. and Earle, D.P. 1971. Changing types of nephritogenic streptococci in Trinidad. J. Clin. Invest. *50*: 1197-1205.

POUCHERT, C.J. 1970. The Aldrich Library of Infrared Spectra. Aldrich Chemical Company.

POULIK, M.D. and Poulik, E. 1958. Isolation of the lethal factor of diphtheria toxin by electrophoresis in starch gel. Nature *181*: 354-355.

RAMMELKAMP, C.H. 1957. Microbiologic aspects of glomerulonephritis. J. Chronic Diseases *5*: 28-33.

RAMMELKAMP, C.H. 1964. Concepts of pathogenesis of glomerulonephritis derived from studies in man. *In* The Streptococcus, Rheumatic Fever and Glomerulonephritis, pp. 289-300. Editor: J.W. Uhr. Williams and Wilkins Co., Baltimore.

RAMMELKAMP, C.H. and Weaver, R.S. 1953. Acute glomerulo-nephritis. The significance of variations in the incidence of the disease. J. Clin. Invest. *32*: 345-358.

RANDALL, H.M., Fowler, R.G., Fuson, N. and Dangl, J.R. 1949. Infrared Determination of Organic Structures. D. Van Nostrand Company, Inc., Princeton.

RAPAPORT, F.T., Markowitz, A.S., McCluskey, R.T., Hanaoka, T. and Shimada, T. 1969. Induction of renal disease with antisera to group A streptococcal membranes. Trans. Proc. *1*: 981-984.

READ, S.E. 1970. Bacteriophages of group A streptococci. Ph.D. Thesis, McGill University, Montreal.

REED, G.B. and Kropp, B. 1944. A simple apparatus for determining peripheral arterial pressure in the rabbit's ear. J. Lab. Clin. Med. *29*: 214-217.

REED, R.W. 1953. An epidemic of acute nephritis. Can. Med. Ass. J. *68*: 448-455.

REED, R.W. 1959. Experimental nephritis in monkeys. *In* Proceedings of the Eleventh Annual Conference on the Nephrotic Syndrome, pp. 83-98. Editor: J. Metcoff.

REED, R.W. and Matheson, B.H. 1954*a*. Experimental nephritis due to type specific streptococci. I. The effect of a single exposure to type 12 streptococci. J. Infect. Diseases *95*: 191-201.

REED, R.W. and Matheson, B.H. 1954*b*. Experimental nephritis due to type specific streptococci. II. The effect of repeated exposure to type 12 streptococci. J. Infect. Diseases *95*: 202-212.

REED, R.W. and Matheson, B.H. 1960. Experimental nephritis due to type specific streptococci. IV. The effect of type 12 nephrotoxin in monkeys. J. Infect. Diseases *106*: 245-249.

REITH, A.F., Warfield, L.M. and Enger, W. 1930. Attempts to produce acute glomerulonephritis in rabbits with the peritoneal lysate of *Streptococcus scarlatinae*. J. Infect. Diseases *46*: 42-52.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. *17*: 208-212.

RICH, A.R., Bumstead, J.H. and Frobisher, M. 1929. Hemorrhagic glomerular lesions produced by filtrates of *Streptococcus viridans* cultures. Proc. Soc. Exp. Biol. Med. *26*: 397-399.

RICKLES, N., Zilberstein, Z., Kraus, S., Arad, G., Kaufstein, M. and Ginsburg, I. 1969. Persistence of group A streptococci labelled with fluorescein isothiocyanate in inflammatory sites in the heart and muscle of mice and rabbits. Proc. Soc. Exp. Biol. Med. *131*: 525-530.

ROCKLIN, R.E., Lewis, E.J. and David, J.R. 1970. Glomerulonephritis: Cellular hypersensitivity to basement-membrane antigen. New Engl. J. Med. *283*: 497-501.

ROITT, I.M. and Doniach, D. 1966. Immunofluorescent tests for the detection of autoantibodies. *In* Immunology Techniques. Edited by Immunology Unit of World Health Organization.

ROREM, E.S. 1959. Ultra-violet fluorescence of quinine sulfate for detection of phosphate ester spots on paper. Nature *183*: 1739-1740.

ROTTA, J. 1969. Biological activity of cellular components of group A streptococci in vivo. Current Topics in Microbiology and Immunology *48*: 63-101.

ROTTA, J. and Bednar, B. 1969. Biological properties of cell wall mucopeptide of hemolytic streptococci. J. Exp. Med. *130*: 31-47.

ROY, S., Wall, H.P. and Etteldorf, J.N. 1969. Second attacks of acute glomerulonephritis. J. Pediat. *75*: 758-767.

RÜHLMANN, K. 1961. Silylierung von Aminosäuren. Chem. Ber. *44*: 1876-1878.

SANFORD, K.K., Earle, W.R. and Likely, G.D. 1948. The growth of single isolated tissue cells. J. Nat. Cancer Inst. *9*: 229-246.

SCHMITT-SLOMSKA, J., Sacquet, E. and Caravano, R. 1967. Group A streptococcal L-forms. I. Persistence among inoculated mice. J. Bacteriol. *93*: 451-455.

SCHWAB, J.H. 1962. Analysis of the experimental lesions of connective tissue produced by a complex of C polysaccharide from group A streptococci. J. Exp. Med. *116*: 17-28.

SEEGAL, B.C., Andres, G.A., Hsu, K. and Zabriskie, J.B. 1965. Studies on the pathogenesis of acute and progressive glomerulonephritis in man by immunofluorescein and immunoferritin techniques. Fed. Proc. *24*: 100-108.

SEEGAL, D. 1935. Acute glomerulonephritis following pneumococcic lobar pneumonia: Analysis of seven cases. Arch. Internal Med. *56*: 912-919.

SHARP, J.T. 1954. L colonies from hemolytic streptococci: New technique in the study of L forms of bacteria. Proc. Soc. Exp. Biol. Med. *87*: 94-97.

SIMON, N.S., Potter, E.V., Siegel, A.C., McAninch, J., Poon-King, T., Humair, L. and Earle, D.P. 1965. Epidemic nephritis in Trinidad. J. Lab. Clin. Med. *66*: 1022.

SMADEL, J.E. 1937. Experimental nephritis in rats induced by injection of anti kidney serum. III. Pathological studies of the acute and chronic disease. J. Exp. Med. *65*: 541-555.

SMITH, C.W., Marshall, J.D. and Eveland, W.C. 1959. Use of contrasting fluorescent dye as counterstain in fixed tissue preparations. Proc. Soc. Exp. Biol. Med. *102*: 179-181.

SMITH, F.G., Weinstein, S.L. and Heimlich, E.M. 1964. Glomerular alterations in the rat produced by homologous kidney, streptococcus and incomplete adjuvant. Life. Sci. *3*: 873-881.

SOLOTOROVSKY, M. and Johnson, W. 1970. Tissue culture and bacterial protein toxins. *In* Microbial Toxins, Vol. I. Bacterial Protein Toxins, pp. 277-327. Editors: S.J. Ajl, S. Kadis and T.C. Montie. Academic Press, New York and London.

SOOTHILL, J.F. 1967. Altered complement component C'3a (β_{1c}-β_{1A}) in patients with glomerulonephritis. Clin. Exp. Immunol. *2*: 83-92.

SPANDE, T.F. and Witkop, B. 1967. Determination of the tryptophan content of proteins with N-bromosuccinimide. *In* Methods in Enzymology, Vol. XI. Enzyme Structure, pp. 498-506. Editor: C.H.W. Hirs. Academic Press, New York and London.

SPICER, S.S. and Warren, L. 1960. The histochemistry of sialic acid containing mucoproteins. J. Histochem. Cytochem. *8*: 135-137.

SPIRA, G., Silberstein, Z., Harris, T.N. and Ginsburg, I. 1968. Toxic effects induced in rabbits by extracellular products and sonicates of group A streptococci. Proc. Soc. Exp. Biol. Med. *127*: 1196-1201.

SPITZER, R.E., Vallota, E.H., Forristal, J., Sudora, E., Stitzel, A., Davis, N.C. and West, C.D. 1969. Serum C'3 lytic system in patients with glomerulonephritis. Science *164*: 436-437.

SRAMEK, J., Vendl, L., Kliment, J. and Panocha, M. 1964. A contribution to the epidemiology of acute glomerulonephritis associated with group A streptococci different from type 12. Zbl. Bakt. *196*: 56.

STAVITSKY, A.B., Stavitsky, R. and Ecker, E.E. 1949. Loss of hemolytic-complement activity and of granulocytes following reinjection of an antigen into the rabbit. J. Immunol. *63*: 389-407.

STEBLAY, R.W. 1962. Glomerulonephritis induced in sheep by injections of heterologous basement membrane and Freund's complete adjuvant. J. Exp. Med. *116*: 253-272.

STEBLAY, R.W. and Rudofsky, U. 1968. Autoimmune glomerulonephritis induced in sheep by injections of human lung and Freund's adjuvant. Science *160*: 204-206.

STEBLAY, R.W. and Rudofsky, U. 1971. Spontaneous renal lesions and glomerular deposits of IgG and complement in guinea pigs. J. Immunol. *107*: 1192-1196.

STERNHEIMER, R. and Malbin, B. 1951. Clinical recognition of pyelonephritis, with a new stain for urinary sediments. Am. J. Med. *11*: 312-323.

STETSON, C.A., Rammelkamp, C.H., Krause, R.M., Kohen, R.J. and Perry, W.D. Epidemic acute nephritis: Studies on etiology, history and prevention. Medicine *34*: 431-450.

STRUNK, S.W., Hammond, W.S. and Benditt, E.P. 1964. The resolution of acute glomerulonephritis. An electron microscopic study. Lab. Invest.*13*: 401-429.

SYMONDS, B.E.R. 1960. Epidemic nephritis in South Trinidad. J. Pediat. *56*: 420-427.

SZYMANSKI, H.A. and Erickson, R.E. 1970. Infrared Band Handbook: Vol. 1, 4240-999 cm^{-1}; Vol. 2, 999-29 cm^{-1}, 2nd Edition. IFI/Plenum Data Corporation, New York.

TAN, E.M., Hackel, D.B., Kaplan, M.H. 1961. Renal tubular lesions in mice produced by group A streptococci grown in intraperitoneal diffusion chambers. J. Infect. Diseases *108*: 107-112.

TAN, E.M. and Kaplan, M.H. 1962. Renal tubular lesions in mice produced by streptococci in intraperitoneal diffusion chambers: Role of streptolysin S. J. Infect. Diseases *110*: 55-62.

TARANTA, A., Spagnuolo, M., Davidson, M., Goldstein, G. and Uhr, J.W. 1969. Experimental streptococcal infections in baboons. Trans. Proc. *1*: 992-993.

TAYLOR-ROBINSON, D., Johnson, K.M., Bloom, H.H., Parrott, R.H., Mufson, M.A. and Chanock, R.M. Rhinovirus neutralizing antibody responses and their measurement. Am. J. Hyg. *78*: 285-292.

TOP, F.H., Wannamaker, L.W., Maxted, W.R. and Anthony, B.F. 1967. M antigens among group A streptococci isolated from skin lesions. J. Exp. Med. *126*: 667-685.

TORII, S. 1960. Experimental studies on the developmental mechanism of acute glomerulonephritis. Ann. Paediat. Jap. *6*: 1-37.

TRESER, G., Semar, M., McVicar, M., Franklin, M., Ty, A., Sagel, I. and Lange, K. 1968. Antigenic streptococcal components in acute glomerulonephritis. Science *163*: 676-677.

TRUMP, B.F. and Benditt, E.P. 1962. Electron microscopic studies of human renal disease. Observations of normal visceral glomerular epithelium and its modification in disease. Lab. Invest. *11*: 753-782.

TRUMP, B.F., Smuckler, E.A. and Benditt, E.P. 1961. A method for staining epoxy sections for light microscopy. J. Ultrastr. Res. *5*: 343-348.

TY, A., Sagel, I., Lange, K. 1970. Identification of type independent nephritogenic streptococcal antigens in acute glomerulonephritis. Am. Pediat. Soc. Proc.: 10.

UNAHUE, E.R. and Dixon, F.J. 1965 . Experimental glomerulonephritis. V. Studies on the interaction of nephrotoxic antibody with tissues of the rat. J. Exp. Med. *121*: 697-714.

UNAHUE, E.R. and Dixon, F.J. 1965 . Experimental glomerulonephritis. VI. The autologous phase of nephrotoxic serum nephritis. J. Exp. Med. *121*: 715-725.

VERNIER, R.L., Papermaster, B.W., Arhelger, R., Mecklenburg, F. and Good, R.A. 1958. Electron microscopy of experimental renal disease. Am. J. Dis. Child. *96*: 547-548.

VON KROGH, M. 1916. Colloidal chemistry and immunology. J. Infect. Diseases *19*: 452-477.

VOSTI, K.L., Lindberg, L.H., Kosek, J.C. and Raffel, S. 1970. Experimental streptococcal glomerulonephritis: Longitudinal study of a laboratory model resembling human acute poststreptococcal glomerulonephritis. J. Infect. Diseases *122*: 249-259.

WADSWORTH, A., Maltaner, E. and Maltaner, F. 1931. The quantitative determination of the fixation of complement by immune serum and antigen. J. Immunol. *21*: 313-340.

WAGNER, V. and Rokop, J. 1956. Auto-antibodies and skin tests with extracts from normal kidney in cases of diffuse glomerulonephritis. Intern. Arch. Allergy *9*: 231-238.

WALDI, D. 1965. Spray reagents for thin-layer chromatography. *In* Thin-Layer Chromatography, pp. 483-503. Editor: E. Stahl. Academic Press, New York and London.

WALTERSDORFF, R.L. and Jackson, R.W. 1970. Induction of kidney disease in rabbits with streptococcal teichoic acid. Bacteriol. Proc.: 117, abst.

WANNAMAKER, L.W. 1970. Differences between streptococcal infection of the throat and of the skin. New Engl. J. Med. *282*: Part 1: 23-31; Part 2: 78-85.

WANNAMAKER, L.W., Skjold, S. and Maxted, W.R. 1970. Characterization of bacteriophages from nephritogenic group A streptococci. J. Infect. Diseases *121*: 407-418.

WATSON, D.W. 1960. Host-parasite factors in group A streptococcal infections: Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxin. J. Exp. Med. *111*: 255-284.

WATSON, M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. *4*: 475-478.

WELLS, A.F., Miller, C.E. and Nadel, M.K. 1966. Rapid fluorescein and protein assay method for fluorescent-antibody conjugates. Appl Microbiol. *14*: 271-275.

WERTHEIM, A.R., Lyttle, J.D., Loeb, E.N., Earle, D.P., Seegal, B.C. and Seegal, D. 1953. The association of type specific hemolytic streptococci with acute glomerulonephritis. J. Clin. Invest. *32*: 359-363.

WILMERS, M.J., Cunliffe, A.C. and Williams, R.E.O. 1954. Type 12 streptococci associated with acute haemorrhagic nephritis. Lancet ii: 17018.

WILSON, C.B. and Dixon, F.J. 1971. Quantitation of acute and chronic serum sickness in the rabbit. J. Exp. Med. *134*: 7s-18s.

WING, A.J., Kibukamusoke, J.W. and Hutt, M.S.R. 1971. Poststreptococcal glomerulonephritis and the nephrotic syndrome in Uganda. Trans. Roy. Soc. Trop. Med. Hyg. *65*: 543-548.

WINKENWERDER, W.L., McLeod, N. and Baker, M. 1935. Infection and hemorrhagic nephritis. Arch. Internal Med. *56*: 297-326.

WITTLER, R.G., Tuckett, J.D., Muccione, V.J., Gangarosa, E.J. and O'Connell, R.C. 1962. Transition forms and L forms from the blood of rheumatic fever patients. Intern. (8) Congr. Microbiol. Abstr.: 125.

WOIWOD, A.J. 1950. Fluorescence of amino-acids, peptides and amines on filter paper. Nature *166*: 272.

WOOD, T. 1955. A reagent for the detection of chloride and of certain purines and pyrimidines on paper chromatograms. Nature *176*: 175-176.

WU, H. 1922. A new colorimetric method for the determination of plasma proteins. J. Biol. Chem. *51*: 33-39.

YUCEOGLU, A.M., Berkovich, S. and Minokowitz, S. 1966. Acute glomerulonephritis associated with ECHO virus type 9 infection. J. Pediat. *69*: 603-609.

ZABRISKIE, J.B. 1964. The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. J. Exp. Med. *119*: 761-780.

ZABRISKIE, J.B., Lewshenia, R., Möller, G., Wehle, B. and Falk, R.E. 1970. Lymphocytic responses to streptococcal antigens in glomerulonephritic patients. Science *168*: 1105-1108.