THE HISTOLOGICAL LOCALIZATION OF RENIN WITH FLUORESCENT ANTIBODY

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THE precise site of production of the pressor enzyme renin is unknown. Since the suggestion by Munoz, Braun-Menendez, Fasciolo and Leloir (1939) that the renal pressor substance of Tigerstedt and Bergman (1898) is a specific proteinase, many attempts have been made to decide where it is produced in the kidney. It is absent from renal medulla but opinions differ widely about the probable component of cortex responsible for its formation.

Goormaghtigh's (1939) demonstration that the juxta-glomerular apparatus in ischaemic kidneys of rabbits and dogs shows microscopical changes suggesting secretory activity, led him to suppose that this structure produces a pressor substance. The hypothesis is supported by a finding of Marshall and Wakerlin (1949) that the amount of renin extractable from dog kidney is correlated with the granularity of the juxta-glomerular apparatus. On the other hand a relationship has also been observed between renin assay and tubular proliferation in developing foetal pig kidneys (Kaplan and Friedman, 1942). The further observation that renin could not be demonstrated in rabbit kidneys in which the proximal tubules had been selectively destroyed by the administration of sodium tartrate (Friedman and Kaplan, 1943) suggested to these authors that the proximal convoluted tubules are the site of renin production or storage. This fits with Taquini's (1950) finding from indirect assay of sections of kidney from the dog, cat, ox and pig, that renin was only demonstrable in the outer 1·5 mm. of cortex which consists of convoluted tubules. However, Yoshimura and Negishi (1954) in similar studies to those of Friedman and Kaplan, were unable to repeat their findings. Furthermore, Cook, Gordon and Peart (1956), by direct assay of sections of rabbit kidney, found renin only where there were glomeruli; there was none in the subcapsular zone. This work had been extended by Cook and Pickering (1958) who devised a magnetic filter to concentrate magnetised glomeruli obtained from kidneys perfused with a suspension of magnetic iron oxide; they found the highest concentration of renin in the fraction containing large glomerular fragments and attached renal tubular segments. Bing and Wiberg (1958) by microdissection and direct renin assay of cortical components of pig, rabbit and rat kidney, also concluded that the glomerular zone was richest in renin though none was detected in the capillary tufts themselves.

The known antigenic properties of renin (Johnson and Wakerlin, 1940) prompted us to try another line of investigation using the fluorescent antibody technique. A tissue antigen may be specifically identified microscopically by virtue of its affinity for the corresponding antibody labelled with a fluorescent

dye. The method, recently reviewed by Coons (1956) and Cruickshank and Currie (1958), has already been applied to the histological localization of normal tissue components, *e.g.* adrenocorticotrophic hormone in the pituitary (Marshall, 1951), kidney antigens (Hill and Cruickshank, 1953) and pancreatic enzymes (Marshall, 1954). The fact that pure renin has not yet been obtained presents difficulties in that a single specific antibody required ideally for the technique is unobtainable. Nevertheless, we have found that many, though not all, of the problems posed by the heterogeneous nature of the antigen and corresponding antiserum may be overcome by suitable serum absorptions. The sensitivity of the fluorescent antibody method is increased by the use of the " immunological sandwich method " in which specific antibody globulin applied to the corresponding tissue antigen is identified by means of a second layer of fluorescent serum antiglobulin. The extra sensitivity thus obtained has been further enhanced in the present experiments by applying the recently introduced technique with lissamine rhodamine B200 (RB 200) as the fluorescent tracer (Chadwick, McEntegart and Nairn, 1958*a* and 1958*b*). These refinements of method have enabled us to show that highly absorbed antirenin serum has an affinity for glomerular tissue which seems to be specific ; the histological findings suggest that the glomerular epithelium may be the site of renin production or accumulation in the renal cortex.

METHODS

The following steps were taken : (1) Preparation of purified renin extract from pig kidneys. (2) Production in rabbits of antiserum to the pig renin. (3) Removal of all precipitins from the antirenin serum by absorption with pig tissue powders, homogenates or renin extract. This left a proportion of the antirenin activity ; the renin/antirenin complex is non-precipitating (Lamfrom, Haas and Goldblatt, 1954) and remains in solution when the renin extract is used for precipitation. (4) Labelling of antiserum. Attempts at direct conjugation of the antiserum with either fluorescein (Coons and Kaplan, 1950) or with RB 200 resulted in a variable loss of antirenin activity, always more than 50 per cent and particularly serious here because the original titre was poor. The direct tracer technique was therefore abandoned in favour of the " sandwich method " with a potent anti-rabbit globulin prepared in a goat. Conjugates of this serum with RB 200 were used for the main study. Comparative experiments with fluorescein labelling gave similar results, but this tracer was found to be insufficiently sensitive for general use with the fully absorbed antirenin serum. (5) Fluorescence microscopy of sections of pig kidney and other organs treated successively with the rabbit antirenin serum and the RB 200-labelled goat antirabbit globulin.

Preparation of renin

The method of Haas, Lamfrom and Goldblatt (1953) was used. Sample (1) used as antigen for the preparation of the antirenin serum was a gift from Dr. Haas ; it had a potency of 90 Goldblatt units/mg. N_2. Sample (2) required for other purposes during the investigation, when the first was exhausted, had a potency of 33 units/mg. N_2.

Production of antirenin

Because pure renin extract was scarce, only two rabbits, adult female chinchills weighing 3 kg., were used to make the antibody; a usable antirenin titre was obtained in one animal after 2 immunizing courses with adjuvant. In the first course, three 27 unit doses of pig renin in Freund's adjuvant excluding *Mycobacterium tuberculosis*, were divided equally in subcutaneous and intramuscular sites and administered at 11 day intervals. Two months later, 5 further 27 unit doses of renin in saline solution were given intramuscularly at approximately 3 week intervals. Serum samples were tested before each inoculation for antirenin activity and for precipitins. The latter, unrelated to the non-precipitating antirenin and

attributable to renal components in the antigen other than renin, provided an extra index of the rabbit's immunological responsiveness to pig protein. The yield from this first immunizing course was poor; most serum samples showed 1 or 2 units of antirenin activity/ml. and only once did the titre rise to 4 units/ml.; one of the rabbits gave a consistently poorer response, about half that of the other rabbit.

After this first immunizing course, it happened that the rabbits were rested for a year and then they were given a second course beginning with 80 units renin in Freund's adjuvant including *Myco. tuberculosis*, intramuscularly, in multiple sites, followed a month later by 8 daily injections of 5 units renin in saline solution intraperitoneally. Five days after the final injection, the antirenin titre in the more responsive rabbit had risen to 20 units/ml., in the other to 10 units/ml.; the precipitin titre in both sera, tested with sample (2) renin at a concentration of 2 units/ml., was 1/20. Except for a few comparative studies, the serum with the higher antirenin titre has been used for all the experiments to be described. Antirenin assay was carried out by the method of Lamfrom, Haas and Goldblatt (1954) using, instead of dogs, " trained " rabbits kept quiet and warm while the blood pressure was recorded in the ear with a Grant capsule (Grant and Rothschild, 1934). The pressor response of the rabbits to renin of known strength, in Goldblatt dog units, was checked frequently and only animals giving consistent and reproducible results were used for bio-assay.

Immunological absorptions of antirenin serum

The precipitins in the antirenin serum tested against sample (2) renin in saline were readily identified by the ring test. They could be reduced by absorption of the serum with acetone-dried pig liver powder, serum powder, kidney medulla powder or wet packed homogenate of medulla; they were eliminated by absorption with kidney cortex powder or homogenate, or sample (2) renin at optimum precipitating proportions (11 units renin/ml. antirenin serum). Further investigation of the precipitins was carried out by simple diffusion of antigen (sample (2) renin, 2–10 units/ml.) into antiserum at 1/5 dilution in 1 per cent agar columns of 1·5 mm. bore (Oudin, 1952). Eight diffusion bands appeared in 48 hr. at room temperature; there was subsequent increase in complexity which could not be readily analysed. Four of the major bands, including the two with greatest density failed to appear when the serum had been absorbed with liver or kidney medulla powders; there were no diffusion bands after absorption of the serum with kidney cortex or renin at optimum precipitating proportions.

Exact determinations of the antirenin activity of absorbed sera were not always possible because of scarcity of serum, but the expected changes occurred. There was little or no reduction in antirenin titre after absorption with pig tissues other than kidney cortex which caused about a 65–80 per cent loss of activity; the acetone-dried cortex powder was more potent in this respect than the wet homogenate. Absorptions with renin at optimum proportions resulted in virtual arithmetical reduction in antirenin titre, *i.e.* removal of precipitins from 1 ml. of serum containing 20 units of antirenin with 0·28 ml. (11 units) of a solution of sample (2) renin, left 9 units (7 units/ml.) of antirenin activity in the supernatant.

In practice, most absorptions were carried out by shaking the sera with the pig tissue preparations (0·1 g./ml. for powders and 0·5 ml./ml. for homogenates) in a mechanical agitator for 1 hr. at room temperature, followed by centrifugation at 10,000 r.p.m. for 30 min. to recover the serum. Sometimes sera were absorbed more than once with the same or different tissue preparations; occasionally, in particular for the precipitation with renin extract, the reaction time was prolonged by incubation for 1 hr. at 37° and refrigeration overnight before centrifugation.

Fluorescent labelling of goat serum (anti-rabbit globulin)

The serum from a hyperimmune goat had an anti-rabbit globulin precipitin titre of 1/20 by the ring test against 1/1000 rabbit globulin; at a 1/5 concentration, it detected rabbit globulin at dilutions up to 1/10,000. Immediately after conjugation with RB 200, it was treated with powdered activated charcoal to remove dye not chemically attached to the serum proteins and then successively with the following acetone-dried pig tissue powders to remove non-specific antibodies: liver, kidney cortex and alcohol-denatured serum. The absorbed conjugated serum globulin was precipitated with ammonium sulphate (40 per cent saturation), the precipitate dissolved in a volume of saline equal to the original serum

volume, and the solution dialysed overnight against buffered saline at pH 7. This conjugate gave brilliant orange fluorescent staining with test bacteria coated with rabbit antisera in smears and sections but had no staining affinity for kidney tissue.

Preparation and microscopy of sections

Kidneys were taken from healthy adult male and female pigs within 20 min. of slaughter ; small blocks (5–10 mm. across and 2 mm. thick) of kidney were quick-frozen in containers immersed in alcohol dry-ice freezing mixture and stored at −20°. Frozen sections were cut at 5–6 μ in a low temperature freezing cabinet at −18° to −20°, and mounted on formol-gelatin coated slides (Coons and Kaplan). After drying for 1 hr. in a current of air, the sections were fixed in 95 per cent ethanol at room temperature. Fixation of the antigen is particularly important in the present experiments because of the need to locate an antigen-antibody complex which is soluble under normal conditions. Unfixed sections gave unsatisfactory ill-defined fluorescence with the unabsorbed antiserum and no significant fluorescence after the precipitins had been removed. To decide the best method of fixation, other fixatives including methanol, absolute ethanol, isopropanol and several other higher alcohols, acetone, ether and weak solutions of formalin were tried alone or in combination and at various temperatures. Of these only isopropanol gave satisfactory fixation without denaturation of test renin smears, permitting identification with fluorescent antibody ; but somewhat better results were obtained with 95 per cent ethanol and, though not an ideal protein fixative, this was adopted as a routine. After fixation, sections were again dried in a current of air at room temperature for 30 min. and were ready for staining. This was usually performed the same day, occasionally on the following day after storage of the sections at 0° ; longer storage sometimes gave anomalous results and old sections were therefore discarded. Sections were treated with a drop of antirenin serum for 60 min. in a damp container to reduce evaporation ; after rinsing and washing for 10 min. with buffered saline (pH 7) they were treated with RB 200-conjugated goat anti-rabbit globulin for 20 min., washed again in buffer for 10 min. and mounted in buffered glycerol. Sections prepared in the same manner from other pig organs including liver, lung, heart, and adrenal, and from kidneys of mice were also examined from time to time.

Fluorescence microscopy of the sections was carried out as soon as possible after mounting, using the Reichert equipment modified for ultraviolet-blue light with a yellow filter above the object (Nairn, Chadwick and McEntegart, 1958 ; Chadwick *et al.*, 1958*b*). Specific orange fluorescent staining of tissue antibody contrasted well with the fainter greenish-khaki background due to tissue autofluorescence. Absence of staining in tissues treated with (*a*) normal rabbit serum or (*b*) antirenin serum fully neutralized with renin antigen, provided tests of specificity.

RESULTS

These are summarized in the Table in which the staining of tissue sections treated with antirenin serum followed by fluorescent goat anti-rabbit globulin is a measure of the affinity of the serum for the tissues. The unabsorbed anti-

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EXPLANATION OF PLATE

Fluorescence photomicrography prints from " Super Anscochrome " colour transparencies.

FIG. 1.—Frozen section of pig kidney, fixed in 95 per cent ethanol and treated first with antirenin serum absorbed with pig liver powder, and then with RB 200-conjugated goat anti-rabbit globulin. Specific fluorescent staining is conspicuous in the cells of the glomrular tuft and capsule ; it is also just distinguishable in the peritubular ground substance and blood vessels. The fluorescence in the tubules is solely autofluorescence. × 120.

FIG. 2.—High-power view of similar preparation to Fig. 3 in which the antirenin serum had been absorbed with pig liver and kidney cortex powders. It shows a single glomerulus with less bright fluorescent staining than in Fig. 1, but the same cellular distribution can be made out. The specific cytoplasmic staining can be seen around non-fluorescent nuclei, and the fluorescence of the surface epithelium can be made out near the lower left corner. The bright yellow spots are fluorescent artefacts used as an aid to focusing. ×280.

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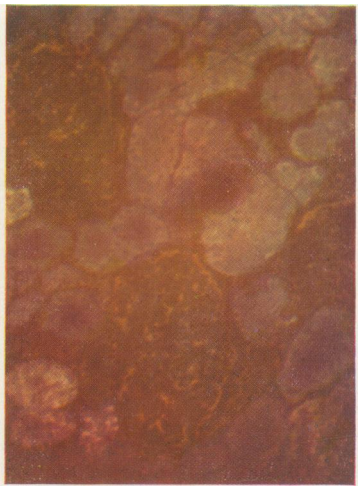

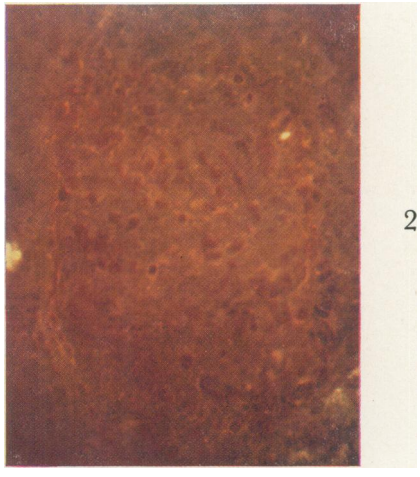

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TABLE.—*Reactions of Unabsorbed and Absorbed Antirenin Serum and of Normal Serum*

Absorptions with pig tissue preparations	Antirenin rabbit serum								Normal rabbit serum
				Liver powder +			Renin		Liver powder + kidney cortex homogenate
	Nil	Liver powder	Kidney medulla powder	Kidney medulla powder	Kidney cortex powder	Kidney cortex homogenate	Optimum precipitating proportions	Neutralizing antirenin activity	
Specific staining with labelled goat antirabbit globulin—									
Kidney glomeruli	+++	+++	++	++	±	+*	±	−	−
Tubules etc.	++	+	±	±	−	−	−	−	−
Other organs	+	±	±	v	v	v	v	v	(v)
Antirenin activity† (units/ml.)	18–20	15–20	..	12–20	3–5	5–8	5–8	0	0
Gel diffusion rings at 48 hr. against renin	8	4	4	..	0	0	0	..	0

* Staining of glomeruli unchanged by a second absorption with homogenate but blocked by neutralizing antirenin activity with renin (8 units renin/ml. serum).

† Only ranges available because of scarcity of material for bioassay.

v Slight and inconstant staining mainly lung and adrenal medulla.

renin serum had strong affinity for kidney tissue, especially the glomeruli, and also some affinity for control tissues (liver, lung, heart and adrenal), but the staining was diffuse and lacked definition. This lack of organ and tissue specificity is not unexpected because the antiserum clearly contains at least 8 precipitating components besides the non-precipitating antirenin. Removal of 4 of the precipitins by a single absorption with pig liver powder caused little or no diminution of antirenin activity but reduced tissue staining in the kidney and in the control organs. In the latter, staining was faint but now more sharply defined and mainly limited to blood vessels and plasma. In the kidney, staining of glomeruli remained conspicuous but elsewhere was diminished and mainly confined to the peritubular ground substance and blood vessels. The appearances in the kidney are illustrated in Figs. 1 and 2 : the specific glomerular fluorescence has the distribution of the cellular elements not the basement membrane. It is not possible to distinguish epithelial and endothelial cells in the centre of the glomerular tuft in these preparations, but the peripheral fluorescence is almost certainly located in the tuft epithelial cells and there are a few fluorescent cells in the position of the capsular epithelium. Thus whilst the fluorescence may be entirely epithelial, the possibility of mixed epithelial and endothelial distribution cannot be excluded. There was no suggestion of specific staining of the " juxtaglomerular " area.

The results after a single absorption with kidney medulla powder were similar except that the kidney staining showed a more uniform reduction, though glomerular fluorescence was still conspicuous. After successive absorptions with liver powder and kidney medulla powder much the same picture was seen in the kidney whilst the staining in the control organs was now only trivial, mainly confined to blood plasma in the adrenal medulla and lung. The minor staining in these latter organs was a variable finding after all double absorptions and could

be neglected ; it was reduced almost to extinction, without effect on the kidney staining, by a further absorption with denatured serum powder, and was presumably partly non-specific since it appeared occasionally in control preparations treated with non-immune rabbit sera. Such normal sera when fully absorbed with pig liver and kidney cortex powder did not induce glomerular fluorescence or any significant staining elsewhere in the kidneys or other organs ; unabsorbed normal sera were avoided for control experiments because they were associated with variable minor non-specific staining of all organs.

Absorptions of the antiserum with liver powder followed by kidney cortex powder or homogenate removed all precipitins and reduced the antirenin activity

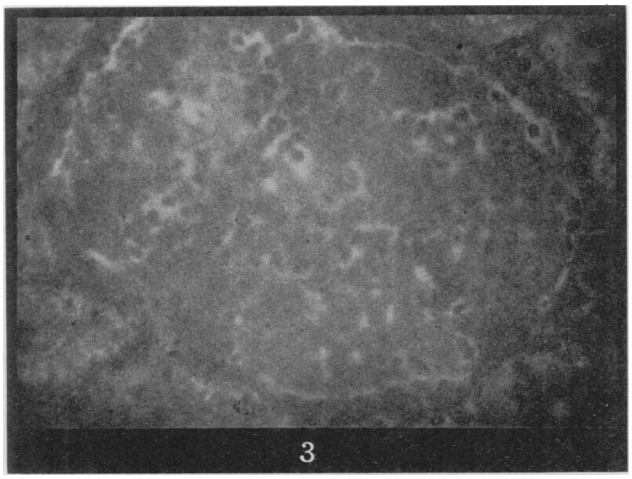

FIG. 3.—Black and white print from a "Super Ansochrome" colour transparency showing a single glomerulus in a similar preparation to Fig. 1. Cytoplasmic fluorescent staining can be seen around non-fluorescent nuclei and the fluorescence of the surface epithelium of the tuft is well shown. ×360.

to 3–8 units/ml. Tissue staining was also eliminated except in the kidney glomeruli where it was diminished but still recognisable (Fig. 3) ; there was no staining in the " juxta-glomerular " areas or in the tubules. The distribution of the glomerular fluorescence looked much the same as that induced by the partly absorbed antisera ; fluorescent cells corresponding in position to the tuft and capsular epithelium were again identified. The glomerular staining was not reduced by a second absorption with cortex homogenate, but when this thrice absorbed serum was treated with renin (8 units/ml.) to neutralize the remaining antirenin activity, its affinity for glomerular tissue was abolished. Similar results were obtained when the precipitins were removed from the serum by treatment with sample (2) renin at optimum precipitating proportions : the supernatant had an antirenin activity of 5–8 units/ml. and showed moderate and consistent affinity for glomerular tissue only. This affinity was blocked by neutralizing the antirenin activity with sample (2) renin.

Unsuccessful attempts were made to restore the glomerular affinity of renin-neutralized sera by recovery of antirenin with alkali : the serum was brought to

pH 11·3 for 1 hr. to detach and destroy the renin in the renin/antirenin complex, and then restored to pH 7 (Lamfrom *et al.*, 1954); in our hands, this resulted in only about 30 per cent recovery of antirenin activity. Absence of glomerular staining with such a reconstituted serum may perhaps be attributable to its final low antirenin titre combined with a possible blocking effect by the denatured renin which is still in the serum.

In brief, elimination of precipitins from the antiserum was associated with loss of general pig protein antibodies and of more specific kidney antibodies, but left a proportion of antirenin activity. This absorbed serum, when applied to kidney sections and followed by fluorescent anti-rabbit globulin, had the property of inducing faint but characteristic fluorescence limited to the cells of the glomeruli; the staining could be blocked by neutralising the antirenin activity of the serum with the calculated quantity of renin.

Mouse kidney sections treated with unabsorbed antirenin serum showed some generalized ill-defined staining which was decreased by absorption with pig liver and kidney cortex powders and abolished by further absorption with homologous tissue powder; the characteristic glomerular staining of the pig kidney was not seen. An attempt was made to introduce pig renin into mouse kidneys by the intravenous injection of 10 units of sample (2) renin and killing the mice at intervals up to 30 min. afterwards. Sections of these kidneys treated first with antirenin serum absorbed with pig liver powder and then with labelled goat anti-rabbit globulin, showed some specific fluorescent staining of the plasma; the staining was very faint but still detectable after re-absorbing the antirenin serum with pig kidney cortex powder. There was no sign of selective localization of the fluorescence in the renal tubules or glomeruli. This experiment is a further indication that the method is capable of locating the renin antigen at low concentration in the tissues.

DISCUSSION

The antibody in the antirenin serum, prepared against a purified sample of renin, has been shown to be predominantly anti-glomerular, as measured by the fluorescent tracer " sandwich " technique. The glomerular affinity of the serum, though reduced in parallel with the antirenin activity by immunological absorptions, was only abolished when this activity was completely neutralized. This is to say, the glomerular affinity and the antirenin activity of the serum could not be dissociated in these experiments. There are two possible explanations for this finding: either the glomerular affinity and the antirenin activity are due to the same antibody, or the serum contains, besides the antirenin, a non-precipitating anti-glomerular component with the same titre as the antirenin. The latter explanation is an unlikely one but cannot be excluded with certainty until either pure renin or pure glomerular proteins become available to carry out specific neutralizing blocking tests.

If it be accepted that the glomerular affinity and antirenin activity of the serum are likely to be due to the same antibody, then the specific glomerular fluorescence obtained with the absorbed serum may be attributed (*a*) wholly to renin, or (*b*) to glomerular protein molecules of which a proportion have renin activity, or (*c*) to glomerular protein, not renin, but indistinguishable immunologically from it. The first two of these hypotheses seem to be the more reason-

able : that the fluorescence obtained in the glomerular cells is an indication of the presence of renin.

The attempt to demonstrate specific fluorescence in mouse kidneys seemed worth making because information about species specificity of renin/antirenin reactions is incomplete and the possibility of a reaction between mouse renin and the antirenin prepared in the rabbit against pig renin, could not be predicted. In the event, the failure to obtain glomerular fluorescence, though an interesting finding, does not affect the conclusion already drawn from the findings in the pig kidney. On the other hand, the demonstration of faint specific fluorescence in the plasma of the mice that had been injected with the renin antigen provides further support for the view that the specific fluorescent staining of the pig kidney could also be due to the presence of this antigen.

We have not been able to determine by the present techniques whether the glomerular fluorescence obtained in pig kidney sections occurs only in the epithelial cells or whether it is also present in the capillary endothelium. It would be unwise to discount a possible secretory function of the endothelial cells since they have been shown by electron microscopy (Rhodin, 1958) to have a small Golgi zone and occasional α-cytomembranes, features usually associated with secretory activity as in pancreatic cells and mucus-producing cells. It might be feasible when a more potent antirenin serum becomes available to decide whether the endothelial cells in fact participate in the specific glomerular staining, by counterstaining the basement membrane of the tuft ; this could be done with a fluorescein-conjugated antiserum to pig reticulin.

SUMMARY

An antirenin serum (20 units/ml.) was prepared by immunizing rabbits with a purified extract of pig renin (90 units/mg. N_2) in Freund's adjuvant. Frozen sections of pig kidney, fixed in 95 per cent ethanol, were treated with the antiserum which becomes attached to the corresponding antigens in the tissue ; the antiserum was itself traced with a potent goat anti-rabbit globulin labelled with lissamine rhodamine B 200, and the sections were examined by fluorescence microscopy. The good contrast of the tracer with the tissue autofluorescence and the extra sensitivity of the " sandwich method " permitted the detection of specific fluorescence in the kidney after repeated immunological absorptions of the antirenin serum to remove all precipitins.

The fluorescent staining with unabsorbed antiserum was mainly glomerular and entirely so after removal of the precipitins. The glomerular fluorescence appeared to be located in the epithelial cells but the possibility of endothelial staining in addition, could not be excluded. Serum absorptions with pig tissue, including kidney cortex preparations, or with renin extract at optimum precipitating proportions, left a proportion of antirenin activity ; this antibody is non-precipitating. Glomerular staining was blocked by neutralizing the antirenin activity with renin extract ; absence of significant staining with fully absorbed antiserum in sections of pig liver, lung, adrenal and heart, and absence of kidney staining with normal rabbit serum provided further tests of specificity.

The results, which show that the antibody in the antirenin serum was predominantly anti-glomerular, suggest that renin is located in the glomeruli, probably in the epithelial cells. Whilst the evidence for this seems strong, it

cannot be conclusive with the present technique until pure renin becomes available to carry out a specific neutralizing blocking test.

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