A STUDY OF ANTIGENIC COMPONENTS OF KIDNEY TISSUE.

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THE phenomenon of " nephrotoxic nephritis," a type of renal damage induced by the injection of an antibody prepared against kidney tissue, has been the subject of many reports. The earliest experiments on these lines were those of Lindemann (1900), but credit for demonstrating nephritic changes clearly attributable to antibodies specific for kidney tissue is generally given to Masugi (1933). In recent years attempts have been made to identify the tissue element in the kidney which stimulates the production of nephrotoxic antibody when introduced into an animal of a different species. Heymann and Lund (1948) reaffirmed an earlier observation (Pearce, 1904) that the cortex of the kidney was very much more active in respect of this antigenic property than the medulla, and Solomon, Gardella, Fanger, Dethier and Ferrebee (1949) discovered that isolated glomeruli could effectively absorb the nephrotoxic antibody from anti-kidney serum. Greenspon and Krakower (1950) found that glomeruli separated from the kidneys of dogs were able to produce a potent nephrotoxic serum when injected into rabbits, while all other components of the renal cortex were ineffective. Krakower and Greenspon (1951) subsequently immunised animals with individual components of glomeruli (separated by sonic vibration), and concluded that the basement membrane of the tufts accounted for most of the antigenic activity of whole glomeruli. The rôle of the glomeruli was again emphasized by Pressman, Hill and Foote (1949), who injected into mice a preparation of anti-mouse-kidney globulin tagged with radio-active iodine and found specific concentration of radio-activity in the glomeruli.

There is therefore good evidence for the importance of the glomeruli and, more specifically, of their basement membrane as antigens in the production of nephrotoxic serum. It is apparent, however, that the antigenic substance is not confined to renal tissue. Both anti-lung (Chikamitsu, 1940) and anti-placenta (Seegal and Loeb, 1946) serum have proved to be nephrotoxic, and anti-lung serum labelled with radio-active iodine has been shown to localise specifically in the kidney as well as in the lung (Pressman and Eisen, 1950).

The concept of a strongly antigenic substance in glomerular basement membrane—a supporting tissue element—seemed to be important not only in helping to elucidate the pathogenesis of experimental nephritis, but perhaps ultimately in relation to the wider problem of the human mesenchymal diseases of unknown

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aetiology. It was decided therefore to prepare an anti-kidney serum, to conjugate it with fluorescein, and to apply this "tagged" serum to sections of kidney. The areas of fixation of fluorescent antibody would be expected to be revealed by green fluorescence when such preparations were examined by ultra-violet light. It seemed likely that by the use of this method it would not only be possible to locate the site of the kidney antigen with considerable histological precision, but also to search for tissue with similar antigenic specificity in other organs. The study was subsequently extended to include experiments with anti-glomerulus and anti-lung serum. The results of these investigations have confirmed the antigenicity of glomerular basement membrane, and have shown that an antibody which reacts with this structure reacts also with the basement membrane of renal tubules as well as with a reticular network in the media of vessels. In addition it is believed that the presence of a separate antigen in the cytoplasm of the cortical tubules has been established. A study of other organs, to be reported later (Cruickshank and Hill, 1953), has disclosed a widespread distribution of structures apparently sharing an antigen similar to or identical with the one contained in renal basement membrane.

A method for the detection of antigenic material in tissue cells, in which antibody labelled with fluorescein was employed as a specific histochemical stain, was reported by Coons, Creech, Jones and Berliner (1942), and described in detail in a later paper (Coons and Kaplan, 1950). Fluorescent antibodies have been used to study the uptake by the tissues of injected bacterial polysaccharides (Kaplan, Coons and Deane, 1950; Hill, Deane and Coons, 1950; Schmidt, 1952) and protein antigens (Coons, Leduc and Kaplan, 1951), to identify viruses or rickettsiae in tissues or in tissue culture (Coons, Snyder, Cheever and Murray, 1950; Watson, 1952) and to demonstrate the ACTH-producing cells of the anterior pituitary (Marshall, 1951).

METHODS.

Preparation of antigens and antisera.

Four antisera, prepared in rabbits by the injection of material from rats, were used. The antigens were a suspension of perfused whole rat kidney, a suspension of isolated rat glomeruli, a suspension of perfused rat lung and a preparation made from rat serum. Each rat used as a source of tissue was anaesthetised with ether and perfused with one litre of saline—introduced into the aorta and allowed to escape through an incision in the inferior vena cava. The whole-kidney and lung preparations were made up as 10–15 per cent suspensions in saline and the dosage schedule was 1 ml. twice weekly intraperitoneally for 4–6 weeks. The isolated glomeruli were injected by the same route, a total of 200 mg. (wet wt.) being given over a period of 8 weeks. The glomeruli were separated by a method based on that of Greenspon and Krakower (1950). Antiserum against rat serum proteins was prepared by the method of Heidelberger and Kendall (1935). The serum referred to hereafter as "anti-kidney serum" (as distinct from anti-glomerulus serum) was the preparation obtained by immunization with *whole* kidney homogenate.

Conjugation of serum to fluorescein: preparation, staining and examination of sections.*

The methods used in these procedures, together with the refrigerated microtome cabinet† and the "window" attached to the blade of the microtome, were essentially similar to those

* "Staining" is not a strictly accurate term for a procedure which results in the appearance in sections of an antigen-antibody complex identifiable in ultra-violet light by virtue of a fluorochrome attached to the antibody. For the sake of brevity it is, however, used throughout this paper—to denote both the procedure and the presence of specific green fluorescence in treated tissues.

† Constructed by the Pressed Steel Company, Oxford.

described in detail by Coons and his collaborators (Coons and Kaplan, 1950; Coons *et al.*, 1951). Conjugates were prepared from whole serum and globulin fractions separated from them by ammonium sulphate precipitation; the fluorescent globulin was suspended in the minimal volume of saline and dialysed against buffered saline (pH 7·6). This procedure was adopted simply to achieve a high globulin content in the final preparation, and did not eliminate the non-specific staining which is characteristically produced by untreated conjugates, and which can be prevented (except in a few sites) by absorption with a washed and dried tissue suspension. The identity of the species selected as the source of material for this absorption procedure is probably immaterial when the conjugate is to be used for tracing an antigen artificially introduced into an animal (Coons and Kaplan, 1950). The present experiments, however, were concerned with the detection of antigenic elements in normal rat tissues, and it was considered desirable to treat the conjugates with dried material prepared from an organ of an unrelated species—generally human skeletal muscle. In some experiments a portion of conjugate was subsequently submitted to a second absorption, this time with a suspension of an appropriate *rat* tissue, to remove its *specific* reactivity. This procedure is discussed more fully below.

No fixative was used in preparing tissues for sectioning. Small pieces of each organ to be examined were removed from freshly killed animals, rapidly frozen in a mixture of alcohol and solid carbon dioxide and stored under solid carbon dioxide. The frozen blocks were transferred to the refrigerated microtome cabinet without being allowed to thaw and sections of 6μ thickness cut at a temperature of −18°. Sections were thawed on to gelatinised slides by warming the lower surface of the slide with a finger-tip, dried under a fan and stored at 5°; they were generally stained and examined within 3 days. Sections were treated with fluorescent conjugates for periods of 2–3 hr. at room temperature, and thereafter washed for 10 min. before being mounted in reagent glycerol containing 10 per cent of the same buffer. The investigation did not include any experiments involving the injection of fluorescent globulin *in vivo*; the globulin-fluorescein conjugates were used simply as histochemical reagents on tissue sections.

The light source for the fluorescence microscope was a 250 watt high-pressure mercury arc.* A Corning 5840 ultra-violet filter was used. With this arrangement there was a tendency for the gelatine eyepiece filter (Wratten 2B) to fluoresce faintly; this was remedied by placing a glass cell containing 2 per cent sodium nitrite below the gelatine disc.

Tests for specificity of fluorescent staining.

The specificity of the green fluorescence in sections which had been exposed to fluorescein-globulin conjugates prepared from anti-kidney serum was tested in several ways. It was necessary to establish that the staining was dependent on a factor present in immune serum but not in normal rabbit serum, and that this factor was active in the absence of the fluorescein "label." This was accomplished by the exposure of sections to the unlabelled antiserum and to normal rabbit serum respectively, and thereafter treatment of both sections with fluorescent globulin prepared from the antiserum. Fluorescent staining failed to develop in the first of these sections (indicating that the reactive groups of the antigen had been specifically bound by the unlabelled antibody), but appeared as usual in the second. As a further check on the absence of the specific factor from the serum of a non-immunised rabbit, a third section was treated with a fluorescein-globulin conjugate prepared from such serum and was found to show no staining. It was also found that a homogenate of rat kidney would absorb the antibody from the anti-kidney conjugate, with resulting loss of its capacity to produce staining, but that similar absorption with rat liver (which contained little stainable material) or with kidney from an unrelated species (pig) was ineffective. The results of absorption experiments with rat lung, of inhibition by pre-treatment with unconjugated anti-lung serum and of other control experiments will be described later.

In the inhibition experiments with unlabelled serum trials were made of various lengths of exposure both to serum and, thereafter, to conjugate; in general, pre-treatment for 3 hr. and staining for 1–1½ hr. gave clear-cut results. In the absorption experiments 1 part of a wet packed organ suspension was mixed with 3 parts of fluorescent conjugate; after 1 hr. the suspension was removed by centrifugation and replaced for a second hour with fresh homogenate in the same proportion.

* Mazda ME/D 26.

RESULTS.

The patterns of staining produced in rat kidney by fluorescent globulin from three antisera—anti-kidney, anti-glomerulus and anti-lung—are summarised in the Table, which also shows the results of pre-treatment with unlabelled sera, and of absorption of the anti-whole-kidney conjugate with organ suspensions.

TABLE.—*Staining Reactions in Rat Kidney.*

Conjugate.	Tissue element.				Figure.
	Glomerular basement membrane.	Tubular basement membrane.	Tubular cytoplasm (cortex).	Media of vessels.	
Anti-rat-kidney globulin (AKG)	+	+	+	+	1, 2, 3
Anti-rat-glomerulus globulin (AGG)	+	+	N	+	4, 5
Anti-rat-lung globulin (ALG)	+	+	N	+	
AKG absorbed with rat kidney	N	N	N	N	
„ „ with rat lung	N	N	+	N	6
„ „ with rat liver	+	+	+	+	
„ „ with pig kidney	+	+	+	+	
AKG after pre-treatment with unlabelled homologous serum	N	N	N	N	
AKG after pre-treatment with unlabelled anti-lung serum	N	N	+	N	
AKG after pre-treatment with unlabelled normal rabbit serum	+	+	+	+	
Normal rabbit globulin	N	N	N	N	

(+ = Specific green fluorescence. N = No specific fluorescence.)

DESCRIPTION OF PLATES.

FIG. 1–6 are photomicrographs of sections of normal rat kidney after exposure to fluorescein-labelled antibody globulin. The lightest areas represent the bright green fluorescence which indicated specific fixation of fluorescent antibody by the homologous tissue antigen: the histological topography in areas where no specific reaction occurred is visible as a darker image, by virtue of the faint bluish auto-fluorescence of the tissues. The sections in Fig. 7 and 8 were photographed under an ordinary light microscope.

FIG. 1.—Treated with anti-kidney globulin. Specific fluorescence is present in the glomeruli and in the cytoplasm of the convoluted tubules. Fluorescence is also present in the tubular basement membrane, but this feature is more easily seen in the high-power view (Fig. 3). × 160.

FIG. 2.—High-power view of a glomerulus from the preparation illustrated in Fig. 1. The basement membrane of the tuft and of Bowman's capsule is distinctly seen as a single bright line; staining is also present in the cytoplasm of epithelial cells. × 480.

FIG. 3.—High-power view of convoluted tubules from the preparation illustrated in Fig. 1 and 2. Specific fluorescence is present in the basement membrane and in the epithelial cytoplasm; no reaction is visible in the nuclei. × 550.

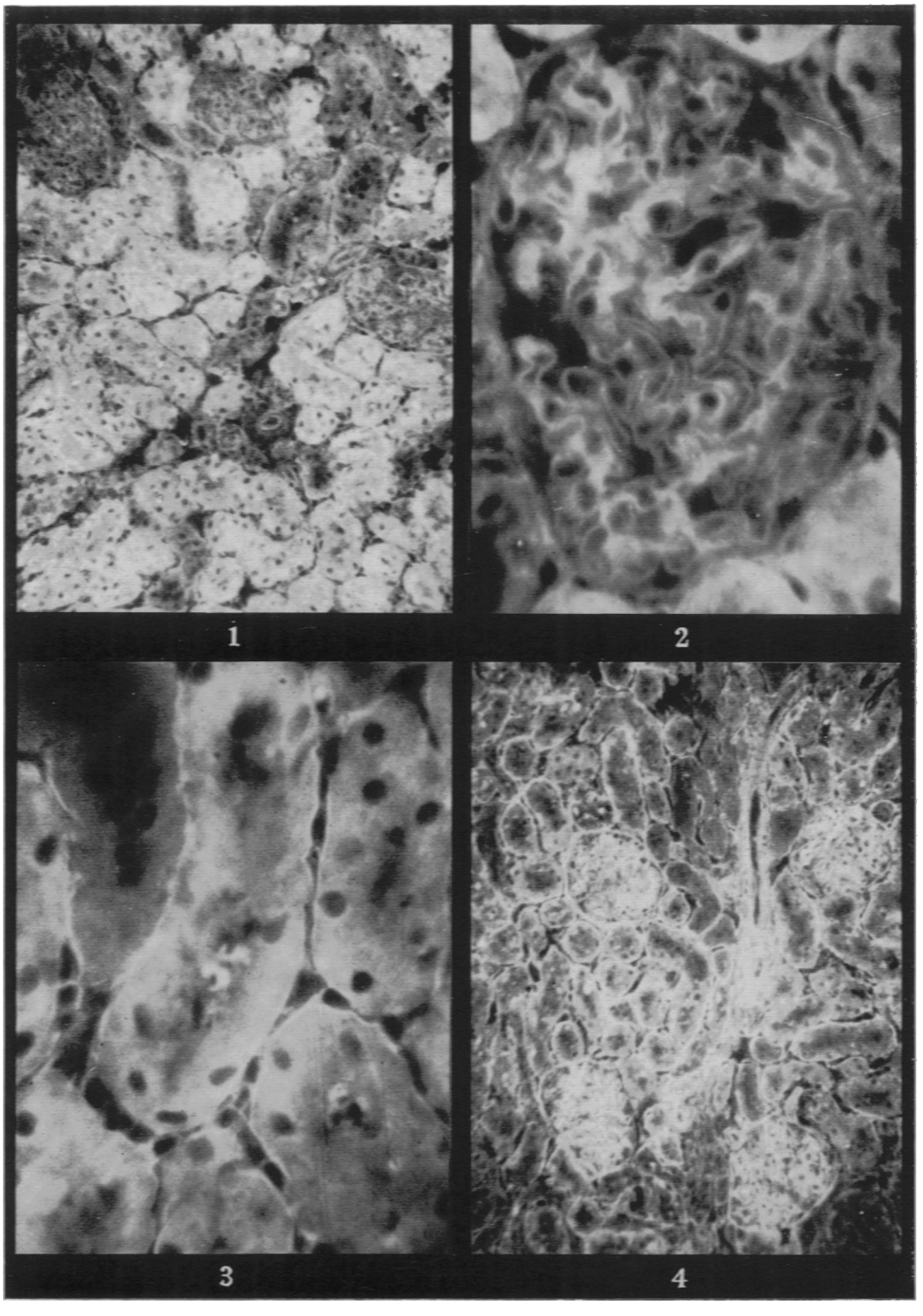
FIG. 4.—Treated with anti-glomerulus globulin. Specific fluorescence is present in the glomeruli and in the basement membrane of the convoluted tubules, but (in contrast to Fig. 1) the tubular epithelium is unstained. × 140.

FIG. 5.—High-power view of convoluted tubules from the preparation illustrated in Fig. 4. The presence of staining in basement membrane and its absence in the tubular cytoplasm is clearly seen (compare with Fig. 3). × 550.

FIG. 6.—Treated with anti-kidney globulin which had been absorbed with a homogenate of rat lung. Fluorescence in the tubular cytoplasm is similar to that seen in Fig. 2, but the glomeruli are unstained. × 160.

FIG. 7.—Section of kidney (stained with haematoxylin and eosin) from a rat killed 4 hr. after an intravenous injection of anti-glomerulus globulin. The two glomeruli show excessive cellularity (an appreciable number of polymorphs were identifiable under the microscope) and a considerable reduction in the number of red cells. × 300.

FIG. 8.—Section of kidney (stained with haematoxylin and eosin) from a rat killed 16 days after an injection of anti-kidney serum. The glomerulus shows generalised thickening of basement membrane with patchy necrosis and markedly reduced vascularity. A cast can be seen in an adjacent tubule. × 300



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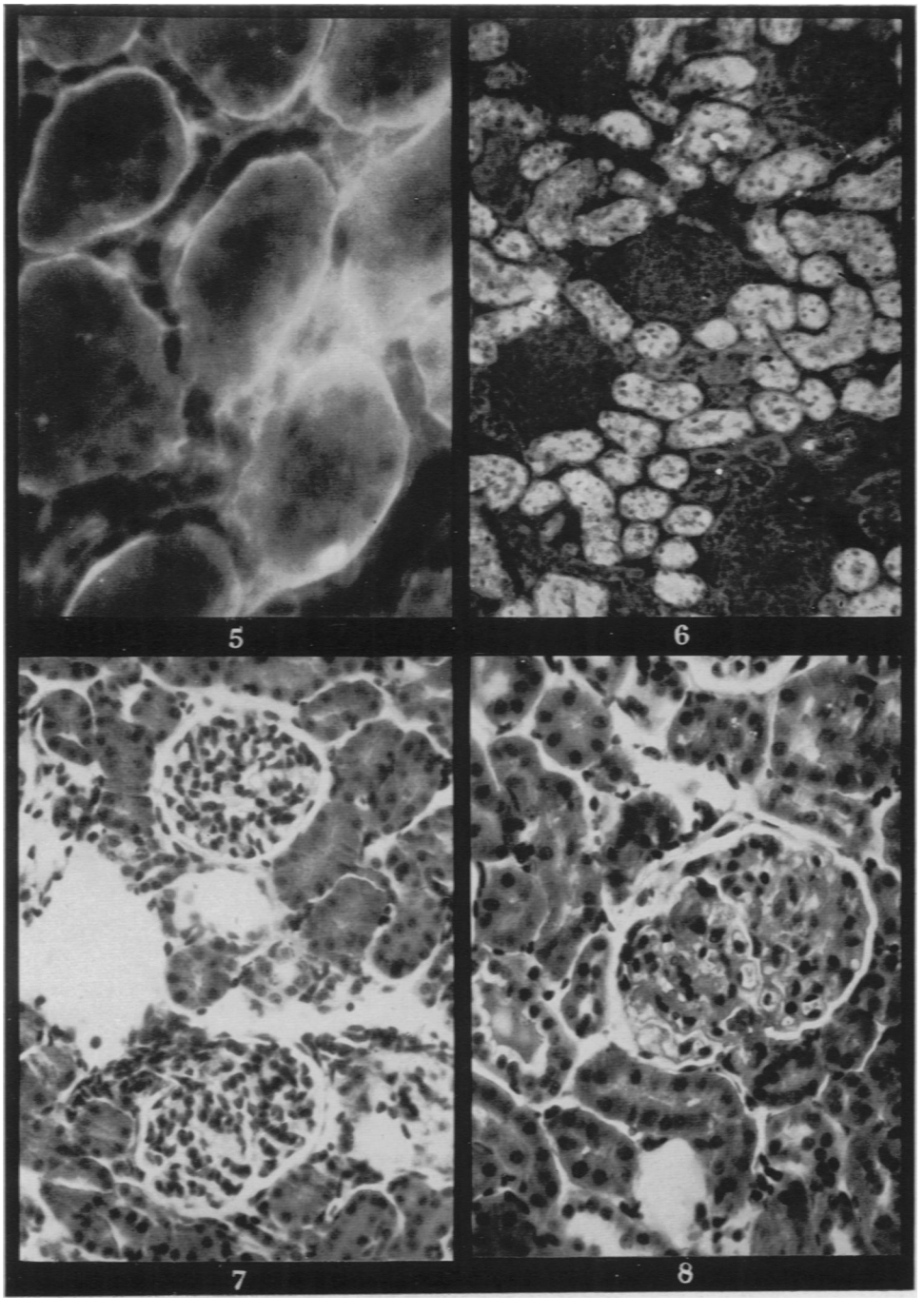

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The anti-kidney preparation reacted with basement membrane, both glomerular and tubular, with the cytoplasm of tubules and with a reticular structure in the media of vessels (Fig. 1–3). In the glomerular tuft a single bright green line outlined the capillary lumina. The endothelium was unstained, but the cytoplasm of the epithelium on the outer surface of the membrane had a fainter green colour. No second membrane was seen beneath this epithelium. The basement membrane of the capsule was also seen as a green line, thinner than that of the tuft. All the cortical tubules showed specific fluorescence, both in their basement membrane and in the cytoplasm of their epithelium : in the medulla, however, staining in these two sites was only occasionally present. It was not possible to identify the few medullary tubules with which the conjugate reacted. Capillaries elsewhere than in the tuft showed the same single fluorescent membrane outside the endothelium. Staining in the media of larger vessels took the form of a distinct reticular pattern surrounding the plain muscle fibres ; the elastic laminae were prominent by virtue of their natural bright bluish-white fluorescence and were readily distinguished from this specific green-staining network. No nuclear staining was seen.

The anti-glomerulus preparation reacted with glomerular and tubular basement membrane and with reticulin in the media of vessels in exactly the same way as the anti-kidney preparation ; unlike the latter it did not, however, react with the tubule cell cytoplasm (Fig. 4 and 5). The anti-lung preparation produced staining which was identical in pattern with that of sections stained with anti-glomerulus globulin but lower in intensity.

The specificity of the two types of antisera—anti-kidney on the one hand and anti-glomerulus or anti-lung on the other—was confirmed by the inhibition experiments. Thus unconjugated anti-glomerulus and anti-lung sera were able to inhibit the staining of basement membrane by anti-kidney conjugate but not the staining of tubule cells, whereas substitution of unconjugated anti-kidney serum resulted in inhibition of staining in all sites. The results of absorption experiments were equally consistent. Whole kidney suspension effectively removed all the staining properties of anti-kidney conjugate ; lung suspension, on the other hand, absorbed out only the basement membrane staining factor (Fig. 6). In all these experiments staining of reticulin in the media of vessels was retained or lost in parallel with basement membrane staining.

Apart from the serum inhibition and absorption experiments and controls with fluorescent normal rabbit globulin the specificity of the staining reactions was checked in various ways. It seemed necessary to eliminate the possibility that staining was simply due to a reaction between serum protein in the tissues and the corresponding antibody in the conjugates. The very weak precipitin reaction which the anti-kidney serum gave with rat serum made this unlikely, but it was thought desirable to prepare a fluorescent conjugate from a potent antiserum against rat serum proteins. This preparation produced some faint fluorescence in sections of rat kidney, but the pattern did not resemble that produced by the anti-organ preparations. The anti-kidney preparation strongly agglutinated rat erythrocytes, but absorption of the agglutinin from the conjugate left its reactivity with kidney tissue unchanged. Finally, to investigate the species specificity of the reaction, anti-rat-kidney conjugate was applied to mouse, guinea-pig, pig, rabbit and human kidney sections. Except in the case of the mouse no reaction occurred : in mouse kidney staining similar to that in the

rat was produced. An immunological cross-reaction between rat and mouse kidney has been noted previously (Pressman, 1949).

The ability of the anti-rat glomerulus serum to produce nephritis was established in an experiment based on the observations of Heymann and Hackel (1952). Rats weighing 50–65 g. were given a single intravenous injection and killed 4 hr. later. Whole anti-glomerulus serum (0·75 ml.) was used in one animal, globulin from the same serum (0·5 ml. of a solution containing 1·7 g./100 ml.) in 3 more, and normal rabbit serum (0·8 ml.) in 2 control rats. In the kidneys of all 4 animals injected with anti-glomerulus serum or globulin there was a considerable increase in the cellularity of the glomeruli, together with decreased vascularity, swelling of endothelium, patchy thickening of the basement membrane in some tufts and blurring of the outline of capillaries in others (Fig. 7). No changes were seen in the tubules except for distension with albuminous fluid in one animal. No pathological changes were found in the kidneys of the rats injected with normal rabbit serum. In other experiments massive proteinuria (up to 1·9 g./100 ml.) was induced by injections of anti-kidney or anti-glomerulus globulin ; in a section from a rat killed 16 days after an injection of the former there was generalised thickening of the basement membrane in the glomeruli with some patchy necrosis and markedly reduced vascularity (Fig. 8).

DISCUSSION.

The earlier experiments in this investigation, in which fluorescein-conjugated anti-kidney globulin was applied to sections of rat kidney, showed the feasibility of using such a conjugate as a histochemical reagent for visualising antigenic tissues. This reagent reacted, however, both with basement membrane (glomerular and tubular) and with the cytoplasm of tubular epithelium ; the nephrotoxic activity of the serum from which it was prepared could therefore not necessarily be attributed solely to an antibody against basement membrane. In order to establish the significance of such an antibody it was clearly essential to prepare a nephrotoxic serum which *in vitro* would react only with basement membrane. Substitution of isolated glomeruli for whole kidney suspension as the immunising material provided a serum which reacted in this way and was also capable of inducing nephritis when injected *in vivo*. Whether or not fixation of antibody by the basement membrane of tubules as well as by that of the glomeruli plays a significant part in the mechanism of this type of experimental nephritis must remain uncertain : early histological changes in glomerular basement membrane are well recognised, but corresponding changes in the basement membrane of tubules have not been described. The results now reported indicate that the epithelium of the cortical tubules contains an antigen distinct from the one which is present in basement membrane, but that serum may possess nephrotoxic activity in the absence of antibody against this tubule antigen. The anti-lung serum resembled the anti-glomerulus preparation in its reaction with kidney tissue sections, an observation consistent with the evidence, quoted in the introductory section of this report, that an identical or similar antigen is to be found in organs other than the kidney.

It would clearly be desirable to know more about the identity of the basement membrane antigen. Its chemical nature is largely speculative, though the reaction of glomerular and tubular basement membrane in the periodic acid—Schiff procedure suggests that it may contain a polysaccharide. This suggestion

is consistent with an observation made by Cole, Cromartie and Watson (1951), who prepared a tryptic digest of kidney suspension and found that it contained a soluble substance capable of absorbing kidney-damaging antibody from nephrotoxic serum. They suggested that the kidney antigen may be a complex molecule containing protein in combination with a polysaccharide or lipid.

It is well known that the immunological activity of anti-kidney serum measured *in vitro* by its reactivity with a supernate from a homogenate of kidney tissue bears little relation to its ability to produce nephritis (Smadel, 1936). If it is correct to conclude that such serum is nephrotoxic by virtue of its ability to react with renal basement membrane this observation is not surprising; the activity of serum assessed *in vitro* against such a supernate may well be independent of its content of basement membrane antibody, for the antigen in basement membrane is probably insoluble in aqueous media (Cole *et al.*, 1951). It is obviously an advantage to be able to demonstrate a reaction between a tissue antibody and an antigenic component of an organ, which may only account for a small proportion of the total bulk of the organ and which may well not be represented in a supernate from homogenised material. The introduction of antibodies labelled with a radio-active tracer into this type of work represented a notable advance. Pressman *et al.* (1949), using this type of preparation, were able to demonstrate fixation of anti-kidney globulin in the glomeruli, but not, apparently, to identify the exact site of the reaction within these structures, nor to demonstrate any reaction with tubular basement membrane. More precise histological identification of the site of activity of anti-kidney antibodies has proved possible in the present investigation, and the results obtained suggest that the fluorescein labelling technique may prove generally useful in the field of tissue antibody reactions.

SUMMARY.

The properties of antibodies prepared against homogenates of whole kidney, isolated glomeruli and lung from normal rats have been investigated. Fluorescein-labelled globulin from each of these antisera has been applied to unfixed sections of normal rat kidney and the sites of specific fixation determined by fluorescence microscopy.

Globulin from the anti-whole-kidney serum reacted with basement membrane in the glomeruli and convoluted tubules and also with the cytoplasm of the tubular epithelium. Antibody globulin prepared by immunisation with lung tissue or glomeruli also reacted with basement membrane, but failed, on the other hand, to react with tubular epithelium. It is concluded that kidney tissue contains at least two distinct antigens—one in basement membrane and the other in the cytoplasm of the convoluted tubules. By the injection of the anti-glomerulus serum into rats it has been shown that an antibody specific for the first of these antigens is capable of producing nephritis.

By the use of fluorescein-labelled antibodies as histochemical reagents it has been possible to locate these antigenic elements in normal renal tissue with considerable precision, and it is suggested that the method may be useful in the study of antibodies against other tissues.

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