ANALYTICAL PATHOLOGY

IV. The Role of Localized Antibodies in the Pathogenesis of Nephrotoxic Nephritis in the Rat*

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Plates 3 to 8

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Studies of the mechanism of experimental nephrotoxic nephritis (1–8) have been concerned largely with: (*a*) the specificity of the nephrotoxins, (*b*) their sites of localization, especially in the kidney, and (*c*) the presence and the role of immune responses in the nephritic animal. In recent years a considerable body of direct evidence relating to these problems has been furnished by the use of labelled antibody techniques, first applied to this field in the form of radioiodinated nephrotoxins by Pressman *et al.* (4, 5, 9).

Among the results achieved by this group was the demonstration that nephrotoxic γ-globulins are preferentially absorbed by the kidney, where they localize, at least in greater part, in the glomeruli, and that the radioactive tracer and, presumably, the intact antibody persist for up to 2 months at the sites of tissue localization. Recently, the potentialities of tracer methods have been more fully realized with the introduction by Mellors *et al.* (10) of fluorescein-coupled antibodies as histochemical reagents for the detection of tissue-localizing antibodies *in vivo*. This new application of the technique developed by Coons *et al.* (11, 12) not only makes it possible to define the sites of localization of nephrotoxic antibodies with a hitherto unattainable precision, but for the first time it permits the demonstration of deposits of autogenous antibodies as well. A highly advantageous characteristic of the technique is that it detects antigenic and, therefore, relatively intact antibodies only.

In a series of papers on experimental and human nephritis studied by this method, Mellors *et al.* (10, 13, 14) have unequivocally confirmed the selective glomerular localization of nephrotoxins in the rat kidney, indicating also that it is primarily membranous, and they have demonstrated the presence of glomerular localizing autogenous antibodies in a foreign protein nephritis of rabbits and in several types of human nephritis. The present investigation is an extension of the work dealing with nephrotoxic nephritis in the rat.

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Materials and Methods

Preparation of Nephrotoxic Sera.—Saline-perfused rat kidneys were collected from 60 adult male rats of the Sherman strain and homogenized in a Waring blendor. The insoluble renal substance was isolated by repeated centrifugation and resuspension in saline until the supernate was clear, and then lyophilized. 5.5 gm. of this kidney sediment was resuspended in saline and injected intraperitoneally into 5 adult male albino rabbits, each animal receiving 100 mg. 3 times a week for a total of 11 injections. At 1, 2, and 4 weeks after the last injection, between 40 and 50 cc. of heart blood was withdrawn from each rabbit, allowed to clot at room temperature, and synerese at 5°C. for 24 hours. The unhemolyzed sera obtained from these bleedings were pooled and fractionated by precipitation of the antibody-active protein with cold alcohol (precipitate A of the procedure outlined by Deutsch (15)). After dialysis against saline, this crude γ-globulin fraction was lyophilized and stored at 5°C. until used.

Preparation of Fluorescein-Coupled Antibodies.—Antisera against rat γ-globulin were prepared by injecting each of 10 adult male albino rabbits with 50 mg. of the lyophilized initial precipitate obtained by cold alcohol fractionation of fresh Sherman rat sera. Each dose of the rat antigen was dissolved in 1.5 cc. of saline, emulsified in 3.5 cc. of Freund's adjuvants, and injected in equal fractions into 5 intramuscular sites. The rabbits were bled by cardiac puncture at 4, 6, and 8 weeks after injection, their sera pooled, alcohol-fractionated as above, and lyophilized. When precipitin tests revealed high titers of antibody against the rat antigen, a suitable amount of the rabbit γ-globulin was coupled to fluorescein by the method of Coons and Kaplan (12). After merthiolate 1:10,000 was added as a preservative, the coupled antibody solution, hereafter designated Rab-anti-Rat, was stored at 5°C. in tightly stoppered test tubes shielded by aluminum foil.

Fluorescein-coupled rabbit anti-human γ-globulin (Rab-anti-Hum) was prepared in the same manner, the antigen in this case being purified human γ-globulin.[1]

The reagent used for the demonstration of rabbit nephrotoxins was kindly supplied to us by Dr. Barbara K. Watson. In its preparation, rabbit globulins, obtained by precipitation of normal rabbit serum with half-saturated ammonium sulfate, were injected into a goat, and the resulting goat antibodies also isolated by ammonium sulfate precipitation. The fluorescein conjugation, preparation, and storage of this reagent (Goat-anti-Rab) were the same as outlined for Rab-anti-Rat.

Experimental Procedure.—Nephrotoxic nephritis was produced in 3 groups of male Sherman rats weighing 250 to 330 gms. Each animal received 3 doses of 40 mg. of the lyophilized nephrotoxic γ-globulins dissolved more or less completely in 1 cc. of saline and administered into the tail veins at 24 hour intervals. It was not necessary to reduce the "primary toxicity" of this material by preliminary heating as recommended for nephrotoxic sera (16).

Of the first group of 20 rats, hereafter referred to as Group II for reasons of chronology outlined below, 5 were sacrificed at 9 days, 5 at 2 weeks, 4 at 3 weeks, 2 at 4 weeks, and 2 at 5 weeks after the first injection of rabbit nephrotoxins. Two rats in this group died during the course of observation, and these were not included in the study of antibody localization.

Four months later, 2 additional groups of rats were injected with the same nephrotoxins in order to provide information on renal changes occurring earlier and later than those found in the initial series. There were 8 animals in the short term group (Group I)—2 each being sacrificed at 2½, 3, 4, and 6 days after the first intravenous injection.

Group III was composed of 2 rats that were permitted to survive to 13 and 15 weeks after their first injection.

Thirteen rats served as controls, of which 3 received 3 intravenous injections of 1 cc. of normal rabbit serum at 24 hour intervals, and the rest were untreated. The injected controls

[1] Kindly provided by the American Red Cross.

were sacrificed 24 hours after the last injection; the others in pairs, at approximately weekly intervals corresponding with the sacrifice schedule of Group II.

All the rats were fed a standard laboratory diet composed of Purina pellets and water *ad libitum*. Weights of each were recorded at the beginning and end of observation, and once a week for each week of survival. They were sacrificed under nembutal anesthesia by exsanguination from the vena cava while simultaneously being perfused with physiological saline through the abdominal aorta. Perfusions were continued until the kidneys were free in the gross of blood—whereupon these organs were removed, weighed, and blocked. A portion of each kidney was fixed in 10 per cent neutral formalin and the remainder wrapped in wax paper, frozen in powdered dry ice, and further wrapped with layers of plastic sheeting and aluminum foil. The frozen blocks were stored at −15°C. until used.

Urinary Protein Determinations.—24-hour urine samples were collected from each rat in Group II and from their corresponding controls once a week for each week of survival; from one of each pair of animals in Group I and the three control rats injected with normal rabbit serum in the 24 hour period preceding sacrifice; and once during the first and last weeks of survival from the Group III rats. During the collection period, the animals were placed in metabolism cages containing water but no food. The urinary protein concentration was determined by treating appropriate dilutions of filtered urine with 3 per cent sulfosalicylic acid and comparing the turbidity with a series of standards (17).

Serum Lipides.—The total lipide content of sera obtained from the Group III rats at the time of sacrifice was determined by the colorimetric oxidative dichromate method (18). Serum from a normal, non-fasting rat of approximately the same size and age served as a control.

Frozen Sectioning.—The technique of frozen sectioning employed, a modification of the Bush and Hewitt gelatin stripping film method (19), has been described in a previous paper (14). It regularly provided large, intact kidney sections of an indicated 7.5 μ-thickness that adhered firmly to unalbuminized slides after 20 minutes of drying *in vacuo*. Frozen tissue blocks stored for long periods at −15°C. appeared to cut as well as those freshly frozen, provided they were well sealed with the plastic and aluminum wrapping.

Fluorescence Staining.—

A. Non-specific staining: Untreated solutions of fluorescein-coupled antibodies have the property of staining many tissue components non-specifically; *i.e.*, in the absence of specific antigen-antibody union. It has been demonstrated empirically that these non-specific reactions can be more or less prevented in many tissues by absorbing the solutions with heterologous liver powders (12). In the present work, mouse liver powder was found to be an effective absorbing reagent for Rab-anti-Rat and rat liver powder for Goat-anti-Rab when employed at concentrations of 200 mg. of powder per 1 cc. of fluorescent antibody. At first, the liver powders were prepared by removing both water- and acetone-soluble material, but later it was determined that the same amounts of powders extracted with acetone alone produced equally effective absorption. Residual particulates present in the fluorescent antibody solution after absorption with liver were removed by passage through a Seitz filter. The latter procedure did not materially reduce the activity of our preparations, but it is not recommended for weak antibody solutions, especially if these are filtered in small volumes.

It was generally necessary to use the fluorescent stains within a few days after absorption with liver powder because of deterioration in their specific activity. The acetone-extracted rat liver powder was particularly prone to reduce specific activity on standing.

B. Staining technique: Frozen sections of the rat kidneys were washed for 15 minutes in Coplin jars with 3 changes of pH 7, 0.01 M phosphate buffered saline. After excess buffer was removed by blotting, the sections were stained for 45 minutes at room temperature with a

few drops of the labelled antibody solution under a Petri dish containing moistened filter paper. The sections were then rinsed in 3 changes of buffer for 6 minutes, mounted in buffered glycerine (1 part pH 7 phosphate buffer, 9 parts glycerin), and sealed with paraffin.

C. Control sections: These consisted of unstained sections, sections stained for 45 minutes with Rab-anti-Hum, and "blocking" preparations. In the latter, an attempt was made to abolish or inhibit staining by pretreatment of a section with uncoupled antibody while simultaneously showing that pretreatment of a companion section with normal homologous serum did not inhibit fluorescence. With Rab-anti-Rat, effective blocking was achieved by pretreatment for 20 minutes and staining for 10 minutes; the corresponding times for Goat-anti-Rab were 45 and 20 minutes.

Fluorescence Microscopy and Photographic Analysis.—Details of the equipment used for fluorescence microscopy and for photographic photometry have been described elsewhere (10). For visual observations, a cell containing 10 per cent $NaNO_2$ was substituted for the Corning 3486 eyepiece filter and an additional 3 mm. path length of Corning 5870 glass was placed at the source. This arrangement transmits all the visible fluorescence of the specimen, permitting one to distinguish blue-white autoflorescence from the green emission of fluorescein.

Histologic Studies.—Sections of the formalin-fixed kidney blocks were cut at 5 μ and stained with hematoxylin and eosin, and with the periodic acid–Schiff reagent.

RESULTS

In the following presentation, we have considered the three groups of rats as a chronologic unit, and dated the duration of nephritis from the time of first injection of nephrotoxins. The term "specific staining" is applied to fluorescence thought to result from union of coupled antibodies with their antigen(s), for reasons described in the Discussion.

Clinical Findings.—There was little visible evidence of disease in the nephritic rats other than a mild listlessness and occasional diarrhea. Two rats that died at 14 and 17 days after injection showed no signs of impending collapse when observed within 24 hours of death. These animals had such marked postmortem autolytic changes in their abdominal organs that they were excluded from the study. Clinical and histologic data were included on a third rat that died within 7 hours of intended sacrifice on the 14th day after injection. Labored respirations were noted in this rat on the day preceding death.

As shown in Table I, a moderate to massive, sustained proteinuria developed in all the treated rats tested, in a range far in excess of the physiological proteinuria present in the controls. The highest levels were recorded within 6 days of injection (2½, 3, 4, and 6 day samples), with lesser concentrations maintained up to 5 weeks, and a considerable decline indicated at 13 and 15 weeks.

Data on the changes in body weight of those animals that survived from 9 days to 5 weeks after injection are summarized in Table II. An excessive weight gain is indicated in the treated rats sacrificed at 9 days, and there is clear cut evidence of an irregular but substantial weight loss in those sacrificed thereafter.

The serum obtained on exsanguination of the rat sacrificed at 13 weeks was milky, while that of the 15 week rat had a pale-grey turbidity. The total lipide content of these sera was 3100 mg. per cent and 579 mg. per cent, respectively.

In contrast, the serum of the untreated control rat was clear and contained 131 mg. per cent total lipide.

Gross Pathology.—Moderate to marked ascites, usually in association with subcutaneous edema, was found in the rats that were sacrificed between 3 and

TABLE I

Urinary Protein Concentrations in Treated and Control Rats

Time	No. of rats sampled	Mg. urine protein/100 gm./24 hrs.	
		Average	Range
<1 wk.	4	100	30–159
9 days	19	67	16–230
2 wks.	14	68	19–136
3 "	7	82	42–134
4 "	3	69	42–90
5 "	2	75	59–92
13 and 15 wks.	2	31	14–48
Controls.....	10	1.6	0.1–2.5

Time, interval between first injection of nephrotoxic antibodies and date of urine collection.
Controls, untreated rats sampled between 9th day and 5th week of observation.

TABLE II

Comparison of Body Weights at Beginning and End of Observation in Group II Rats and Their Controls

Time	Experimentals		Controls	
	No. of animals	Average weight gain or loss	No. of animals	Average weight gain or loss
		gm.		*gm.*
9 days	5	+25	2	+15
2 wks.	5	−38	2	+17
3 "	4	−28	2	+22
4 "	2	−91	1	+25
5 "	2	−13	1	+35

Time, interval between first injection of nephrotoxic antibodies and sacrifice, for the experimentals, and a comparable period for the untreated controls.

Weight gain or loss, the difference between the initial weight and the weight 24 hrs. before sacrifice.

6 days after injection. To a lesser degree it was also present in 2 rats sacrificed at 9 days, 1 rat sacrificed at 2 weeks, and 2 rats sacrificed at 3 weeks after injection. No correlation of the presence or absence of effusion with the level of protein excretion was established, but when edema was demonstrated after 6 days, it occurred, paradoxically, in those rats showing the greatest loss in body weight.

Numerous scattered petechial hemorrhages were a characteristic finding in the lungs and, to a much lesser extent, the kidneys of rats sacrificed within 9 days of injection, but they were only occasionally seen thereafter, and they were absent in the Group III rats.

Comparison of the kidney weights, expressed in per cent of body weights, of treated and control rats (Table III) reveals an approximately 28 per cent enlargement of the nephritic kidneys through most of the experimental period with the exception of a drop to control levels at 9 days in Group II and an increase to 56 per cent in Group III. The kidneys of the latter were strikingly

TABLE III

Weights of Perfused Kidneys of Treated and Control Rats

Group	Time	No. of animals	Kidney weight*	
			Average	Range
I	<1 wk.	8	0.58	0.42–0.80
II	9 days	5	0.44	0.38–0.48
	2 wks.	4	0.61	0.51–0.69
	3 "	4	0.58	0.38–0.70
	4 "	2	0.58	0.55–0.61
	5 "	2	0.62	0.61–0.63
III	13 and 15 wks.	2	0.72	0.65–0.78
Controls.....	—	11	0.46	0.39–0.55

Time, interval between first injection of nephrotoxic antibodies and sacrifice (Groups I–III).

Controls, 3 rats sacrificed 24 hours after third injection of normal rabbit serum, and 8 untreated rats sacrificed between 9th day and 5th week of observation.

* Kidney weight expressed as per cent of body weight.

hypertrophied, particularly in the 13 week rat, with granular cortical surfaces. On sectioning these kidneys, the enlargement was found to result from uniform, disproportionate cortical thickening.

Histopathology.—Extensive structural alterations were demonstrated in the glomeruli and the convoluted tubules of every treated rat kidney examined microscopically. The glomerular change in the acute stage of the nephritis was already fully developed in the first rats sacrificed, and it was still detectable at 6 days after injection. It consisted of a marked, diffuse swelling of the noncellular substance of the glomerular tufts, or of what will be referred to for the present as the capillary basement membranes, with narrowing of the capillary lumens and a more or less complete obliteration of Bowman's spaces (Figs. 1 to 3). The swollen membranes were lightly stained with eosin and they exhibited a fine, irregular fibrillation that gave the tufts a soft or "fluffy" ap-

pearance. Pronounced swelling was also observed in the nuclei of numerous tuft and capsular epithelial cells, usually in association with diminished basophilia.

Evidence that the membranous swelling was subsiding was suggestive at 6 days and definite at 9 days after injection, marking the appearance of an intermediate stage in the evolution of the nephritis. In this period, which extended through the 5th week of the study, the glomerular tufts were less enlarged, and the capillary channels reopened to some extent, coincident with a progressive hyaline condensation and thickening of the basement membrane (Figs. 4, 5). In addition, there was hyperchromatism and, frequently, indentation or lobulation of the enlarged epithelial cell nuclei that had previously been vesicular and oval. By 4 to 5 weeks, delicate, focal adhesions were visible between the tuft and capsule of occasional glomeruli.

Early and late examples of a final, sclerosing stage were found in the two long term survivors (Group III). The rat sacrificed at 13 weeks showed a far advanced glomerulosclerosis characterized by diminished cellularity of the tuft, extreme thickening of the capillary basement membranes, and a concentric, hyaline sclerosis of Bowman's capsule associated with extensive to complete fusion of tufts to capsule (Fig. 6). In the 15 week rat, the membranous thickening was less marked, the capsular involvement was limited to a narrow, band-like hyaline expansion, and the adhesions were dense but rarely diffuse (Fig. 7).

No arteriolar lesions, fibrinoid necrosis or exudation, and only minimal proliferation were found in the glomeruli at any stage of the nephritis. The existence of a low order of proliferative activity, barely suggested by visual estimates of cellularity, was established by the demonstration of eleven mitotic figures in the glomerular tufts of 6 rats—nine mitoses occurring within 5 days of injection—while none was detected in the controls (Figs. 2, 3). We were unable to categorize precisely the identity of these dividing cells, but in several instances it was possible to show that they were extravascular; *i.e.*, that they were not endothelial.

Diffuse fibrin thrombosis of the afferent arterioles and the glomerular capillaries was the outstanding lesion observed in the unperfused rat that died of its disease 14 days after injection. It was not present, even as a focal process, in any other rat, although isolated, necrotic glomeruli were found as a rare, sporadic occurrence in the absence of thrombosis.

The coexisting damage to the proximal convoluted tubules was also divisible into three stages, each corresponding in duration to those described for the glomerular lesions. Unlike the glomerular lesions, which showed diffuse involvement and striking uniformity in a given rat, the tubular changes were characteristically patchy and diversified in each treated rat kidney throughout the period of study. Classification into stages was possible, however, on the basis of the relative amounts of injury and regeneration present.

The characteristic tubular lesion—hyaline droplet degeneration—was defined inadequately by hematoxylin and eosin; its extent and early appearance were far better revealed with the periodic acid-Schiff (PAS) stain. Normally, PAS-positive material is found in the proximal convoluted tubules as a homogeneous, continuous, wavy band on the lumenal aspect of the epithelium (corresponding to the brush border), and as a barely perceptible stippling through the remainder of the cytoplasm. The tubular basement membrane appears as a delicate single line of uniform thickness. By combining the findings obtained with each stain, the sequential development of the tubular damage was seen to proceed as follows: marked swelling of the tubular epithelium with disappearance of the basal, mitochondrial striations and the PAS-positive lumenal band, followed by a progressive enlargement and coalescence of intensely eosinophilic, PAS-positive cytoplasmic droplets culminating, frequently, in cell rupture and extrusion of the droplets (Figs. 8 to 10).

The hyaline droplet change was intense during the first 6 days after injection, relatively inconspicuous at 9 days, increasingly marked through the 5th week, and minimal in the long term survivors. In the acute stage of the nephritis it was accompanied by waves of mitotic activity in the proximal convoluted tubular epithelium that involved degenerating as well as apparently intact tubules (Figs. 8, 9). Thereafter, mitoses were scarcely more abundant than in the controls; nevertheless, increasing numbers of regenerated tubules, instantly recognizable by their scanty cytoplasm and closely spaced nuclei, were observed in the intermediate stage. The most extensive regeneration was thus present at 4 to 5 weeks after injection, and it was at this time, also, that focal, irregular, hyaline thickening of the basement membranes of the regenerated tubules became apparent.

The tubular pattern in the Group III rats was distinguished from that of the preceding groups by the presence of hypertrophy in many tubules and atrophy with marked basement membrane sclerosis in others. The lesions were again much more prominent in the rat sacrificed at 13 weeks (Figs. 11, 12). Comparatively few tubules showed recent regeneration and, fewer still, the hyaline droplet changes.

A slight to moderate, generally diffuse interstitial lymphocytic infiltration was present in about half of the treated rats. The reaction had no apparent relation to the duration of the disease and, in all but one rat, it was limited to the cortex. Large, focal accumulations of lymphocytes were present only in the renal cortex of the rat sacrificed at 13 weeks (Fig. 12).

Albuminous (hyaline) tubular casts were a finding common to every nephritic kidney, in keeping with, but not quantitatively related to, the universal incidence of proteinuria. Infrequently, these casts were also present in Bowman's space.

No lesions were demonstrated in the control kidneys.

Fluorescence Staining.

When a freshly prepared, unstained section of rat kidney was examined in the fluorescence microscope, the glomeruli appeared as round, empty spaces outlined by a faint, blue-white autofluorescence in the epithelium of the proximal convoluted tubules (Fig. 13). After a few days of storage, the tubular autofluorescence increased and, eventually, the entire section brightened markedly, although details of glomerular structure were not filled in. The one exception to this pattern was the occurrence of coarse, dull orange and brilliant white particulates in the cortical tubules of unstained sections of the 13 week rat. Intrinsic green fluorescence was never encountered.

Ideally, sections of control kidneys exposed to our fluorescein-coupled antibody solutions would have fluoresced at the level of the unstained sections, and this was virtually the case with Goat-anti-Rab after absorption with rat liver. A somewhat higher, but still negligible, level of non-specific staining was imparted by Rab-anti-Rat absorbed with mouse liver. No differences were noted between the staining properties of controls receiving injection of normal rabbit serum and those that were untreated.

As in previous work (10, 13, 14), our inability to demonstrate specific staining of the tubules, and the constancy of the distribution and the degree of non-specific staining made it possible to obtain quantitative data by relating the fluorescence intensity of the glomeruli to that of the tubules (glomerular: tubular ratio). Relatively few measurements were required to establish this ratio in the control kidneys after staining with either Goat-anti-Rab or Rab-anti-Rat as the glomerular staining was invariably and uniformly below that of the tubules.

A. Goat-anti-Rab: A strikingly selective, glomerular localization of specific staining was demonstrated in rat kidney sections chosen randomly from each survival category and stained with the Goat-anti-Rab reagent. In every instance, the glomeruli stood out as brilliant beacons of green fluorescence in a surround of hazy, barely perceptible tubules. It was apparent, furthermore, as can be judged from the illustrations (Figs. 15 to 17, 19, 21), that there was a preferential deposition of the fluor in the membranes of the glomerular tuft with very little, if any, staining of the arterioles of the stalk or of Bowman's capsule.

The quantitative data, summarized in Table IV, amply confirmed the visual impression that high levels of specific glomerular fluorescence were maintained in all stages of the nephritis. Despite the relatively small number of animals studied, the data also indicated a significant increase in this fluorescence with increasing duration of disease. Possible explanations of this change are discussed below.

B. Rab-anti-Rat: All the nephritic kidneys that yielded technically satisfactory preparations after staining with the Rab-anti-Rat reagent were analyzed

visually and photometrically. Additional data were secured from 3 kidneys that showed excessive tubular autofluorescence; in these cases the average tubular fluorescence of the foregoing group was used in the calculation of glomerular: tubular ratios.

The only demonstrably significant, quantitative deviation from the control pattern encountered was a variable elevation in the level of specific glomerular fluorescence. As indicated in Table IV and in Text-fig. 1, the elevation was

TABLE IV

Glomerular:Tubular (G/T) Ratio of Specific Fluorescence Intensity in Sections of Rat Kidneys Stained with Goat-anti-Rab (for Localized Nephrotoxic Antibodies) and Rab-anti-Rat (for Localized Autogenous Antibodies)

Time	G/T ratio: Goat/anti-Rab				G/T ratio: Rab-anti-Rat			
	No. of animals	AV	CI	N	No. of animals	AV	CI	N
2½ days	1	2.4	2.2–2.6	16	2	1.5	1.3–1.7	28
3 "	1	2.5	2.2–2.8	15	2	1.3	1.2–1.4	29
4 "	2	2.7	2.4–3.0	28	2	1.3	1.2–1.4	26
6 "	1	2.1	1.9–2.3	15	2	1.5	1.4–1.6	33
9 "	2	2.5	2.3–2.7	24	5	1.9	1.8–2.0	67
2 wks.	2	2.7	2.5–2.9	27	4	2.0	1.9–2.1	63
3 "	2	2.8	2.6–3.0	30	3	2.0	1.9–2.1	43
4 "	2	3.5	3.1–3.9	27	2	2.0	1.8–2.2	25
5 "	1	3.2	1.9–3.5	12	2	2.0	1.9–2.1	23
13 "	1	3.6	3.1–4.1	11	1	3.1	2.7–3.5	15
15 "	1	3.2	3.0–3.4	17	1	2.4	2.3–2.5	10
Controls.....	1	0.7	0.6–0.8	6	3	0.6	0.5–0.7	37

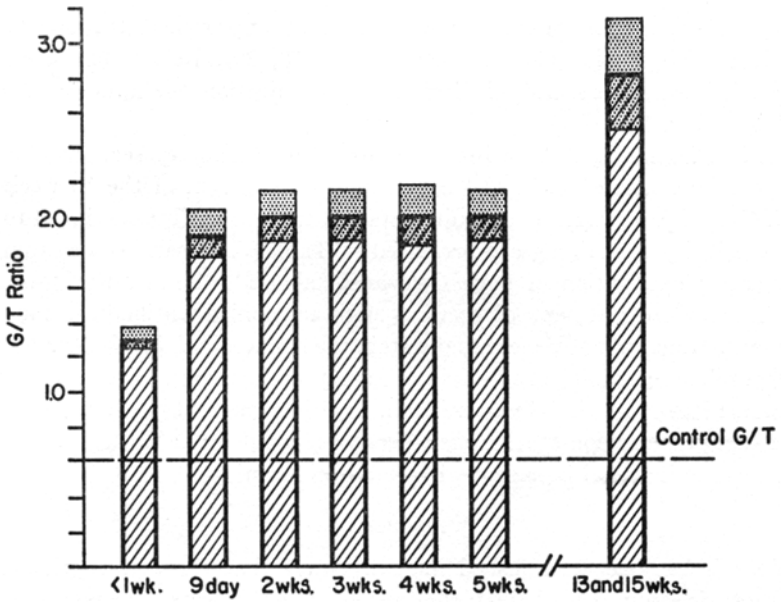
Time, interval between first injection of nephrotoxic antibodies and sacrifice.

Controls: Goat-anti-Rab, rat sacrificed 24 hours after third injection of normal rabbit serum; Rab-anti-Rat, untreated rats.

AV, average; CI, confidence interval which includes 99 per cent of the estimates of the average; N, No. of measurements.

substantial during the 1st week of disease, even in the earliest rats studied, and progressively more marked at 9 days and 2 weeks. Subsequently, the glomerular concentration of the fluor was remarkably constant until a final rise was recorded in the long term survivors. There was a notable uniformity in the staining pattern within individual glomeruli and from one glomerulus to the next in most kidneys. The one outstanding exception was the finding of irregularly shaped foci of unequal stain density in many glomeruli of the 13 week rat. It was in this animal, also, that a suggestion of staining of Bowman's capsule was observed (Fig. 22).

With glomerular:tubular ratios of 2.0 or above, the glomeruli showed, gen-



TEXT-FIG. 1. Glomerular: tubular (G/T) ratio of specific fluorescence intensity in sections of rat kidneys stained with the Rab-anti-Rat fluor to detect rat globulins. Time is dated from the first injection of nephrotoxic antibodies. The 99 per cent confidence interval for the average is indicated by the stippled areas on the bar graph.

TABLE V

Glomerular: Tubular Ratio of Specific Fluorescence Intensity in Sections of Rat Kidneys after the Blocking Procedure and After Staining with Rab-anti-Hum

Fluor	Time	Pretreatment of section	Glomerular: tubular ratio		
			AV	CI	N
Goat-anti-Rab	2½ days	Uncoupled Goat-anti-Rab	0.8	0.7–0.9	16
		Normal goat serum	1.8	1.7–1.9	19
Rab-anti-Rat	2 wks.	Uncoupled Rab-anti-Rat	0.8	0.7–0.8	24
		Normal rabbit serum	1.5	1.4–1.6	14
Rab-anti-Rat	4 wks.	Uncoupled Rab-anti-Rat	0.9	0.8–1.0	14
		Normal Rabbit serum	1.6	1.5–1.7	16
Rab-anti-Hum	4 wks.	None	0.9	0.8–1.0	12

Time, interval between first injection of nephrotoxic antibodies and sacrifice. One rat analyzed in each category.

AV, average; CI, 99 per cent confidence interval; N, No. of measurements.

erally, a crisp membranous fluorescence that approached the spectacular displays elicited with Goat-anti-Rab (Figs. 18, 20). Below this level, and especially in the rats sacrificed within 6 days of injection, the tufts were stained diffusely (Fig. 14).

No localization of Rab-anti-Rat was found in the kidney sections of 3 rats injected intravenously with 2.4 cc of the lipemic serum of the 13 week rat.

C. Control procedures: The results obtained from procedures designed to test the specificity of staining are presented in Table V. It can be seen that the glomerular localization of both Goat-anti-Rab and Rab-anti-Rat was fully blocked by pretreatment of sections with uncoupled antibodies, and that Rab-anti-Hum did not preferentially stain the glomeruli of diseased rat kidney.

As a rule, the staining of tubules was more prominent and more variable with Rab-anti-Rat than with Goat-anti-Rab. In either case and in whatever degree, it was considered a non-specific effect because it could not be blocked effectively, and because it was also present with Rab-anti-Hum.

DISCUSSION

Since it has been established in many ways that the active principle of nephrotoxic serum is antibody globulin (5–7), there appears to be no reasonable doubt that the union of this antibody with the renal antigen or antigens *in situ*, if not in itself an injurious event, is at least the essential initiating reaction that leads to nephrotoxic nephritis. In the pathogenetic mechanism originally formulated by Masugi (20, 21), the nephritis was considered to be a degenerative and reparative process stemming wholly from a direct assault on renal antigenic structure by the injected nephrotoxic antibodies; *i.e.*, a form of reverse anaphylaxis. While this concept might explain adequately the nephrotoxic nephritis of rats, its failure to account for the characteristic latent period of the rabbit nephritis induced by duck antisera led Kay (1, 2) to search for an alternative, indirect mechanism in the latter condition. On the basis of evidence which need not be detailed here, he concluded that (*a*) the nephrotoxic duck antibodies were innocuous *per se*, but that they did localize and remain antigenic in the kidney, (*b*) the latent period was one of active immunization of the rabbit to circulating normal duck serum antigens, and (*c*) the onset of detectable nephritis coincided with the union of *rabbit* antibody with *duck* antigen in the kidney in an Arthus type reaction.

Subsequently, it has been shown that an incubation period can also occur in the rat (22) but, in either case, the question of whether or not a heterologous nephrotoxic antibody directly damages the kidney is a significant issue only in nephrotoxic nephritis while the proposed mechanism for the participation of autogenous antibodies in the development of renal lesions has a potentially broad applicability to many other forms of experimental nephritis and to human nephritis also. For the Kay hypothesis suggests, in effect, that any

antigenic substance of heterologous origin, specific antibody or otherwise, that localizes in critical areas of the kidney *may* elicit a nephritis indirectly and regardless of primary toxicity, provided the antigen has induced an immune response in the recipient animal. Evidence has been presented in earlier fluorescent antibody studies from this laboratory (13, 14) that the mechanism postulated by Kay does, in fact, operate in the foreign protein (bovine γ-globulin) nephritis of rabbits and in several types of human nephritis. In the present investigation, we have applied the fluorescent antibody method to the nephrotoxic nephritis of rats with the principal objectives of securing data on (*a*) the persistence, as antigen, of localized rabbit nephrotoxin, and (*b*) the presence and role of autogenous antibodies. However, the newness of the fluorescent antibody approach and the bewildering variety of clinical and histologic findings that have been described in this condition (23–27) made an expansive characterization of our own findings necessary for the interpretation of the data.

The course of nephrotoxic nephritis in the rat can be influenced profoundly by the manner in which the renal antigen is prepared, the potency and dose of the nephrotoxic serum, the age, strain, and diet of the rats, and, doubtless, many other factors (16, 28, 29). In this connection, our main methodological departures from previous work were the use of renal sediment for the production of nephrotoxins, of the crude γ-globulin fraction of nephrotoxic serum for inducing nephritis, and of perfused kidneys from Sherman rats for analysis. Under these experimental conditions, the nephritis was characterized clinically by an abrupt onset, massive, persistent proteinuria, and hyperlipemia; and histologically by early, marked glomerular swelling followed by progressive membranous thickening and eventual glomerulosclerosis, all in association with severe tubular changes. This pattern is clearly of the type described by Smadel (25, 26) and Heymann and Lund (16), and, aside from the extremely artificial manner in which it is induced, it closely resembles the lipemic (membranous) nephritis of man (30). The essential glomerular lesion of this type of experimental nephritis consists, in the view of the aforementioned authors, of a simple, progressive thickening of the capillary basement membrane, and, admittedly, there is little else to consider if one accepts the classic concept of glomerular structure (30). In our opinion, this interpretation and the concept on which it is based cannot be fully reconciled with the observations. We believe that the appearance of the swollen glomerular tufts in the early nephritis (Figs. 2, 3) strongly suggests the additional presence of edema and that it is only when this edema has subsided that one can convincingly demonstrate membranous thickening (Figs. 4, 5). In this regard, note should be taken of the ease with which one can overlook the glomerular swelling or underestimate its magnitude if there is uniform involvement in a given kidney. For example, a generalized 20 per cent increase in glomerular area, reflecting, of course, even larger alterations in volume, may require micrometry for certain detection.

The answer to how edema can occur *within* a glomerular capillary basement membrane when elsewhere in the body it involves the interstitial spaces is, perhaps, best supplied by the work of Jones (31). By the use of highly refined techniques, this investigator has demonstrated that the human glomerular capillary wall may actually consist of tightly apposed epithelial and endothelial basement membranes enclosing a potential "connective tissue space," the presence of which is made apparent only in disease. It is obvious that such a space would furnish a likely site for the accumulation of edema fluid. The rat kidney would seem to be a good place to test this concept despite the delicacy of the membranes involved. Electron microscopic studies, to date restricted to the normal rat glomerulus, have failed to resolve the issue (32).

If, in the rat, the Arthus-type reaction of the Kay mechanism succeeds an initial reverse anaphylaxis in the production of renal injury, one might expect to distinguish between one effect and the other, on clinical and histologic grounds alone, by the demonstration of significantly separated peaks of activity or, in the event of an insensible transition, by the finding of clear cut qualitative differences. A careful analysis of our data did not permit us to make this distinction although there is good evidence, in the morphologic and kidney weight changes, that the severity of the nephritis subsided after reaching its maximum in the 1st week after injection, and there is some indication of a gradual intensification of renal injury between the 2nd and 5th week. Too little is known about the persistence of hyaline droplets to ascribe their presence at 3 months to continuing acute renal injury. It should be noted, in passing, that the phenomenon of increasing nephrotoxicity of frozen antisera on prolonged storage (16) may have contributed to the dramatic changes noted between the Group I and early Group II rats.

Immunologic Properties of the Fluorescent Stains.—Our fluorescent antibody solutions were prepared against impure γ-globulin fractions and they were not absorbed with cross-reacting antigens. Under any circumstances, of course, they would still have reacted equally well with antibody or non-antibody γ-globulin. It follows that the certain identification of antigen cannot be based on the results obtained with these reagents alone. However, the fluorescent antibodies can be characterized sufficiently well to permit a meaningful analysis when their reactions are considered in the light of other evidence.

The serum fractionation methods we have employed regularly yield solutions that contain, in addition to γ-globulin, certain antigenic contaminants, principally β-globulin and albumin (15), all of which will be represented to a greater or lesser degree by complementary antibody in the corresponding heterologous antisera. This fact was well borne out by the appearance of multiple discrete lines of precipitation when Goat-anti-Rab and Rab-anti-Rat were tested against normal rabbit serum and normal rat serum, respectively, by a modified Ouchterlony agar diffusion technique (33). It was demonstrated,

further, that a faint but definite line of precipitation formed on reaction of Goat-anti-Rab with normal rat serum, but, as one would expect, no cross-reaction occurred between Rab-anti-Rat and normal rabbit serum.

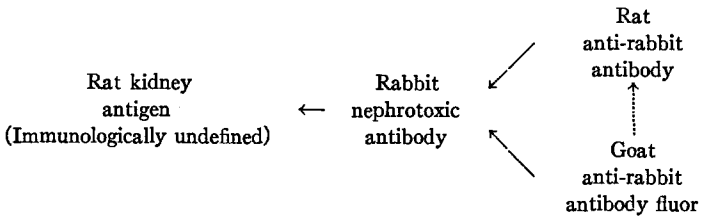
Non-Specific Staining.—The nature of the immunologically non-specific staining of tissues by fluorescent antibodies appears to be no better understood today than when it was first described (12), and the means by which it is recognized and removed are also essentially unchanged (34). When the antigen under consideration is a foreign substance, such as the rabbit nephrotoxins in the rat, any part of the staining pattern that is shared by the tissues of treated and normal animals can, with reasonable certainty, be considered to be non-specific; if native antigen is being studied, the non-specific staining can be identified by its relative inability to be blocked by pretreatment of the sections with unconjugated antibody. With either type of antigen, the non-specific staining can usually be reduced or abolished by absorption with powders of liver or other tissues.

Our experience with fluorescent antibodies prepared against a variety of antibody antigens has shown them to vary considerably in their ability to impart non-specific staining and in the degree to which the non-specific staining can be reduced by absorption with a given tissue powder. The most effective absorption was achieved by treating Goat-anti-Rab, a reagent which produced unusually intense and diffuse non-specific staining, with rat liver (Figs. 23, 24). It should be emphasized that non-specific staining is superimposed on specific staining, and that it interferes with the detection and resolution of the latter in much the same way that background "noise" obscures an electronic signal. Significant results can still be obtained with unabsorbed fluorescent antibodies, as has been shown in previous work (10, 13), especially if there is a high concentration of antigen in the sections studied.

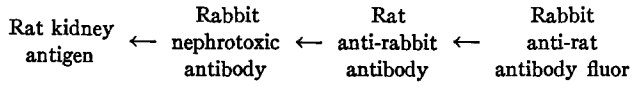
The Specific Reactions.—The residual, glomerular staining of nephritic kidneys by the absorbed fluorescent antibodies was considered to be immunologically specific, *i.e.* the result of fluorescent antibody-antigen combination, because it could be inhibited (blocked) by pretreatment of the sections with unconjugated antibody, but not by normal serum. The results with the anti-human fluorescent antibody corroborated but did not amplify those of the blocking experiments. Throughout this paper, references to "specific staining" have been made with the full realization that this term was not restricted to the reactions of a single antigen.

The Quantitative Data.—Until the quantitative immunochemical characteristics of the reactions between localized antigens and fluorescent antibodies have been defined, no significance will be ascribable to absolute levels of specific fluorescence intensity in tissue sections stained with coupled antibodies. The formidable complexity of this problem, as it pertains to the present study, is indicated by consideration of only one of its aspects, the probable antigen-

antibody linkages present in the stained sections. In the case of Goat-anti-Rab these were:—



with Rab-anti-Rat:



Fortunately, the absence of perceptible specific staining in the renal tubules made it possible to circumvent this issue by furnishing a convenient reference point for the analysis of *relative* fluorescence intensities, provided variation in autofluorescence, non-specific staining, and section thickness were kept within reasonable limits. As the combining characteristics differed for each of our stains, it was necessary to limit the direct comparison of variations in glomerular:tubular ratios to those detected by the same fluorescent antibody. Such variations were judged to be significant only if the statistical confidence intervals did not overlap.

The Nephrotoxic Antibodies.—Nephrotoxic serum differs from normal rabbit serum only in that it is obtained from animals immunized against rat kidney. Since renal localization of antigens was demonstrated in rats receiving alcohol-fractionated nephrotoxic serum, but not in those injected with normal rabbit serum or in untreated controls, the conclusion seems inescapable that, if the localized antigens are of rabbit origin, they are nephrotoxic antibodies. Furthermore, the possibility that these antibodies are being demonstrated while traversing the glomerular membranes can be excluded for the following reasons: (*a*) The nephrotoxins were still present after prolonged perfusion. (*b*) Nephrotoxins are effectively removed from the circulating blood within a few minutes of injection (35, 36). It is inconceivable that they would be present in the glomeruli for many weeks afterwards if they were merely being excreted. (*c*) Thick dried smears of rabbit blood containing a much higher concentration of rabbit γ-globulin than would be present in rat glomerular filtrate at the moment of sacrifice showed very faint, diffuse green fluorescence on staining with Goat-anti-Rab. In short, the evidence is strong that the rabbit nephrotoxic antibodies are both fixed and concentrated in the rat kidney.

It can also be shown that the antigens stained with Goat-anti-Rab are not primarily cross-reacting rat antibodies because: (*a*) The marked qualitative

differences in the appearance of glomeruli stained with Goat-anti-Rab and those stained with Rab-anti-Rat in the early phase of the nephritis precludes the possibility that the same antigens are being demonstrated. (*b*) Both stains had approximately equal antibody titers against their respective antigens, and Goat-anti-Rab cross-reacted very weakly *in vitro* with rat serum. Under these conditions, it is very unlikely that the cross-reaction alone would result in much higher glomerular:tubular ratios than were obtained with the fluor specifically directed against rat antigens.

Consideration of the Goat-anti-Rab data in Table IV reveals not only that the localized nephrotoxic antibodies persisted as antigens in rat kidneys for at least 3 months after injection, but that there was an apparent steady increase in their concentration over this period. Obviously, if the nephrotoxins are absorbed by the kidney with the rapidity indicated above, and there is no source of additional rabbit antigen, the latter finding cannot represent an increase in the total amount of localized foreign material. It is necessary, therefore, to seek factors that might cause an increase in the nephrotoxin concentration per unit area once they have become fixed in the kidney—unless one makes the completely unsupported assumption that nephrotoxins which cross-reacted with other tissues immediately after injection were subsequently released to localize in the kidneys or that, with time, changes in the renal antigen-nephrotoxin bonds made more reactive groups available for staining by Goat-anti-Rab. A satisfactory explanation for this paradox is not provided in the present work. The increasing glomerular:tubular ratios demonstrated by Goat-anti-Rab were not paralleled in the Rab-anti-Rat data and besides they were of too great a magnitude to be the result of cross-reaction alone. Similarly, if the increases were due to progressive glomerular condensation, they should also have been elicited by Rab-anti-Rat. However, the failure to resolve this problem does not, in our opinion, affect the validity of the observation that there is a remarkable persistence of rabbit nephrotoxins, as antigens, in rat kidney. This persistence suggests the feasibility of similarly demonstrating foreign antigens in human nephritis.

Hill and Cruickshank (37), using a *in vitro* application of the fluorescent antibody technique, have shown that rabbit nephrotoxic serum contains antibodies against the basement membranes of the convoluted tubules and the cytoplasm of the tubular epithelium as well as against the glomerular basement membranes of rat kidneys. The present work unequivocally confirms the previous observation (10) that the tubular antigens do not combine with antibody *in vivo*. It appears that only the glomerular localizing antibodies have a significant role in the evolution of rat nephrotoxic nephritis. The extensive tubular pathology is thus secondary to glomerular injury, presumably the result of ischemia.

The Autogenous Antibodies.—In the evaluation of the Rab-anti-Rat data,

the absence of cross-reactions with rabbit protein immediately permitted the identification of the localized substances as being of autogenous origin. Considering, for the moment, only the experimental period from 6 days to 15 weeks after injection, it is also possible to marshal strongly presumptive evidence that these autogenous proteins, antigens to the stain, were rat antibodies as follows:—

(*a*) They were fixed and concentrated in the glomerular membranes, judging by the same perfusion and smear criteria outlined for the nephrotoxic antibodies.

(*b*) Their membranous localization was preceded by that of antigenic rabbit antibodies and, in the period cited, there was a virtual point for point correspondence of these localization patterns (Figs. 19, 20).

(*c*) The localization patterns of injected and autogenous protein became qualitatively equivalent at 6 to 9 days after injection, in keeping with an immunizing process.

As we have shown, significant elevation of the glomerular-tubular ratio was demonstrated by the anti-rat fluor even in the 2½ to 6 day rats, and the fluorescent staining in this early stage differed markedly from that of Goat-anti-Rab (Figs. 14, 16). Since this is the period that histologically included marked glomerular edema, it is logical to propose that the diffuse fluorescence patterns resulted from the staining of non-antibody globulins in the edema fluid.

Analysis of the quantitative data on rat antibody localization (Table IV, Text-fig. 1) reveals that the treated animals can be divided into early (under 1 week), middle (9 days to 5 weeks) and late (13 and 15 weeks) groups on the basis of significantly separated glomerular: tubular ratios. Because of the small number of animals in the late group, we wish only to lay stress on the presence of a low level of specific glomerular fluorescence in the 1st week after injection, which is probably due to non-antibody globulins, and a higher level thereafter that represents the localization of autogenous antibodies.

SUMMARY AND CONCLUSIONS

The principal objective of this investigation was to define the roles of injected and autogenous, kidney-localizing antibodies in the pathogenesis of rat nephrotoxic nephritis by relating data obtained with fluorescent antibody techniques to clinical and histologic observations.

Such an analysis of the nephritis that developed in 28 rats after injection with the crude γ-globulin fraction of nephrotoxic serum has led us to the following conclusions:—

The renal localization of nephrotoxic antibodies is primarily and, perhaps, exclusively in the membranes of the glomerular tufts. These antibodies are demonstrable, as antigens, in the glomeruli for up to 3 months after injection.

In the acute stage of the nephritis, non-antibody, autogenous globulins are present in the glomerular tufts, probably as components of edema fluid. From 6 and 9 days to 3 months after injection, autogenous antibodies are localized in the glomerular tufts in a pattern that corresponds closely to that of the nephrotoxins.

The essential requirements for the operation of the mechanism postulated by Kay are fulfilled in rat nephrotoxic nephritis.

We wish to acknowledge our indebtedness to Dr. Leonhard Korngold and Dr. Wilbur F. Noyes for much helpful advice; to Mr. John Hlinka for the photometric analyses and the photomicrographs; and to Miss Betty Corcoran for assistance in the general conduct of this experiment.

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EXPLANATION OF PLATES

FIGS. 1 to 12. Perfused rat kidneys. Sections stained with hematoxylin and eosin unless otherwise specified. The interval between the first injection of nephrotoxic antibodies and sacrifice is indicated in days or weeks.

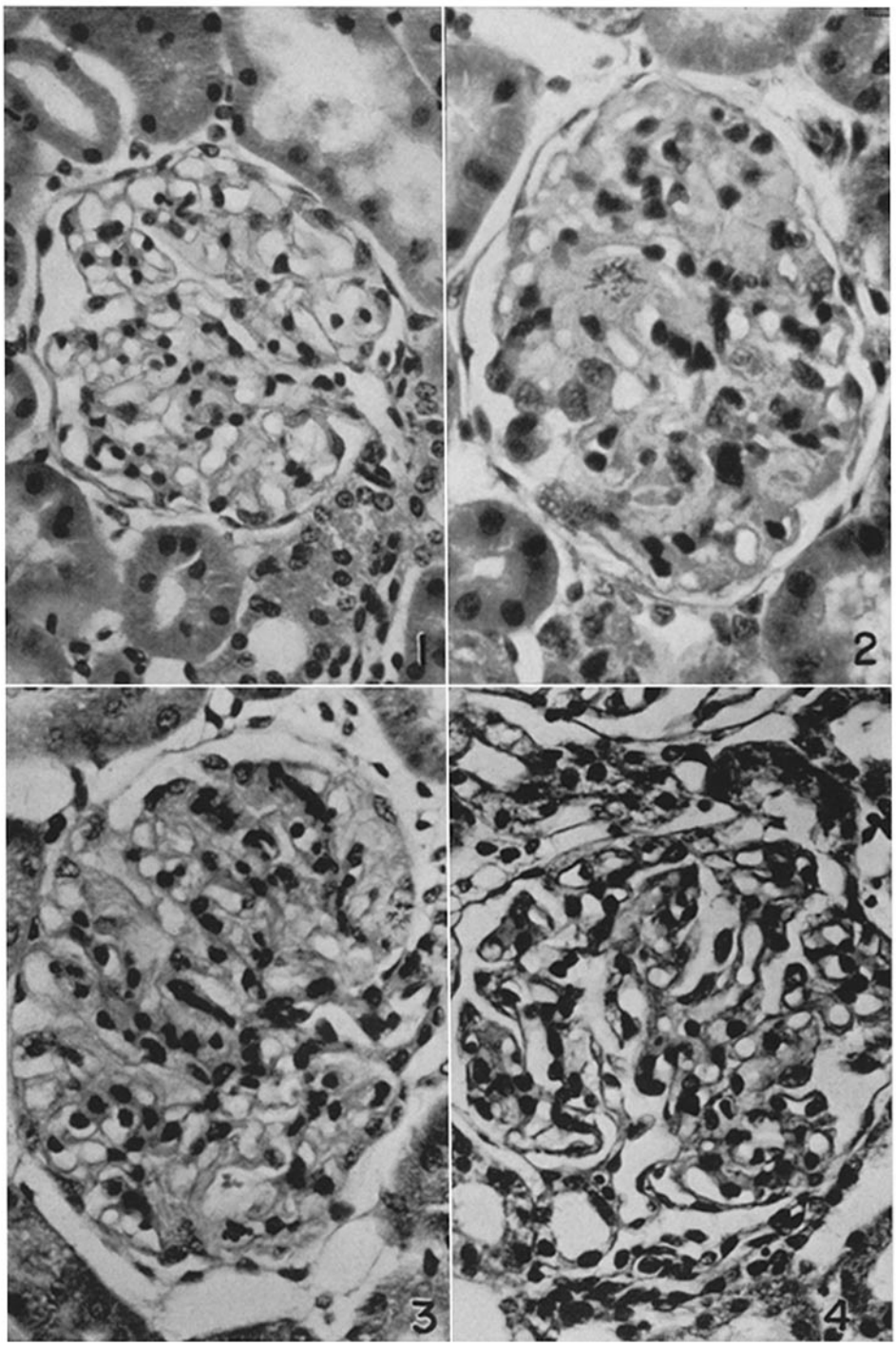
PLATE 3

FIG. 1. Control rat injected with normal rabbit serum. × 500.

FIG. 2. 3 days. Marked "soft" swelling of the glomerular tuft with narrowing of capillary lumens and enlargement of many epithelial cell nuclei. A metaphase mitotic figure is present near the tuft center. × 500.

FIG. 3. 4 days. Swollen tuft illustrating the maximum cellularity encountered. A dividing cell is visible at 2 o'clock. × 420.

FIG. 4. 2 weeks. Moderate membranous thickening of the glomerular tuft. × 430.



(Ortega and Mellors: Antibodies and nephrotoxic nephritis)

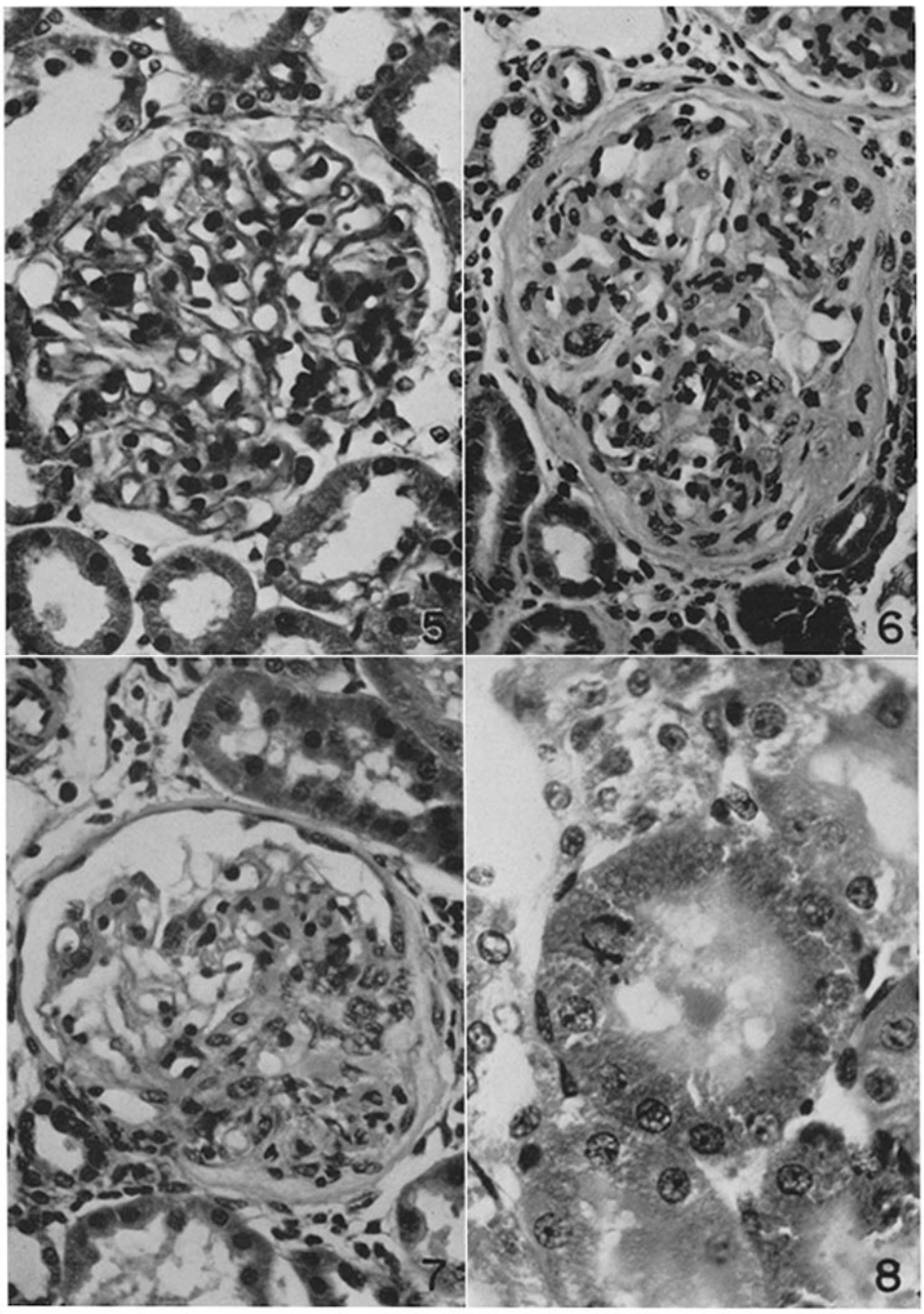
PLATE 4

FIG. 5. 4 weeks. A glomerular tuft showing more advanced membranous thickening but, as yet, no involvement of Bowman's capsule. × 430.

FIG. 6. 13 weeks. The late stage of nephrotoxic nephritis: glomerulosclerosis. × 315.

FIG. 7. 15 weeks. Advanced membranous thickening with focal adhesion of tuft to capsule. Elsewhere, the capsule shows narrow, hyaline thickening. × 340.

FIG. 8. 4 days. Early formation of hyaline droplets in proximal convoluted tubules. The central tubule contains an anaphase mitotic figure. × 560.



(Ortega and Mellors: Antibodies and nephrotoxic nephritis)

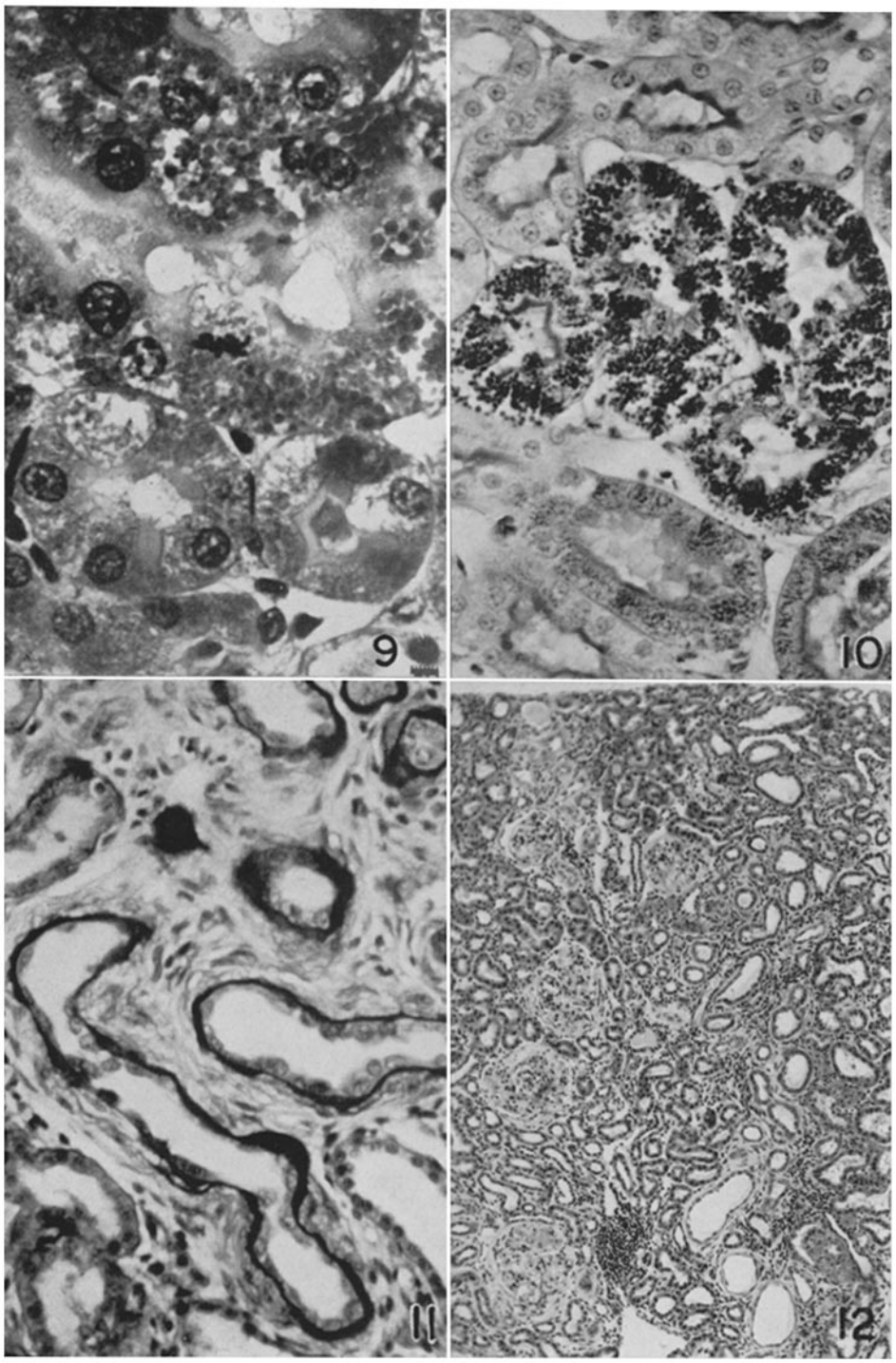
PLATE 5

FIG. 9. 4 days. Severe hyaline droplet degeneration. A metaphase plate is present near the center of the figure. × 660.

FIG. 10. 4 days. PAS stain. The central tubule shows fully developed hyaline droplet degeneration. Normal tubules are present above, and tubules showing early hyaline droplet change below. × 300.

FIG. 11. 13 weeks. PAS stain. There is sclerosis of the basement membranes of many tubules. × 300.

FIG. 12. 13 weeks. Late nephrotoxic nephritis with glomerulosclerosis, alternating tubular atrophy and hypertrophy, and patchy, lymphocytic interstitial infiltration. × 95.



(Ortega and Mellors: Antibodies and nephrotoxic nephritis)

PLATE 6

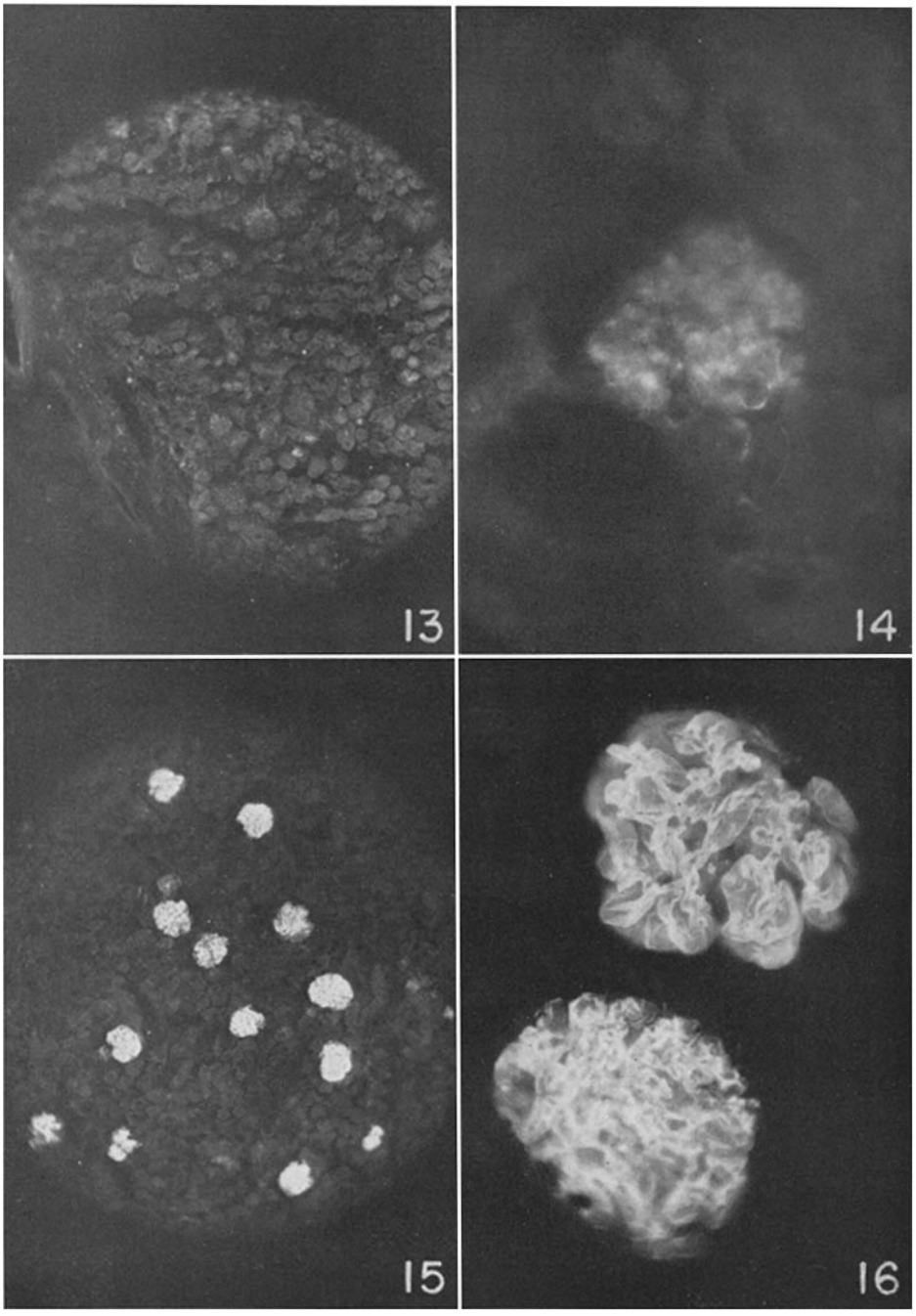
FIGS. 13 to 24. Fluorescence photomicrographs of sections of perfused rat kidneys. The interval between the first injection of nephrotoxic antibodies and sacrifice is indicated in days or weeks. All sections cut on gelatin film at an indicated 7.5 μ unless otherwise specified. G/T = average glomerular:tubular ratio of specific fluorescence intensity.

FIG. 13. 4 days. Stained with Rab-anti-Rat. G/T = 1.2. The non-specific staining is lower than that of most sections stained with this fluor. The numerous glomeruli present, while scarcely discernible, have a fluorescence intensity well above that of the glomeruli in stained control kidney sections. At the same magnification and exposure, fresh unstained sections of rat kidney are virtually invisible. × 37.

FIG. 14. 6 days. Stained with Rab-anti-Rat. G/T = 1.3. Diffuse staining of a glomerular tuft. The specific fluorescence intensity of this tuft is higher than that of other tufts in the section, but the staining pattern is representative. × 225.

FIG. 15. 6 days. Stained with Goat-anti-Rab. G/T = 2.1. This is the characteristic fluorescence pattern of all the nephritic kidney sections treated with this fluor. × 37.

FIG. 16. 6 days. Stained with Goat-anti-Rab. The tuft at the top shows predominantly membranous localization of nephrotoxic antibodies. The other tuft is partly out of focus and shows a crushing artifact. × 205.



(Ortega and Mellors: Antibodies and nephrotoxic nephritis)

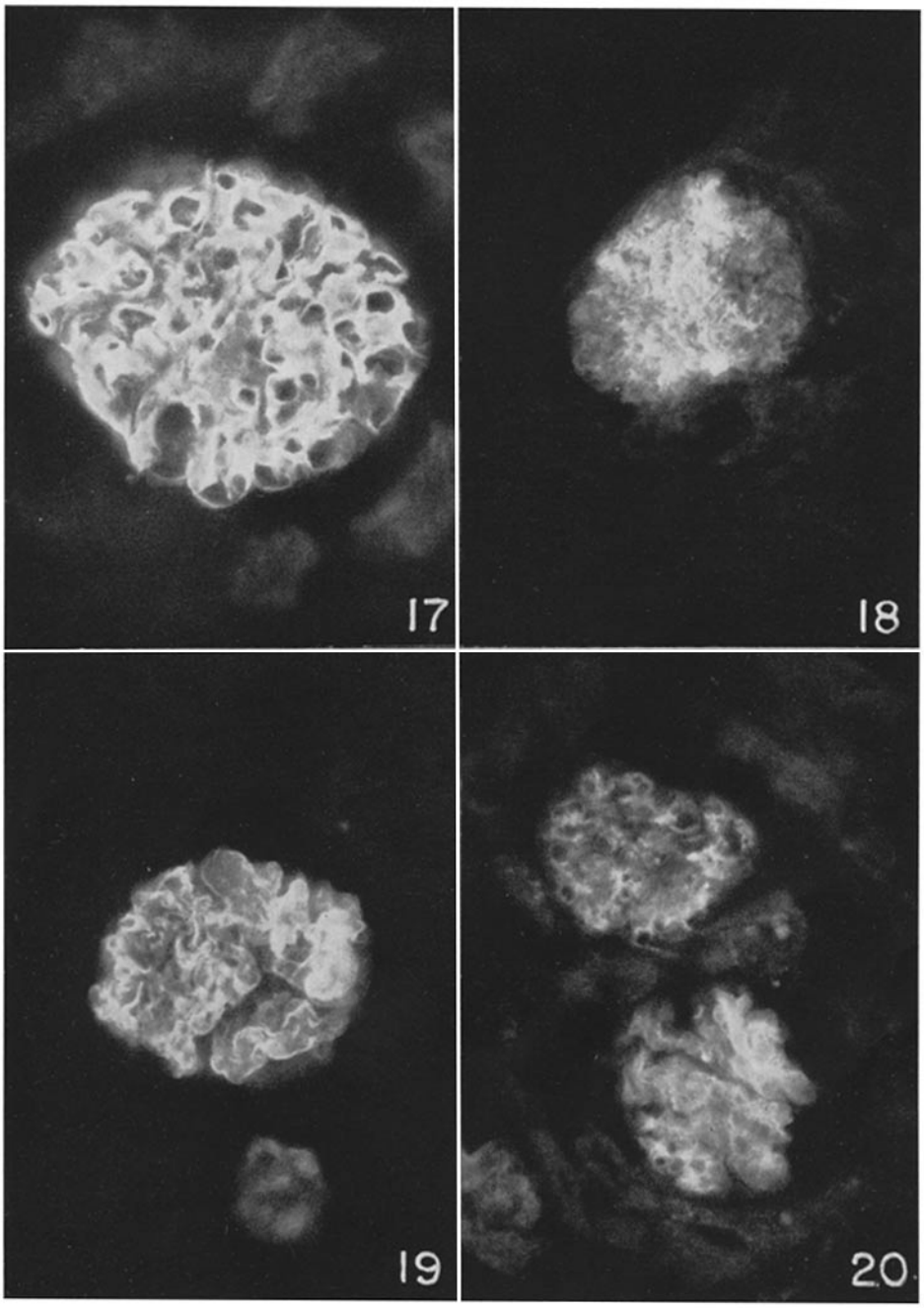
PLATE 7

FIG. 17. 9 days. Stained with Goat-anti-Rab. G/T = 2.4. × 360.

FIG. 18. 9 days. Stained with Rab-anti-Rat. G/T = 2.0. Early membranous localization of autogenous antibodies. × 175.

FIG. 19. 4 weeks. Stained with Goat-anti-Rab. G/T = 3.1. One intact glomerulus and two glomerular fragments are stained. × 200.

FIG. 20. 4 weeks. Stained with Rab-anti-Rat. G/T = 2.2. There is a predominantly membranous localization of autogenous antibodies. × 210.



(Ortega and Mellors: Antibodies and nephrotoxic nephritis)

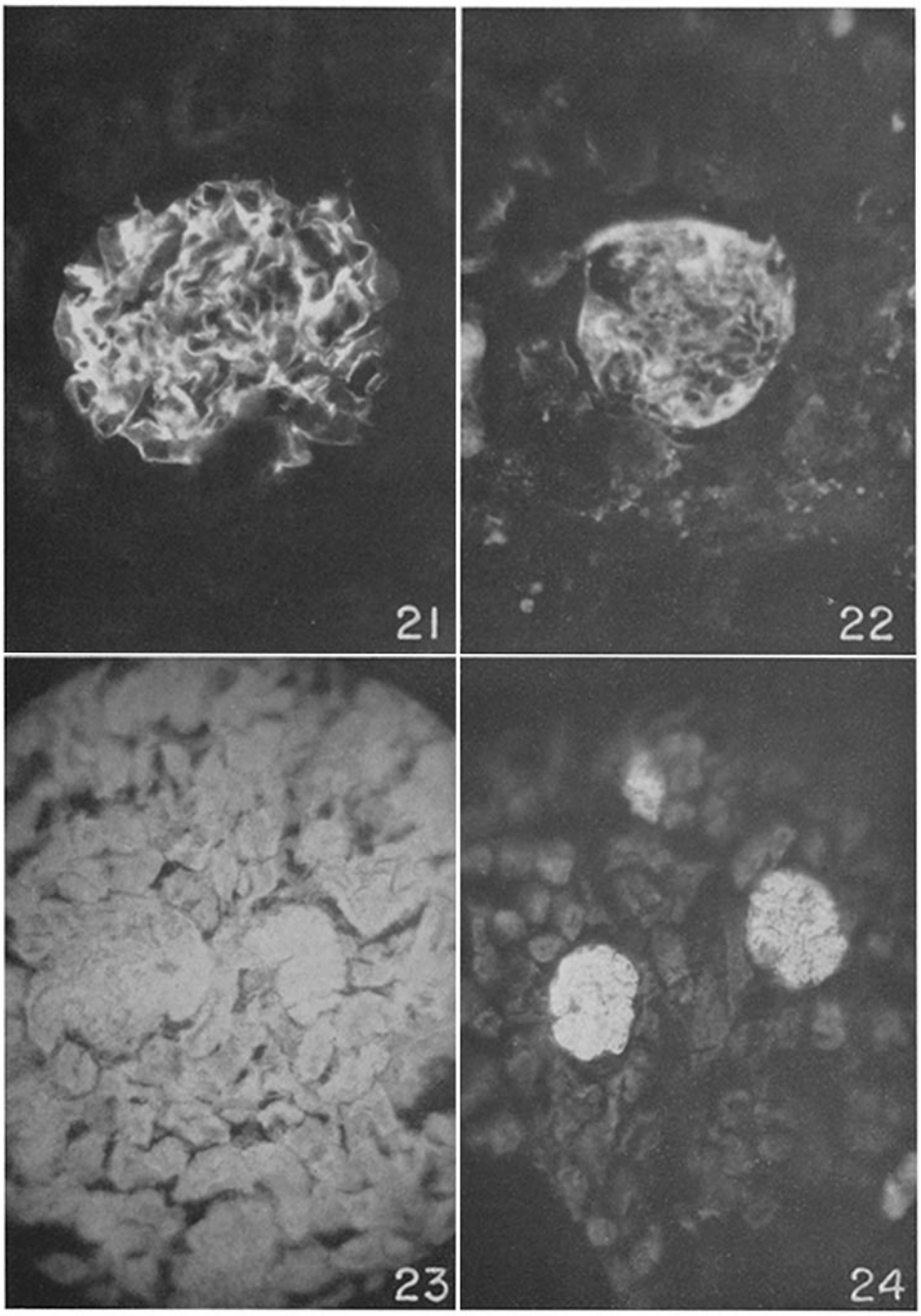
PLATE 8

FIG. 21. 13 weeks. Section cut in cryostat at 5 μ and stained with Goat-anti-Rab. G/T = 3.6. The pattern of nephrotoxic antibody localization is essentially unchanged. × 225.

FIG. 22. 13 weeks. Stained with Rab-anti-Rat. G/T = 3.1. The central glomerulus is one of several in this section that showed a higher peripheral concentration of autogenous antibodies. Numerous autofluorescent particulates are present in the tubular epithelium. × 220.

FIG. 23. 4 days. Stained with unabsorbed Goat-anti-Rab. Two glomeruli are present in the center of the field but they are barely distinguishable from the tubules. × 100.

FIG. 24. A section adjacent to that depicted in Fig. 23, but stained with absorbed Goat-anti-Rab. Note the marked reduction in non-specific staining and the corresponding enhancement of glomerular fluorescence relative to tubules. × 100.



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