COMPARISON OF REACTIONS OF ANTIBODIES TO RAT COLLAGEN AND TO RAT KIDNEY IN THE BASEMENT MEMBRANES OF RAT RENAL GLOMERULI*

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The pattern of immunofluorescence in the basement membranes of the kidneys appeared to be similar after antibody either to rat tail collagen (1) or to rat kidney (2) had been injected into the circulation of normal rats. Yet these antibodies reacted by complement fixation with different antigens and induced different microscopic renal lesions in rats (3).

In attempts to define the antigens with which these antibodies react, application of the antisera to frozen normal rat kidney sections was found to give satisfactory immunofluorescence. This in vitro method allowed pretreatment of the kidney sections with various enzymes to determine whether selective destruction would permit identification of the antigens. In addition, comparisons of the two antibodies were made by cross-absorption studies with subsequent immunofluorescence in normal rat kidney sections and in kidneys of rats injected with the antisera.

By comparing the antisera to rat kidney and to rat collagen, we have attempted to clarify their relationship to each other and to identify the antigens in the renal basement membranes with which each of these antisera reacts.

Materials and Methods

The preparation of purified acid-soluble rat tail collagen, rabbit anti-rat collagen sera, and the technique of the complement fixation tests have been described in detail (4). The anti-collagen serum had complement fixation titers of 1:256 to 1:512. Rabbit anti-rat kidney sera were prepared by intradermal and subcutaneous immunization of rabbits with a suspension of cortices from saline-perfused rat kidneys. The suspension was mixed, 2:1, with complete Freund adjuvant. Each rabbit was given 4 ml of the mixture in divided doses each week for 3 wk; blood was taken 5 days after the last injection. These antisera promptly induced severe proteinurea and nephrotoxic nephritis in normal rats.

For absorption of antisera with collagen, three parts of serum were mixed with one part of packed, washed, finely minced native rat tail collagen. For absorption with rat kidney, serum

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was mixed with lyophilized rat renal cortices in the proportion of 12 ml of serum to 1 g of kidney powder. These mixtures were incubated at 37°C for 2 hr with frequent mixing, stored overnight at 4°C, centrifuged in the cold at 3500 rpm for 10 min, and then at 40,000 rpm at 0°C for 90 min.

The rats used were young adults of the Long-Evans strain of both sexes, weighing 180 to 340 g.

Trypsin was a twice-crystallized salt-free preparation obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, containing 10,000 units/mg. The enzyme was dissolved in 0.01 M phosphate saline buffer, pH 8.0, 1 mg/ml.

Pepsin was a thrice-crystallized preparation obtained from Nutritional Biochemicals containing 2500 units (Anson method) per milligram. The enzyme was dissolved in 0.2 M HCl-KCl buffer, pH 2.2, 1 mg/ml.

Pronase was a highly purified preparation from *Streptomyces griseus*, obtained from Enzyme Development Corp., New York, containing 45,000 Pronase units (Kaken) per gram. This was dissolved in Veronal saline buffer without Ca^{++} or Mg^{++} ions, pH 7.1, 2 mg/ml, and was activated just before use by the addition of equal volumes of 0.005 M $CaCl_2$ so that the final enzyme concentration was 1 mg/ml.

Papain was obtained from Sigma Chemical Co., St. Louis, Mo., as a suspension of 16 mg of twice-crystallized enzyme in each ml. of 0.05 M sodium acetate, pH 4.5. Before use, this suspension was dialyzed against 0.02 M acetate buffer to bring the pH to 5.4. The papain was activated by adding an equal volume of 0.01 M L-cystine and 0.2% ethylene diaminotetracetic acid so that the final concentration of papain was 8 mg/ml.

Hyaluronidase from bovine testes was obtained from Worthington Biochemical Corp., Freehold, N. J., and contained approximately 300 USP units/mg. This was dissolved in Veronal saline buffer, pH 7.0, 2 mg/ml.

Collagenase was a lyophilized purified collagenase A preparation from *Clostridium histolyticum* containing 400 units/mg and was provided by the courtesy of Dr. Sam Seifter, Department of Biochemistry, Albert Einstein College of Medicine, New York. The absence of nonspecific proteolytic activity was determined by treating Group A streptococcal M protein with the collagenase (4); no serological activity of the M protein against its antibody was lost. The collagenase was dissolved in Veronal saline buffer, pH 7.2, and mixed with an equal volume of 0.01 M $CaCl_2$. The final concentration of collagenase was 200 units/ml.

Neuraminidase was obtained from several sources. A preparation from *Vibrio cholerae* (strain Z 4) was obtained from General Biochemicals, Chagrin Falls, Ohio, as a solution in 0.05 M acetate buffer, pH 5.5, with 500 units/ml; 1 unit liberates 1 μM sialic acid in 15 min at 37°C. This preparation was stated to be free of proteolytic, aldolase, and glucosidase activity. The buffer solution contained 0.1% $CaCl_2$. A preparation from influenza virus B was also obtained from General Biochemicals as a virus suspension in 0.05 M tris(hydroxymethyl) aminomethane-HCl buffer, pH 7.0, containing 100 units/ml.; the units were the same as those for the *V. cholerae* preparation. A preparation derived from *Clostridium perfringens* was obtained from Worthington Biochemical Corp., Freehold, N. J., as a dry powder containing 0.5 units/mg; 1 unit is equivalent to 1 μM N-acetylneuraminic acid per min/ml of substrate. The powder was suspended in acetate buffer, pH 5.0, 2 mg/ml. Another preparation from *C. perfringens* was obtained through the courtesy of Dr. Saul Roseman, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Md., as a solution in acetate buffer, pH 6.0, containing 2 of the same units per milliliter as used for the *C. perfringens* preparation above.

Chloromycetin was added to each enzyme solution as 0.1 mg of the dry powder per milliliter of solution.

When antisera were injected intravenously, they were first heated to 56°C to reduce primary toxicity. The serum was given in divided doses on 3 consecutive days with the volume of the first dose usually one-half that of the others to prevent fatal reverse anaphylaxis. The rats were

sacrificed 7 days after the last injection. The kidneys were perfused with cold saline before duplicate blocks of kidney were taken. One set was fixed in Zenker-formol for paraffin sections and stained with hematoxylin and eosin. The other was frozen in petroleum ether at −70°C. After frozen sections were cut in a cryostat at −20°C, they were treated with fluorescein-conjugated globulin from sheep immunized with rabbit globulin.[1] The details of preparing sections and examining and photographing them under ultraviolet light have been previously reported (1).

When antisera were applied to frozen kidney sections, normal rat kidneys were perfused and frozen sections prepared in the same way as those from serum-injected rats. Unconjugated anti-collagen serum, 0.1 ml., was put on the kidney sections for 1 hr at room temperature in a moist chamber. The slides were washed for 10 min in 0.01 M phosphate buffered saline, pH 7.2, and air dried. Conjugated sheep globulin, 0.1 ml., containing antibody to normal rabbit globulin was then applied for 30 min at room temperature. The slides were washed twice for 10 min each in the buffered saline, dried, and mounted in buffered glycerine, pH 7.2, under a cover slip. The globulin fraction of anti-rat kidney serum was conjugated with fluorescein isothiocyanate and applied to kidney sections for 1 hr at room temperature before washing and mounting.

Specificity of immunofluorescence was controlled by the blocking technique of Coons and Kaplan (5), using the appropriate unconjugated globulin. Heterologous conjugated rabbit anti-sheep globulin was also used as a fluorescence control.

EXPERIMENTAL RESULTS

Similarity of Renal Immunofluorescence of Antibodies to Rat Collagen and to Rat Kidney.—For comparison of the fixation of these two antibodies under the same conditions, each unconjugated serum was injected intravenously into three rats; 0.5, 1.0, and 2.0 ml, respectively, were given on each of 3 successive days. Frozen sections from the kidneys of these rats were then treated with fluorescein-conjugated antibodies to normal rabbit globulin. Under ultraviolet light, the basement membranes of the renal glomeruli were outlined by yellow-green fluorescence (Figs. 1 and 2). With both sera, the fluorescence at the site of the antigen-antibody reaction was a continuous, regular outline following the capillary loops. Little fluorescence occurred by this method in the basement membranes of the renal tubules in either preparation.

The immunofluorescence of these two antisera was also compared after direct application to frozen sections of normal rat kidney. This was done in two steps; the treatment with unconjugated antiserum was followed, after washing, by the conjugated sheep globulin containing antibody to rabbit globulin. Again, the fluorescence appeared the same for the two antisera (Figs. 3 and 4). By this method, however, not only the renal glomeruli but also the tubular basement membranes and Bowman's capsule were brightly fluorescent. This difference in the degree of fluorescent staining of the tubules is probably caused by differences in the amount of antibody reaching them.

Antibody to rat kidney was also used as the globulin fraction conjugated

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[1] The sheep serum containing antibody to rabbit globulin was supplied by the courtesy of Mr. L. L. Roach and Dr. Henry D. Piersma of Lederle Laboratories, Pearl River, N. Y.

with fluorescein isothiocyanate and applied to the kidney sections in a single step. The fluorescence obtained by this procedure was indistinguishable from that of the two-step method. Anti-collagen serum was not of high enough titer for satisfactory immunofluorescence using direct conjugation and the single-step method.

These studies show that, in the rat kidney, the immunofluorescence of the antibody to rat collagen and that to rat kidney, when applied by the same method, appears the same.

Immunofluorescence after Cross-Absorption of Antisera to Rat Collagen and to Rat Kidney.—Samples of anti-rat collagen and anti-rat kidney sera were each absorbed with the homologous and heterologous antigens, respectively, rat collagen and rat kidney cortex. Each of these absorbed sera were then tested by injection into the circulation of two rats, in three daily injections with a total volume of 5.5 ml. The unabsorbed sera were also tested at the same time in the same way. To sections from the kidneys of these rats conjugated antibody to rabbit globulin was applied. The renal glomerular basement membranes from rats given either anti-rat collagen or anti-rat kidney serum absorbed with the heterologous antigen showed fluorescence like that in rats given the unabsorbed serum. The rats given anti-kidney serum, unabsorbed or absorbed with collagen, developed proteinurea and the histopathologic findings of nephritis. However, homologous absorption of each antiserum completely removed the antibodies so that fluorescence did not appear and nephritis did not occur.

Absorbed sera were also applied directly to normal rat kidney sections. In this experiment the anti-rat kidney globulin had been conjugated before absorption. Again, the absorption of the anti-kidney globulin with rat kidney removed all detectable antibody (Fig. 5), and absorption of this globulin with collagen had no apparent effect on fluorescence in the glomerular and tubular basement membranes (Fig. 7) when compared with the unabsorbed globulin. The anti-rat collagen serum was tested by the two-step method and also showed removal of antibody by homologous absorption with collagen (Fig. 6) and no effect of heterologous absorption with rat kidney (Fig. 8).

To determine whether one of these antisera would block the other when applied to normal kidney sections, an additional experiment was done. Unconjugated anti-rat collagen globulin was applied to frozen sections of normal rat kidney for 2 hr at room temperature. After washing the section, conjugated anti-rat kidney globulin was applied for 30 min, and the slide was then washed and mounted. There was no blocking of the fluorescence, which was like that after the conjugated anti-kidney globulin alone. If the slide was treated initially with unconjugated anti-rat kidney globulin, blocking of the fluorescence was complete. It was not possible to determine whether the anti-rat kidney globulin would block the fixation of anti-rat collagen globulin because fixation of the

anti-collagen globulin could be detected only by the use of conjugated anti-rabbit globulin which would, of course, react with the rabbit globulin associated with either of these antibodies.

These experiments show that although, in the kidney, the immunofluorescence of both of these antibodies appears in the basement membranes, they react with different antigens.

Enzyme Pretreatment of Normal Rat Kidney Sections.—Treatment of normal rat kidney sections with purified enzymes before application of antibodies might alter the antigens with which these antibodies react and permit their identification.

A solution of each enzyme (0.1 ml.) was applied to a frozen normal rat kidney section and incubated at 37°C in a moist chamber. The slides were then washed, air dried, treated with fluorescein-conjugated globulin containing antibody to rat kidney or with rabbit serum containing antibody to rat collagen at room temperature in a moist chamber for 30 min. The slides treated with anti-collagen serum were further treated with fluorescein-conjugated antibody to rabbit globulin. The degree of specific fluorescence was determined under ultraviolet light. Each experiment included control slides treated with no enzyme and others treated with the corresponding heat-inactivated enzyme.

Renal glomerular basement membranes have been shown to consist of complex glycoprotein material, the major protein constituent of which is collagen or a collagen-like protein (6–8). Several proteolytic enzymes were therefore tested (Table I). Pepsin was found to act on the renal basement membranes so that subsequently neither antibody to rat kidney nor to rat collagen became fixed, and no fluorescence was visible. Trypsin, however, had no effect, and both antibodies were fixed as well as in untreated sections (Figs. 9 and 10). Pronase and papain are proteolytic enzymes of wide substrate specificity. Treatment with Pronase, like that with pepsin, altered the membranes so that no fixation of either antibody occurred (Figs. 11 and 12). Slides treated with papain showed diminution of immunofluorescence of both antibodies (Figs. 13 and 14).

Bovine testicular hyaluronidase acts on the mucopolysaccharides, hyaluronic acid and chondroitin sulfate A and C (9). Treatment of kidney sections with this enzyme did not affect the subsequent fixation of either antibody (Table I, Figs. 15 and 16).

Highly purified collagenase, free of nonspecific proteolytic activity, acts only on collagen and gelatin. This enzyme acted on the collagen component of the renal basement membranes so that no antibody to collagen became fixed and specific fluorescence was absent (Table I, Fig. 20). However, it had no effect on fixation of the antibody to rat kidney (Fig. 19).

Neuraminidase is an enzyme which splits sialic acid from glycoproteins. Some slides treated with neuraminidase derived from *V. cholerae* had dimin-

ished immunofluorescence, slightly greater in some of those treated with antibody to rat kidney than with antibody to rat collagen. However, a repetition of these experiments, using neuraminidase from influenza virus and two preparations from *C. perfringens* showed no effect on fixation of either antibody (Table I, Figs. 17 and 18).

Treatment with collagenase completely prevented fixation of antibody to rat collagen and had no effect on fixation of antibody to rat kidney. The other

TABLE I

Immunofluorescent Reactions of Antibodies to Rat Kidney or to Rat Collagen in Basement Membranes of Normal Rat Kidney Sections Previously Treated with Enzymes

No. of experiments	Enzyme treatment		Specific immunofluorescence*	
	Enzyme	Duration	Antisera prepared against	
			Rat kidney	Rat collagen
		hr		
	None (controls)‡		++++	++++
3	Trypsin	3½	++++	++++
3	Pepsin	3	-	-
3	Pronase	3	-	-
3	Papain	18	++ to +++	++ to +++
3	Hyaluronidase	18	++++	++++
4	Collagenase	8 to 15	++++	-
	Neuraminidase from:			
5	*V. cholerae*	12 to 18	+ to +++	++ to ++++
3	Influenza virus	18	++++	++++
7	*C. perfringens*	18	++++	++++

* Intensity and extent of fluorescence in renal glomerular and tubular basement membranes is indicated on a ++++ to ± scale; - indicates no specific fluorescence.

‡ Each experiment included, as controls, slides not treated with enzymes and others treated with heat-inactivated enzyme; all of these controls showed ++++ fluorescence.

enzymes tested did not act differently on the antigens with which these two antibodies react, although some of them diminished or prevented antibody fixation.

DISCUSSION

Much confusion has arisen because of the similarity of immunofluorescence in the kidney and other organs when antibodies to kidney and to collagen are injected into rats. Antigens from which anti-kidney nephrotoxic serum can be prepared are present not only in various parts of the kidney, but also in placenta (10), lung (11), and liver (12). Cruickshank and Hill (13, 14) found by immunofluorescence that anti-glomerulus globulin reacted in vitro with epithelial and vascular basement membranes and with reticulin fibers in spleen,

lymph nodes, thymus, sarcolemma, and neurilemma, but not with collagen fibers. When injected intravenously into rats, anti-kidney serum has been found by immunofluorescence in kidney, adrenal, ovary, and spleen, and also, in lower concentrations, in thyroid, lymph node, and liver, but not in testes, lung, skin, brain, or heart (15). This distribution differs from that of injected anti-collagen serum which was found widely distributed in heart, lung, liver, spleen, adrenal, kidney, jejunum, lymph node, thymus, joint synovia, peripheral nerve, aorta, skeletal muscle, eye, meninges, and choroid plexus at sites where collagen and reticulin are normally present (16).

Both the anti-collagen and anti-kidney sera give uniform linear fluorescence along the glomerular capillary outlines and are indistinguishable. This fluorescence differs from the irregular granular appearance of the capillary wall which is seen when antigen-antibody complexes are deposited in the kidney, as, for example, in serum sickness nephritis (17). The specific site of reaction of ferritin-conjugated nephrotoxic antibody has been demonstrated by electron microscopy within the renal glomerular basement membranes (18–20).

Injections of nephrotoxic antibody cause an immediate proteinurea and acute and chronic glomerulonephritis and frequently, also, the nephrotic syndrome. Antibody to collagen causes no renal change in normal rats unless they have been prepared with Freund adjuvant, when they develop a delayed type of renal lesion (3).

The antigen to which the nephrotoxic antibody is directed is still unidentified. However, a fraction of the protein in the urine of rabbits with nephrotoxic nephritis and also of normal rabbits has been reported to react with anti-glomerular basement membranes serum (21). These urinary proteins may make possible the chemical identification of the renal antigen or antigens to which the nephrotoxic sera are directed.

The presence of collagen in renal glomerular basement membranes has been inferred from the finding of large amounts of hydroxyproline (22–24). Recent studies on the chemical composition of renal glomerular basement membranes have shown that they consist of glycoprotein with large amounts of collagen or collagen-like protein, hexosamines, sialic acid, neutral sugars, and small amounts of lipids, but no mucopolysaccharides (6–8). Although collagen fibers with typical periodicity have not been demonstrated in normal renal basement membranes by most workers, they have been shown in the frog by Yamada (25) and in the rat by Latta (26). Kefalides has recently isolated collagen from canine glomerular basement membranes which he was able to reconstitute in the form of fibers of variable dimensions (27). Immunologic studies have also demonstrated, using antibody directed specifically to collagen, that collagen is present in these membranes (1).

Enzymes have been used in the past in attempts to identify the nephrotoxic antigen (13, 14, 28) and in studies of the composition of the renal basement membranes (24). The method of immunofluorescence in kidney sections pro-

vided an opportunity for the use of enzymes on basement membrane antigens *in situ*. Although some of the proteolytic enzymes of wide substrate specificity acted on the antigens and others did not, neither they nor hyaluronidase or neuraminidase differentiated the antigens to which the antibodies to rat kidney or collagen are directed. However, collagenase sharply differentiated these antigens: collagen was hydrolyzed so that its specific antibody no longer reacted with it, and no fluorescence was subsequently visible in the basement membranes; the antigen with which the anti-kidney sera reacted was not affected, and fluorescence was bright.

The best evidence that these antigens are not related are the cross-absorption studies. In an earlier study using complement fixation, we were able to show that purified rat collagen did not react with antisera against rat kidney or glomeruli, but an emulsion of whole rat kidney fixed complement slightly with antisera to rat collagen (3). Native collagen in the whole kidney emulsion was considered to be the cause of this slight cross-reaction. It seems probable that complement fixation is more sensitive than the immunofluorescent reaction, which showed no cross-reactions when the sera were tested in vivo or in vitro. Furthermore, the anti-kidney serum retained its nephrotoxic potency after absorption with collagen. The antibody to rat collagen and that to rat kidney are apparently directed to different antigens, both of which are present in the renal basement membranes.

SUMMARY AND CONCLUSIONS

By in vivo and in vitro methods of immunofluorescence, antibody to rat collagen and to rat kidney show the same regular, linear fluorescence following the outlines of the renal glomerular capillaries. Absorption of each antiserum with its homologous antigen completely removed the antibody for immunofluorescence, while absorption with the heterologous antigen had no effect. The nephrotoxicity persisted in the anti-kidney serum absorbed with collagen.

By pretreatment of frozen normal rat kidney sections with various enzymes followed by immunofluorescence, it was shown that trypsin and hyaluronidase had no effect on the subsequent fluorescence of either antibody; papain reduced the fluorescence; and pepsin and Pronase acted on both antigens so that no fluorescence was present. One preparation of neuraminidase, derived from *V. cholerae*, reduced fluorescence of both antibodies in some preparations, but the same enzyme derived from influenza virus or *C. perfringens* had no effect on either. Collagenase completely prevented fluorescence of the antibody to collagen and had no effect on that to rat kidney.

The findings in this study show that the antibody to collagen is directed to collagen in rat renal glomerular basement membranes and that the antibody to rat kidney reacts with some antigen other than collagen in these membranes.

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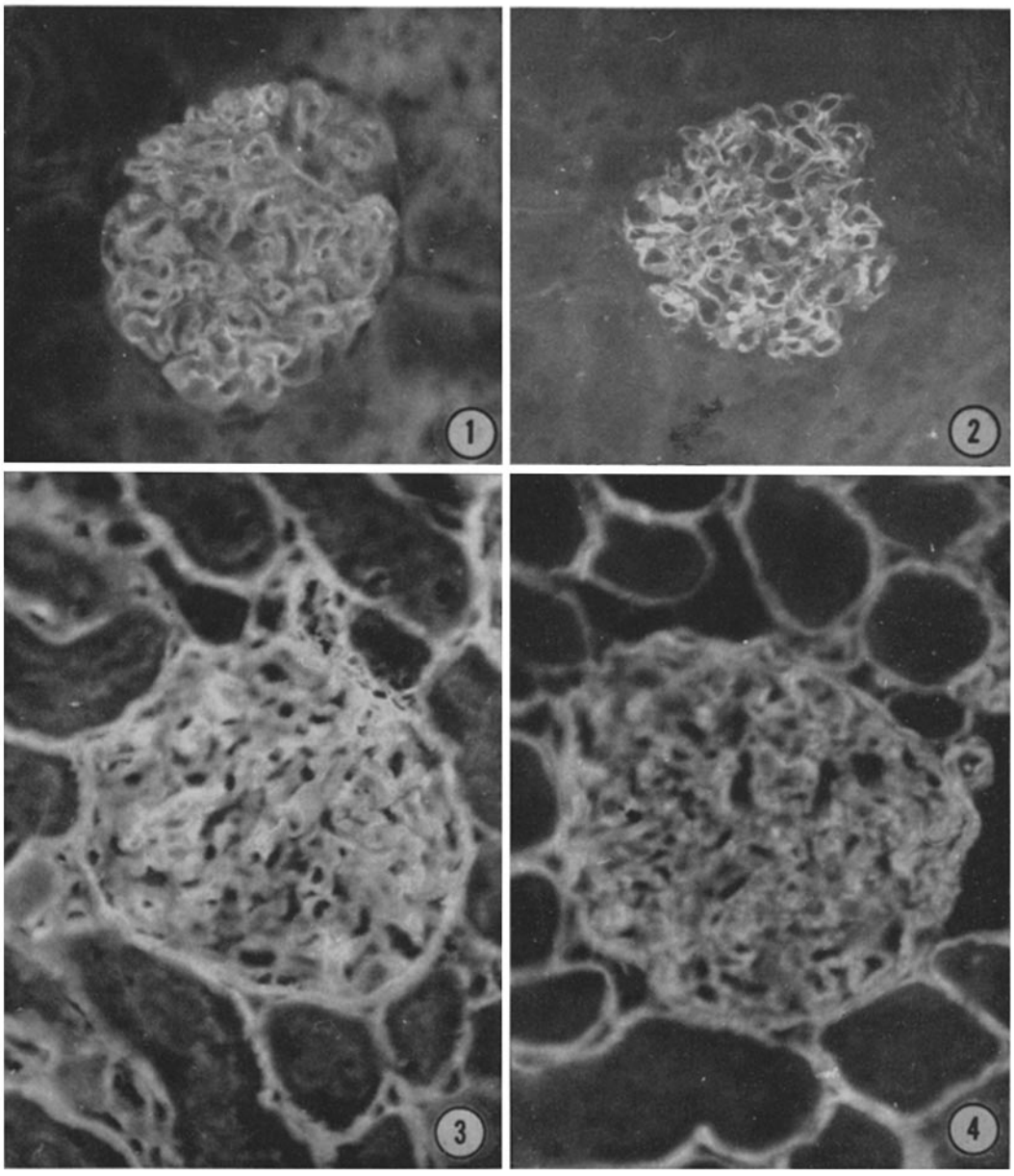
FIG. 1. Kidney section from a normal rat injected intravenously with rabbit anti-rat kidney serum was treated with fluorescein-conjugated sheep globulin containing antibody to rabbit globulin. The fluorescence at the site of antigen-antibody reaction shows a regular linear pattern outlining the glomerular capillaries. Bowman's capsule and the tubular basement membranes show no fluorescence. ×357.

FIG. 2. Kidney section from a normal rat injected intravenously with rabbit anti-rat collagen serum was treated with the fluorescein-conjugated antibody to rabbit globulin. The fluorescence, like that in Fig. 1, outlines the glomerular capillaries. ×333.

FIG. 3. Normal rat kidney section was treated with rabbit anti-rat kidney serum and then with the conjugated anti-rabbit globulin. The regular linear pattern of fluorescence again outlines the glomerular capillaries and also Bowman's capsule and the tubular basement membranes. ×333.

FIG. 4. Normal rat kidney section was treated with rabbit anti-rat collagen serum and then with the conjugated anti-rabbit globulin. The fluorescence is like that shown in Fig. 3. ×357.



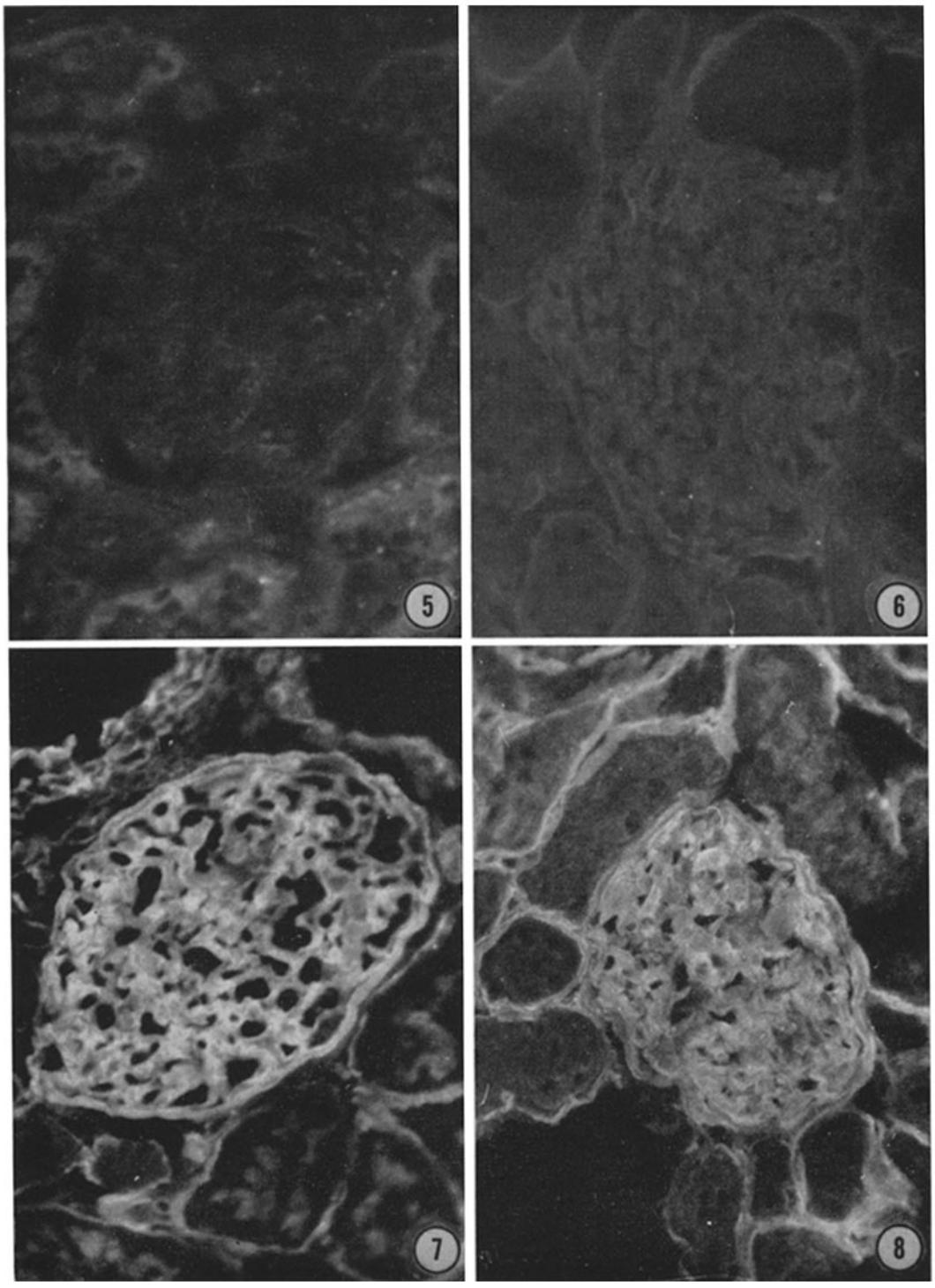
FIGS. 1–4.

FIG. 5. Normal rat kidney section was treated with conjugated anti-rat kidney globulin which had been absorbed with rat kidney. The absence of fluorescence shows that the antibody had been removed by the homologous absorption. ×333.

FIG. 6. Normal rat kidney section was treated with anti-rat collagen serum which had been absorbed with collagen. No specific fluorescence is present after this homologous absorption, but the outlines of a glomerulus and tubules can be seen. ×375.

FIG. 7. Normal rat kidney section was treated with conjugated anti-rat kidney globulin which had been absorbed with collagen. The heterologous absorption failed to remove the antibody, so that strong immunofluorescence is evident. ×333.

FIG. 8. Normal rat kidney section was treated with anti-rat collagen serum which had been absorbed with rat kidney. The immunofluorescence has not been diminished by the heterologous absorption. ×357.



FIGS. 5–8.

FIG. 9. This normal rat kidney section was treated with trypsin before the application of anti-rat kidney serum. The trypsin treatment has not altered the fluorescence. ×357.

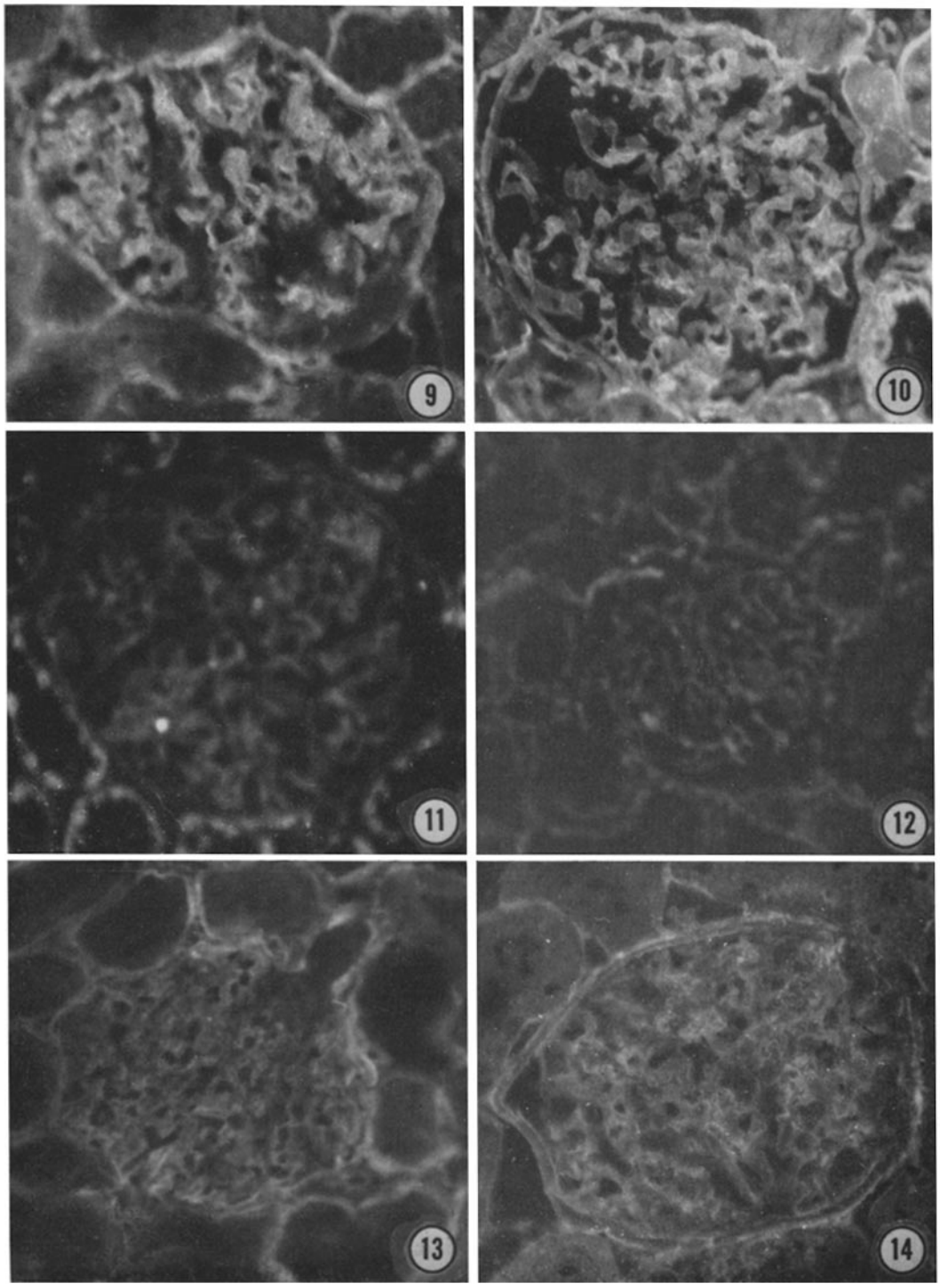
FIG. 10. This normal rat kidney section was treated with trypsin before the application of anti-rat collagen serum, and, as in Fig. 9, the fluorescence is unaltered. ×357.

FIG. 11. Pronase was used to treat this normal rat kidney section before the application of the anti-kidney serum. Here, as in sections treated with pepsin, fluorescence is absent except for a few small particles showing weak fluorescence. ×375.

FIG. 12. Pronase was also used to treat this normal rat kidney section before the application of anti-collagen serum. As in Fig. 11, fluorescence is almost completely absent. ×357.

FIG. 13. Papain pretreatment of this normal rat kidney section before the anti-kidney serum has uniformly reduced the brightness of the fluorescence, but the glomerular capillaries and tubules are still outlined. ×333.

Fig. 14. Papain pretreatment of this rat kidney section before the anti-collagen serum has reduced the fluorescence in the same way as in Fig. 13. ×333.



FIGS. 9–14.

FIG. 15. This normal rat kidney section was treated with hyaluronidase before treatment with anti-kidney serum. The immunofluorescence appears unaltered. ×357.

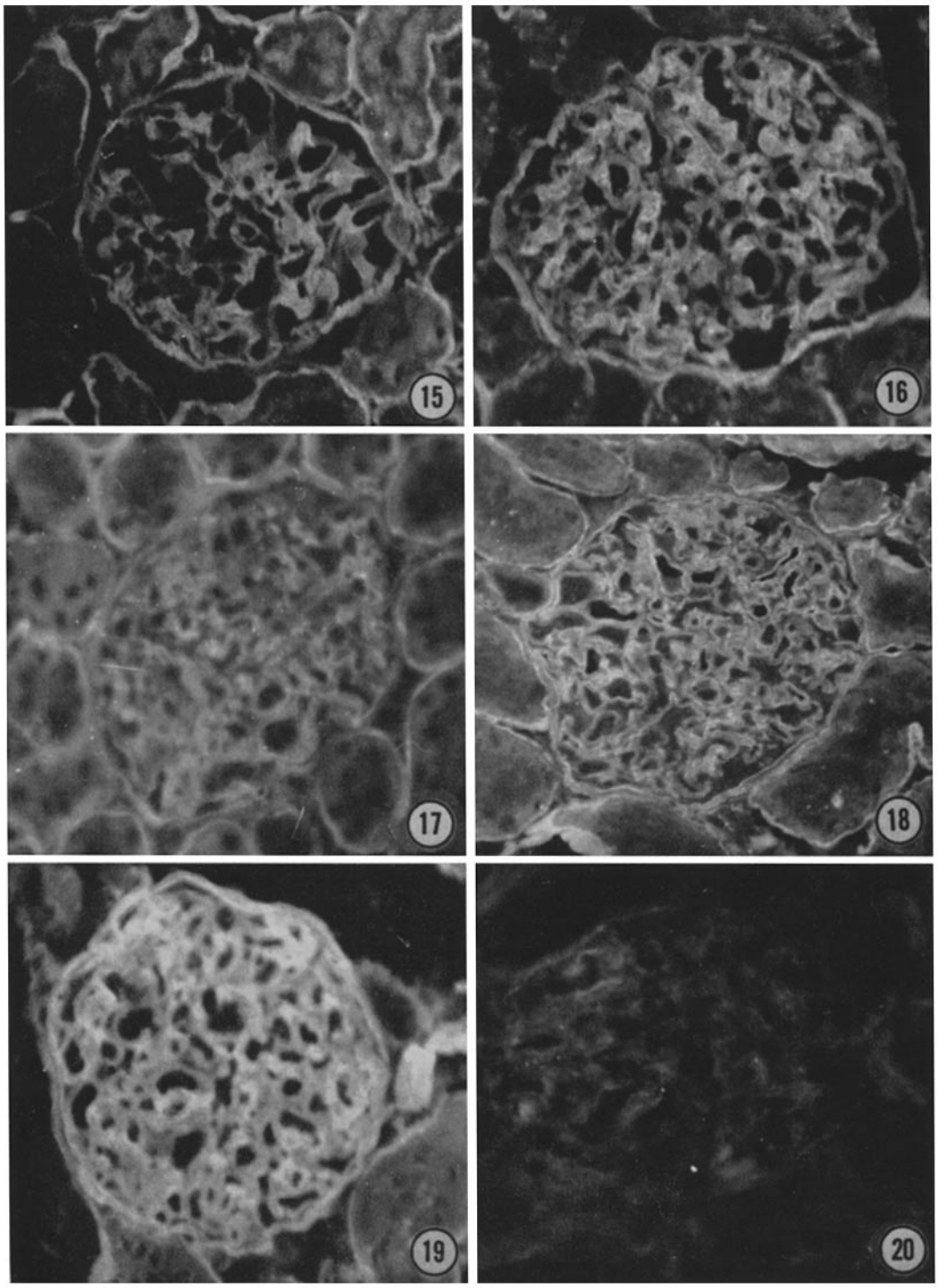
FIG. 16. A normal rat kidney section was treated with hyaluronidase and then with anti-collagen serum. As in Fig. 15, the fluorescence is not altered. ×357.

FIG. 17. A normal rat kidney section was treated with neuraminidase from *C. perfringens* before anti-kidney serum. The fluorescence does not appear to be reduced ×320.

FIG. 18. This normal rat kidney section was treated with neuraminidase from *C. perfringens* and then with anti-collagen serum. Fluorescence does not seem altered. ×333.

FIG. 19. Collagenase was used to treat this rat kidney section before anti-kidney serum was applied. The fluorescence has remained bright. ×333.

FIG. 20. Collagenase was used to treat this rat kidney section before anti-collagen serum. In contrast with Fig. 19, fluorescence is almost completely absent. ×333.



FIGS. 15–20.