EXPERIMENTAL GLOMERULONEPHRITIS

IV. PARTICIPATION OF COMPLEMENT IN NEPHROTOXIC NEPHRITIS*, ‡

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In nephrotoxic serum nephritis (NTN) the initial step in the disease, the reaction of heterologous antibodies with glomerular antigens, is followed by a number of host-mediated processes. One of the most significant of these, the participation of host complement (C') in the glomerular based antigen-antibody reactions, has already been the subject of several studies.

Serum C' levels have been known to drop after the injection of mammalian nephrotoxic sera (NTS) into experimental animals (1, 2). Glomeruli of rats injected with rabbit NTS will fix heterologous C' *in vitro* (3). Host C' has been detected by fluorescent antibody techniques in the glomeruli of rats injected with rabbit NTS (4, 5). De-complementation prior to the injection of rabbit NTS produces an amelioration of the initial kidney disease in rats (6, 7). Pepsin- or papain-degraded fragments of rabbit NTS have neither the ability to cause renal damage nor to fix C' (8, 9). The injection of duck NTS neither lowers circulating C' greatly (10), nor results in C' binding in glomeruli (6, 11) which has been linked with the inability of some duck NTS to produce renal injury (11–13).

The studies to be reported extend these observations on the role of C' in NTN produced in the rat by either rabbit NTS or duck NTS. In addition, the course of C' reactions in the glomeruli in NTN is used as a measure of the course of immunologic events in this disease. These observations give the first indication of the duration of immunologic reactions in the chronic form of this nephritis. Finally, the differences among the roles played by C' in NTN produced by various rabbit and duck NTS suggest the possibility of different mechanisms of mediation of injury caused by these sera.

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Materials and Methods

In producing NTN, we have followed the same methods previously reported (6). In all the experiments, the nephritis was induced by one intravenous injection of NTS. Sprague-Dawley rats (Holtzman Company, Madison, Wisconsin), weighing 130 to 180 gm, were used. Collection and determination of protein in the urine were done as in the previous report (6). Concentration of protein in the urine was expressed as milligrams per 24 hour period, and in normal rats of the size employed it never exceeds 15 to 20 mg.

In order to obtain NTS, rabbits and ducks were immunized with the insoluble fraction of perfused rat kidney (6). The rabbits were immunized by intraperitoneal injection of the antigen in 0.15 M NaCl, the ducks were immunized by intramuscular injection of the antigen in complete Freund's adjuvant (with *Mycobacterium butyricum*) as recommended by Hasson *et al.* (14). The rabbit NTS were tested by injecting 1.0 ml into rats and examining for proteinuria. The NTS which induced an immediate and continuous proteinuria were pooled. This pool we have labeled as potent NTS and used in these experiments in doses of 0.6 to 1.0 ml which gave immediate proteinurias of 80 to 150 mg. The rabbit NTS which induced a proteinuria after a lag period of 7 to 10 days were also pooled. This second pool of rabbit NTS we have labeled as weak NTS and used in doses of 1.0 to 2.0 ml which gave a delayed proteinuria comparable to that seen with potent NTS. In experiments to be reported, it was found by labeling NTS with I^{131} that the per cent of gamma globulin fixing in the rat kidney was two times greater in the potent NTS than in the weak NTS. Two individual duck NTS were used in these experiments. Both induced an immediate and continuous proteinuria of 100 to 350 mg at doses ranging from 0.5 to 1.0 ml. At a 0.3 ml dose, proteinuria was present for only the first 2 to 3 days. All the NTS were decomplemented by heat (56°C for 30 minutes) and absorbed with rat serum and rat erythrocytes. They were stored frozen until use.

Rat gamma globulin (rat GG) was prepared by DEAE cellulose fractionation as recommended by Sober et al. (15). A sodium phosphate buffer at pH 6.4 with a molarity of 0.0175 was used as the eluant. Also, rat GG and beta globulins were obtained by preparative electrophoresis in a pevikon block (16). Rat fibrinogen was prepared by the method of Kekwick (17) for human fibrinogen. Rat albumin was prepared by the method of Schwert (18). Rabbit gamma globulin (rabbit GG) was obtained commercially (Pentex Inc., Kankakee, Illinois, lot 28). Human fraction II was also obtained commercially (E. R. Squibb and Sons, New York, lot 1979). Duck gamma globulin (DGG) was obtained by a 40 per cent ammonium sulphate fractionation of pooled normal duck serum. Antisera against rat GG, albumin, fibrinogen, human fraction II, and DGG were prepared in rabbits by injections of the antigens in incomplete Freund's adjuvant (total of three injections containing 5 to 10 mg of protein each at weekly intervals). Antiserum against rabbit GG was prepared in sheep and rats by similar procedures. All the antisera were tested by immunoelectrophoresis, using the microtechnique of Scheidegger (19). The antiserum against rat GG contained antibodies to 7S gamma globulin and to a beta-2-protein. The antiserum against fibrinogen contained antibodies against fibrinogen and also traces of antibodies against rat GG. The antisera against rabbit GG and DGG had antibodies against 7S gamma globulins and beta globulin.

The antiserum against rat beta-1-C (β1C) was prepared in rabbits by repeated injection of a precipitate formed by equivalent amounts of rabbit anti–bovine serum albumin (BSA) and BSA in the presence of fresh rat serum. The precipitate was allowed to form for 1 hour at 37°C and 4 hours in the cold; it was then washed five times with veronal buffer (pH 7.2 containing Ca. and Mg. ions) and incorporated into incomplete Freund's adjuvant for immunization. The individual antisera that showed lines by immunoelectrophoresis against β1C only were selected for further studies. There is evidence that β1C in the rat, as in the human and guinea pig, is part of the third component of C' (20, 21). On immunoelectrophoresis, β1C in fresh rat serum shows an arc in the middle of the beta area and converts to a β2 protein on aging, or after ad-

dition of an immune precipitate to the rat serum. Rabbit anti–guinea pig $\beta 1C$ was prepared in a similar way. In all the experiments, the detection of fixed $\beta 1C$ in the tissues was interpreted as a part of the events in the fixation of C'. It is assumed that $\beta 1C$ fixes to an immune reactant only if there is previous fixation of C'142. Rabbit anti–guinea pig $\beta 1C$ did not crossreact with rat $\beta 1C$ as tested by immunoelectrophoresis. Kidneys of rats with NTN which contained rat $\beta 1C$ in the glomeruli were negative when stained with fluorescent anti–guinea pig $\beta 1C$.

For the fluorescent antibody technique, we employed the methods as reported previously (6). All the antisera were conjugated to fluorescein isothiocyanate, and were absorbed with liver powder before use. The sheep anti-rabbit GG, the rabbit anti-rat fibrinogen, and the rabbit anti-DGG were absorbed with rat GG prior to their use. The rabbit anti-rat GG was absorbed with rat beta globulins before use so only antibodies to rat 7S gamma globulin remained. The specificity of the rabbit anti-rat $\beta 1C$ was controlled by absorbing out the antibody with a precipitate formed as described above; *i.e.*, a precipitate formed by adding equivalent amounts of BSA and rabbit anti-BSA in the presence of fresh rat serum. The antiserum was also absorbed with a similar precipitate except that heated, instead of fresh, rat serum was used. A kidney slide from a rat with NTN of 1 week duration that stained positive with fluorescent anti-rat $\beta 1C$, also stained with anti-$\beta 1C$ absorbed by a precipitate formed in the presence of heated rat serum. The staining reaction of the anti-rat $\beta 1C$ was abolished by absorption with a precipitate formed in the presence of fresh rat serum (Figs. 1 a and 1 b).

Kidneys from rats receiving the different preparations of NTS were examined by fluorescent antibody methods daily during the first 2 weeks following the injection and every 2 to 3 weeks thereafter. Each kidney was examined for localization of rat GG, fibrinogen, and albumin, and for the heterologous nephrotoxic gamma globulin.

Three methods were used to study C' and C' fixation in the glomeruli. First, direct examination of the kidney section with fluorescent anti-rat $\beta 1C$ was done to determine the presence or absence of host C'. Secondly, at varying times after the injection of NTS, 5 ml of fresh guinea pig serum was injected intravenously into the rats. The recipients were then sacrificed 3 hours later and their kidneys examined with fluorescent antibody to guinea pig $\beta 1C$. As controls, rats were injected with similar amounts of guinea pig serum that had been heated at 56°C for 1 hour. To establish that there was fixation of guinea pig $\beta 1C$ to the glomeruli, three criteria were followed: (a) the majority of the glomeruli had to contain detectable guinea pig $\beta 1C$; (b) the detectable guinea pig $\beta 1C$ had to be present in a capillary pattern (see Results for description) (Figs. 2 a and 2 b); and (c) the control NTN rats injected with heated guinea pig sera had to have little, if any, detectable $\beta 1C$ in a capillary pattern. This procedure revealed the presence or absence of C'-fixing materials in kidneys, capable of reacting with circulating guinea pig C'. Thirdly, frozen kidney sections were exposed *in vitro* to fresh guinea pig serum (diluted 1 to 10) and then stained with fluorescent antibody to guinea pig $\beta 1C$. This is a similar procedure to the one described by Goldwasser and Shepard (22) and by Klein and Burkholder (5) for detection of tissue reactants capable of fixing C' *in vitro*. By this procedure, the sectioning of the tissue may have exposed materials anatomically not available for reaction with *in vivo* circulating C'.

Also studied by fluorescent antibody were kidneys of rats made nephrotic by daily injections of aminonucleoside of puromycin (Nutritional Biochemicals Corporation, Cleveland). Rats were injected subcutaneously with 1 mg of aminonucleoside daily for 6 to 10 days until proteinuria developed. They were then sacrificed for the fluorescent antibody studies.

The participation of C' in NTN was prevented by either removing the host's serum C' with heat-aggregated human gamma globulin (HGG) or zymosan (Fleischmann Lab., New York, type A, lot 7B651), or by impairing the C'-fixing ability of the rabbit NTS. Heat-aggregated HGG was prepared by the method described by Christian (23). It was injected intravenously in amounts from 2.5 to 3.6 mg of N. Zymosan was activated as described in Kabat and Mayer

(24) and suspended in 0.15 M NaCl at a concentration of 2 mg/ml. The potent rabbit NTS was treated with 2-mercaptoethanol to reduce its C'-fixing properties. In a preliminary experiment, a potent rabbit NTS was fractionated in a sucrose gradient according to the technique described by Kunkel (25). The different layers were collected and injected into rats to determine their nephrotoxic effect. It was established that all the nephrotoxic activity was localized in the light antibody (7S) fraction. The potent rabbit NTS was dialyzed against 0.1 M mercaptoethanol for 2 to 24 hours at room temperature and then dialyzed against 0.01 M iodoacetamide for 48 hours in the cold. A control preparation was dialyzed against 0.15 M NaCl at room temperature and then against 0.01 M iodoacetamide in the cold. Each preparation was tested by quantitative C' fixation with homogenized kidney using guinea pig C'. Most of the C'-fixing properties were lost in the mercaptoethanol-treated NTS, regardless of the time dialyzed against mercaptoethanol. In a typical experiment 38 $C'H_{50}$ units were present in the system; 21.3 units were fixed by the control NTS and 2.0 by the mercaptoethanol treated NTS. These treated rabbit NTS were then injected into rats to determine their biologic activity.

The hemolytic titers of serum complement ($C'H_{50}$) were determined by using the 50 per cent end point, as described in Kabat and Mayer (24). Rats were bled either from the tail vein or the retrograde venous plexus of the eye into acid-cleaned tubes. After 1 hour at room temperature, the blood was centrifuged and the serum removed and frozen at $-40°C$. Experiments were performed to determine the changes in serum C' at various times after the injection of NTS. For this purpose, representative rats receiving duck NTS or rabbit NTS were bled daily; however, no rat was bled more than twice during the course of this experiment. Control groups injected with normal rabbit serum and normal duck serum were included in these experiments. An additional group given a daily injection of aminonucleoside of puromycin, as previously described, until proteinuria developed, was bled periodically to determine the variation of the C' levels in relation to non-immunologically induced proteinuria.

Quantitative C' fixation by NTS reacting with kidney antigen was measured by the method of Mayer et al. (26) using guinea pig serum as a source of C' and incubating at 37°C for 1 hour. The total volume of the reaction was 10 ml. In each experiment, control tubes were set up with kidney antigen or the NTS alone. The number of $C'H_{50}$ units fixed by the antigen and the NTS alone were subtracted from the number of $C'H_{50}$ units fixed in the whole system. The antigen was the insoluble fraction of homogenized kidney which was sonicated (Raytheon sonic oscillator of 10 kc for 20 minutes), washed five times with veronal buffer before use, and employed at a concentration of 10 mg per ml. The kidney antigen had anticomplementary activity. NTS from duck and rabbit were diluted 1 to 10 with veronal buffer. Preliminary tests showed these concentrations to be the optimum for the fixation of C'. The rabbit NTS had some anticomplementary activity that was abolished by centrifuging at 105,400 g for 1 hour and discarding the lower fourth of the tube. The duck NTS showed anticomplementary activity which was not abolished by ultracentrifugation for 1 hour, or absorption with mouse liver powder (50 mg powder/ml serum) (27).

<div align="center">RESULTS</div>

Experiments on Normal Rats.—

Immunohistochemical studies: In rats injected with potent rabbit NTS, rat $\beta1C$ was detected concentrated in the glomeruli as early as 2 hours after the injection. There was an increase in the amount of stainable $\beta1C$ by the 2nd or 3rd day after which the staining intensity did not vary. $\beta1C$ was deposited as a fine line that followed the glomerular capillary walls. Rabbit GG was deposited in a similar pattern. This pattern, which we will refer to as "capillary

pattern," appears to result from the uniform fixation of immunologic reactants along the glomerular capillary wall (Figs. 3 and 4). Thus, the early reaction of host C' during the first days of NTN is anatomically related to the fixation of heterologous nephrotoxic antibody in the glomeruli. During the first 5 to 8 days, rat GG was deposited in a focal manner in the axial or mesangial region of the glomeruli and not along the capillary walls (Fig. 5). Additional observations showed that with a heavy proteinuria of non-immunologic nature (aminonucleoside nephroses) rat GG, rat β1C, and rat fibrinogen were always present in this mesangial pattern. The amount of protein detected in a mesangial pattern was in direct proportion to the degree of proteinuria. After 5 to 8 days following the injection of NTS, rat GG was deposited in a capillary pattern. Observations made 4 months after the injection of rabbit NTS showed that rabbit GG, rat GG, and rat β1C were still bound apparently undiminished in a capillary pattern.

In rats injected with weak rabbit NTS, a small amount of rat β1C was also detected early in the glomeruli in the absence of immediate proteinuria. At 24 hours, rat β1C was detected in 70 per cent of the glomeruli in a faint capillary pattern and in the remaining 30 per cent in a mixed mesangial and capillary pattern. The development of heavy staining for rat β1C in a capillary pattern coincided with the appearance of proteinuria and rat GG in a capillary pattern by 5 to 8 days after injection of NTS.

In rats injected with 1 ml of duck NTS, rat GG and rat β1C were observed in the glomeruli during the first 5 to 7 days only in a mesangial pattern. Subsequently, they appeared in a heavy capillary distribution. Rat β1C, rat GG, and DGG persisted in the glomeruli throughout the whole length of the disease. If the dose of duck NTS was lowered so only an initial, transitory proteinuria was induced, rat GG or rat β1C were found only in very small amounts in a mesangial pattern.

The kidneys of rats receiving potent or weak rabbit NTS and duck NTS were also stained for fibrinogen and albumin. Fibrinogen or fibrin did not appear to be involved in the primary lesion of the glomerular capillary walls. Rather when present early the fibrinogen was in mesangial and extraglomerular sites. In the advanced nephritic kidneys fibrinogen was concentrated in the areas of hyalinization or fibrosis, particularly periglomerular crescents. Rat albumin was rarely localized in the glomeruli except in areas of scarring or hyalinization.

Studies of *in vitro* tissue fixation of guinea pig C', in general, bore out the observation made by direct staining for rat β1C. *In vitro* C' fixation was strongly positive in a capillary pattern in the glomeruli of rats that had received a full dose of potent rabbit NTS, regardless of the time lapsed after injection. C' fixation was positive but to a lesser degree in rats receiving a subnephritogenic dose of potent NTS.

In rats receiving duck NTS there was no *in vitro* C' fixation within the first

few days after injection. Fixation occurred in the kidneys taken in the chronic stage only when rat GG became localized in a capillary pattern in the glomeruli (Fig. 6). In rats injected with a dose of duck NTS, insufficient to provoke a

TABLE I

Relationship between in Vitro Complement Fixation and Host $\beta 1C$ in Glomeruli of Rats with NTN

Nephrotoxic sera	Dose	Time after injection	Nephrotoxic GG	Rat GG	Rat $\beta 1C$	In vitro fixation of guinea pig $\beta 1C$
	ml					
Rabbit NTS	1.0	2 hrs.	Pos.	Neg.	Pos.	Pos.
Rabbit NTS	1.0	day 3	Pos.	Neg.	Pos.	Pos.
Rabbit NTS	0.25	day 3	Pos.	Neg.	Neg.	Pos.
Rabbit NTS	1.0	day 30	Pos.	Pos.	Pos.	Pos.
Duck NTS	1.0	2 hrs.	Pos.	Neg.	Neg.	Neg.
Duck NTS	1.0	day 11	Pos.	Pos.	Pos.	Pos.
Duck NTS	0.3	day 11	Pos.	Neg.	Neg.	Neg.
Duck NTS	1.0	day 20	Pos.	Pos.	Pos.	Pos.

Heterologous globulin, rat GG, and rat $\beta 1C$ detected by fluorescent techniques.

Positive refers to capillary pattern (see text). Tissue C′ fixation done by exposing slides to guinea pig serum (1 to 10) and staining with fluorescent anti–guinea pig $\beta 1C$.

TABLE II

Complement Levels in Rats Given NTS ($C'H_{50}$ units/ml serum)

Time after injection	Rabbit NTS (potent)	Rabbit NTS (weak)	Duck NTS	Normal rabbit serum	Normal duck serum
3 hrs.	9.9(2)	—	31.3(2)	39.3(2)	38.8(2)
day 1	10.7(5)	12.5(2)	28.3(4)	45.8(2)	33.5(2)
day 2	15.4(2)		40.0(1)	—	—
day 3	14.5(2)	16.6(2)	31.2(1)	39.6(2)	40.8(2)
days 5–6	6.2(4)	29.8(3)	18.4(3)	29.8(2)	36.8(2)
	30.4(4)		44.6(1)		
day 7	27.1(6)	44.0(3)	29.9(2)	43.0(2)	45.0(2)
day 12	38.2(3)	42.5 (2)	42.8(2)		

(Figures in parenthesis indicates number of animals.)

host antibody response (0.3 ml), *in vitro* C′ fixation never occurred. These results are summarized in Table I.

Serum complement levels: Some of the results of the serum C′ determinations are shown in Table II. The rats given aminonucleoside, which causes a presumably non-immunologic nephrosis (28–30), had an average drop in the $C'H_{50}$ levels from normal values of 40 to 50 to 27 coincident with the onset of pro-

teinuria. Although proteinuria persisted, the C' levels returned to normal within 4 or 5 days.

There was a marked difference in the C' levels between the group receiving duck NTS and those receiving rabbit NTS. By 24 hours after injection, C' levels of rats receiving either the potent or weak rabbit NTS fell approximately 70 per cent and recovered only slightly during the next 5 to 6 days. C' levels returned to normal between 7 and 12 days after injection. Four of eight rats receiving potent NTS experienced an additional sharp transitory fall in C' levels at 5 to 6 days after NTS injection possibly related to the appearance of the host antibody response to rabbit serum. Twenty-four hours after injection, C' levels of rats receiving duck NTS fell approximately 30 per cent, which may

TABLE III

Quantitative Complement Fixation with Nephrotoxic Serum

Antigen	Antibody	Total $C'H_{50}$ units fixed	$C'H_{50}$ units fixed by Ab
Kidney homogenate	VB	10.9	—
VB	Rabbit NTS (1/10)	0.5	—
Kidney homogenate	Rabbit NTS (1/10)	32.2	20.8
VB	Duck NTS (1/10)	9.7	—
Kidney homogenate	Duck NTS (1/10)	24.4	3.8

Source of C' is guinea pig serum, 38 $C'H_{50}$ units used throughout. Kidney homogenate at a concentration of 10 mg per ml. Volume of 10 ml each tube. Fixation at 37°C for 1 hour. Results are average of duplicate tubes. VB, veronal buffer.

be related to the proteinuria exhibited by these rats. After the initial fall, C' levels were maintained near normal for 5 to 6 days after the injection. At that time, three of four rats experienced a transitory drop in C' again probably related to a host antibody response. C' levels were back to normal by 7 to 10 days after the injection of duck NTS.

The control rats injected with normal duck serum showed a drop of C' to 33 $C'H_{50}$ within 24 hours followed by a return to normal. The group of animals injected with normal rabbit serum had normal C' levels except for a transitory slight drop at day 5.

Quantitative complement fixation: Several experiments were done to determine the ability of potent and weak rabbit NTS and duck NTS to fix guinea pig C' in the presence of kidney homogenate. The results of one of such experiments are noted in Table III. In this experiment, potent rabbit NTS fixed 21 of 38 $C'H_{50}$ units of the system while duck NTS showed fixation of only 3.8 $C'H_{50}$ units after subtracting the anti-complementary activity of the antigen and the duck serum. There was a difference in the amount of C' fixed between the potent and

weak rabbit NTS. In one experiment of 95.2 $C'H_{50}$ units in the system, 50 were fixed by the potent NTS and 34.1 by the weak NTS.

Experiments on Decomplemented Rats.—Rats were decomplemented using the method previously reported by Hammer and Dixon (6); *i.e.*, by the intravenous injection of heat-aggregated HGG. This procedure removes nearly all circulating C' within 15 minutes and levels remain low for 6 to 12 hours. One hour after injection of the aggregates, the rats were challenged by the intravenous injection of potent NTS. It was important for a time interval to elapse

TABLE IV

Effect of Aggregated HGG on Rats Receiving Rabbit NTS

Injection	No. of rats	Urinary Protein/24 hrs.		
		Day 1	Day 3	Day 6
		mg	*mg*	*mg*
1. Rabbit NTS...................	14	2.8	11.8	11.6
2. Agg.-HGG.....................	10	1.7	2.5	4.1
3. Rabbit NTS + agg. HGG........	15	65.8	26.6	21.6
4. Rabbit NTS + fr. II............	4	6.7	4.3	4.8
5. Agg.-HGG + rabbit NTS........	6/12	7.9	38.0	69.0
	6/12	43.9	47.0	86.3
6. Rabbit NTS...................	9	114.4	88.2	90.6

1. Potent rabbit NTS, 0.25 ml.
2. Heat aggregated-HGG, 3.5 mg N.
3. Potent rabbit NTS, 0.25 ml and Agg.-HGG, 3.5 mg N, injected simultaneously.
4. Potent rabbit NTS, 0.25 ml and human fraction II, 4.0 mg N, injected simultaneously.
5. Agg.-HGG, 3.5 mg N, injected 1 hour before 1.0 ml of potent rabbit NTS.
6. Potent rabbit NTS, 1.0 ml.

between injection of aggregates and NTS presumably to allow for elimination of circulating aggregates and serum C'. If NTS was injected simultaneously with the aggregates, the renal injury was actually enhanced. The simultaneous injection of aggregated HGG with a subnephritogenic dose of NTS (0.25 ml potent NTS) produced proteinuria as noted in Table IV. Neither 0.25 ml of NTS nor the aggregated HGG alone was capable of producing proteinuria.

In most rats challenged with potent rabbit NTS 1 hour after the injection of aggregated HGG, there was amelioration of the initial kidney injury as judged by proteinuria. Proteinuria, if present at all, was minimal to moderate in amount for the first 4 to 5 days. This varied from rat to rat and occasionally heavy proteinuria appeared by the 2nd or 3rd day after the injection of NTS. One of such experiments is shown in Table IV. Twenty-four hours after decomplementation and rabbit NTS injection, six of twelve rats showed normal amounts of protein in the urine. The remaining six rats showed moderate pro-

teinuria though still far less than the control groups which were not decomplemented prior to NTS injection. Fluorescent antibody studies were done on tissues taken daily in the 1st week of the disease from these and other similarly treated rats. In rats preinjected with aggregated HGG rat β1C was slow in binding to the glomeruli in spite of the immediate fixation of rabbit GG to the glomeruli. At 24 hours little β1C was demonstrable in the glomeruli. When present, it was found predominantly in a mesangial distribution. No relationship could be established in these rats with borderline proteinuria between the amount of C′ in the glomeruli and the level of proteinuria. In the majority of rats, β1C staining became intense and capillary in pattern by 6 days after the NTS injection. This intense C′ staining usually correlated with the appearance of rat GG and heavy proteinuria. However, in a few rats, β1C in a capillary pattern was present 1 to 2 days before rat GG could be seen. Also, in a few, proteinuria preceded the appearance of a heavy staining reaction of rat GG or β1C by 1 to 2 days.

Serum C′H$_{50}$ levels were determined in rats decomplemented by aggregated HGG and challenged with NTS. At 24 hours after the injection of NTS, the C′H$_{50}$ levels averaged 10.8 units. For the next 2 to 4 days, the C′ levels fluctuated around 20 to 25 units. Five to 6 days after injection the C′ levels dropped to an average of 6.4 C′H$_{50}$ units in four of seven rats. C′ levels returned to normal by day 7 and experienced no further drop. Rats decomplemented but not challenged with NTS had normal C′ levels at 24 hours after injection and for the next 10 days of observation.

An experiment was done to determine the importance of the host antibody response to the injected rabbit GG in the appearance of the delayed heavy proteinuria in the decomplemented rats. Twenty-four hours after the injection of the aggregated HGG and NTS, the rats were challenged with an intravenous injection of rat serum containing antibody to rabbit GG (4 ml containing 400 μg of antibody nitrogen per ml). Large concentrations of rat GG and β1C were found in the glomeruli within 24 hours after injection of anti-rabbit GG and proteinuria appeared within 48 hours, several days before the host's own anti-rabbit GG response would have induced proteinuria. Thus, when rabbit NTS reacts with glomerular antigens in the absence of serum C′, full blown NTN does not develop even with return of considerable serum C′ until antibody to rabbit GG appears.

To further evaluate the role of C′, another method of decomplementation was tried. It was an intravenous injection of activated zymosan. Two hours after the injection of 10 mg of zymosan, at a time when at least a 50 per cent fall in serum C′ would be expected (31), the rats were given NTS. The combination of zymosan and NTS was toxic and of 14 rats, seven died with disseminated hemorrhages. However, the remaining seven had an ameliorated kidney disease similar to the rats decomplemented with HGG aggregates.

Evaluation of the role of C′ was also made using rabbit NTS that had been

treated with mercaptoethanol to reduce its C'-fixing properties. Rats were injected with mercaptoethanol-treated NTS (0.9 ml) and control NTS treated with NaCl (0.9 ml). Rats from each group were bled 6 hours after injection to determine their $C'H_{50}$ levels. In four rats given the control NTS, the serum C' levels dropped to 8 to 15 $C'H_{50}$ units, with an average of 11.3. The serum C' levels of the four rats given mercaptoethanol-treated NTS dropped to 20.7 to 28.5 $C'H_{50}$ units, with an average of 24.8. An additional group of eight rats injected with mercaptoethanol-treated NTS showed that six had an initial mild disease while two responded similarly to the controls, given untreated NTS. One week after injection all these rats developed heavy proteinuria which persisted for a further period of observation of three weeks. These observations are summarized in Table V. The kidneys 24 hours after injection of mercapto-

TABLE V

Proteinuria of Rats Receiving Rabbit NTS Treated with Mercaptoethanol

	Treatment	Dose	No. of rats	Urinary protein/24 hrs.		
				D 1	D 3	D 6
		ml		*mg*	*mg*	*mg*
NTS	None	0.9	9	139.3	110.0	126.8
NTS	Mercaptoethanol	0.9	7/9	19.0	31.6	80.3
			2/9	135.0	95.0	88.5

ethanol-treated NTS showed on fluorescent antibody study small amounts of β1C in mesangial and focal capillary patterns. Rabbit GG was detectable in the glomeruli in a capillary pattern. Tissue C' fixation on the kidneys of these animals showed weakly positive results.

Studies on the Duration of the Immunologic Reaction in the Kidneys.—Experiments were done in which fresh guinea pig serum was administered intravenously to rats with NTN induced by rabbit NTS. The fixation of guinea pig β1C in the glomeruli in a capillary pattern was taken as evidence of an immunologic reaction taking place in the glomerular capillary wall. Preliminary observations showed that in the 1st week of NTN, injected guinea pig β1C fixed to the glomeruli within 3 hours in a capillary pattern similar to that for rabbit GG, rat GG, or rat β1C. That the guinea pig β1C was immunologically fixed in the glomerulus was suggested by both its pattern and its persistence in this site for at least 40 days.

Control experiments employing heated (56°C for 30 minutes) guinea pig serum showed no stainable guinea pig β1C in the glomeruli. Finally, to establish the immunologic nature of the C' fixation with guinea pig serum, similar observations were carried out in rats made nephrotic by injection of aminonu-

cleoside of puromycin. Only small amounts of guinea pig β1C were seen and these were in a mesangial pattern quite different from that seen above.

In the first experiment, rats, during the first 2 weeks after injection of potent NTS, showed clear evidence of binding of injected guinea pig β1C in a capillary pattern. Eighteen or 21 days after injection of NTS, this binding was borderline. Thereafter, all observations, which extended through a 4 month period revealed guinea pig β1C only in a non-specific, mesangial pattern (Figs. 2 a and 2 b).

A second experiment was done in which rats with NTN of 32, 67, and 106 days duration were challenged with an intravenous injection of rat anti-rabbit GG (7 ml containing a total of 1.75 mg of Ab N) and 6 hours later were given guinea pig serum. In previous experiments it was established that the passively administered antibody promptly fixes to the rabbit nephrotoxic gamma globulin in the glomerular capillary walls. Control rats were injected with normal rat serum and then given the guinea pig serum. Other controls were given only guinea pig serum. All rats were sacrificed 3 hours after the injection of guinea pig serum. The kidneys of the rats given the passive antibody revealed guinea pig C' fixed in the glomeruli in a capillary pattern. Rats receiving no rat serum, or rats receiving normal rat serum, had no fixation of guinea pig β1C.

In a third experiment, guinea pig serum was given intravenously to chronically nephritic animals (71 days after NTS) which were being immunized with repeated subcutaneous injections of rabbit GG in incomplete Freund's adjuvant. These immunized rats showed binding of the injected β1C in the glomeruli. Negative results were obtained in this and the other groups, when the rats were given heated guinea pig serum.

DISCUSSION

Our experiments show that in NTN host C' fixes to the glomeruli during two periods or stages of the disease in general agreement with earlier observations. The first period of C' fixation is dependent upon the type and amount of the NTS and begins promptly after injection when the nephrotoxic antibody is reacting with its antigen in the glomerular capillary wall. The experiments with the potent rabbit NTS showed: (a) within hours localization of rat β1C in the glomeruli by the fluorescent antibody technique; (b) within hours a drop in the serum $C'H_{50}$ levels; and (c) soon after NTS, fixation of injected guinea pig β1C to the glomeruli. Also, with the rabbit NTS, in $vitro$ C' fixation was detected in tissue sections by fluorescent antibody and in kidney homogenates by quantitative C' fixation. The injection of weak rabbit NTS caused early fixation of much smaller amounts of β1C in the glomeruli than did potent NTS.

Further, our experiments show continuing fixation of C' to the glomeruli for a period longer than the minutes or hours usually assumed necessary for the binding of nephrotoxic antibody to tissue antigens. The evidence in favor of

this is as follows: (a) by fluorescent antibody, the amount of rat $\beta 1C$ detectable in the glomeruli increased progressively over the first 2 to 3 days after injection of rabbit NTS; (b) guinea pig $\beta 1C$ injected at any time within the first 2 to 3 weeks after injection of NTS promptly localized in the glomeruli; (c) in a few rats decomplemented before NTS injection, rat $\beta 1C$ appeared in the glomeruli 2 to 3 days after NTS and several days before rat GG was seen; and (d) the serum $C'H_{50}$ levels remained below normal during the first 4 days after the injection of the rabbit NTS. The reason for this prolonged period of C' fixation by a presumably relatively short lived antigen-antibody reaction is not clear. The level of serum C' presumably reflects immunologic reactions between NTS and appropriate antigens not only in the kidney but also in other tissues (1, 32, 33).

As previously reported, the duck NTS induced an immediate proteinuria in normal rats without detectable participation of C'. Prior decomplementation by aggregated HGG does not abolish the initial proteinuria induced by duck NTS (6). No rat $\beta 1C$ was demonstrable in the glomeruli in a capillary pattern by fluorescent antibody methods up to 5 to 7 days after the injection. During the first 7 days after injection there was no tissue C' fixation in the kidneys of rats injected with duck NTS. Also, there was little, if any, serum C' fixed with duck NTS and kidney homogenate as measured by the quantitative C' fixation technique. Benson (34) has shown that in order for guinea pig C' to fix to an antigen–avian antibody complex, it is necessary that avian $C'1$ be present in the system, which may account for our negative results. These observations raise the question of whether mediators other than hemolytic C' may be involved in the tissue injury produced by duck NTS. While it seems unlikely that such mediators could have been transferred in the small amounts of decomplemented duck NTS employed, this possibility is not entirely ruled out. Clearly, the duck NTS neither involves nor depends upon the rat's hemolytic C' for producing tissue injury in the same manner as rabbit NTS.

In the group of rats decomplemented and given rabbit NTS, it was expected that within a day after the NTS injection, $\beta 1C$ would be localized in the glomerular lesion along with the appearance of heavy proteinuria. However, what was found was a gradual fixation of small amounts of $\beta 1C$, as detected by fluorescent antibody, through a several day period without heavy proteinuria, despite significant levels of hemolytic C' in the serum. It may be that the optimal fixation of C' must take place concomitantly with the reaction between NTS and glomerular antigen(s), and the fixation of C' to a preformed immune complex in the glomerulus is slower and less injurious. This explanation is supported in part by certain *in vitro* experiments which have shown that with rabbit antibody, guinea pig C' fixes better to an antigen-antibody aggregate during its formation than to a preformed precipitate (35–37).

The experiments with rabbit NTS treated with mercaptoethanol showed that when the C'-fixing property of this serum was impaired, the initial kidney in-

jury was ameliorated. The secondary stage of the disease appears not to be affected, for rats became proteinuric 1 week after the injection. Impairment of *in vitro* C'-fixing ability of 7S gamma globulin without loss of antibody activity by mercaptoethanol treatment has been shown for the rabbit and human (38, 39). Pepsin and papain fragments of rabbit NTS which fix little if any C' produce no kidney injury early or late, although fixing briefly in the glomeruli (8, 9, 40).

The passive administration of guinea pig C' was used to allow us to follow the dynamics of the immunologic events in NTN; *i.e.*, to determine whether there was at any given time an antigen-antibody complex in the kidney capable of fixing detectable amounts of circulating C'. The assumption was made that guinea pig C' and rat C' would react similarly with antigen-antibody complexes. As long as the antigen-antibody reaction was proceeding the fixation of C' seemed likely. Once the antigen-antibody reaction reached a standstill the complex should become saturated with C' in a matter of days and fixation should stop. The results demonstrate that the fixation of detectable amounts of circulating C' is limited to the first two to three weeks of NTN. After this there appears to be a marked decline or cessation of C'-fixing immunologic activity in the glomeruli. Circulating C' will fix to the glomeruli of rats with NTN of longer duration only if the immunologic reaction is reactivated. Thus, C' fixes to the glomeruli of rats with long standing NTN if they have been recently given rat antiserum to rabbit GG or if they are being actively immunized to rabbit GG. The rat β1C detected in the glomeruli as late as 4 months after injection of NTS probably represents C' deposited during the first 2 to 3 weeks of NTN. Evidence in favor of this is given by the experiment in which injected guinea pig β1C was still detectable 7 weeks after injection. The immunologic reactants,—nephrotoxic gamma globulin, glomerular antigen(s), host antibody to nephrotoxic gamma globulin and C',—appear to be relatively inert, once deposited in the kidney, and persist for long periods. In this regard, they behave similarly to the antigen-antibody complex deposits in the kidneys in daily injection serum sickness (41).

It seems possible from these and other observations (6) that the immunologic events which take place in NTN during the initial 2 to 3 week period are capable of producing an irreparable damage to the glomeruli. The production of permanent and irreversible lesions appears to depend on the degree of immunologic trauma to the glomerular capillary wall. While not ruling out the possibility, these observations provide no support for the thesis that there is continuous antikidney antibody formation in NTN (12, 42, 43). If there is a late antikidney antibody response, it does not cause C' fixation in the kidney detectable by available methods and would therefore be either much smaller than the host antinephrotoxic gamma globulin response of the 2nd and 3rd weeks of the disease or perhaps involve a non–C'-fixing antibody.

The fluorescent antibody technique has been used extensively to identify

immunologic reactants in both clinical and experimental kidney diseases (44–50). On the basis of some of the present observations the interpretation of positive results obtained by this technique in relation to the nature and activity of the disease must be made with caution:

1. The concentration of host gamma globulin and β1C in glomeruli is not in itself presumptive evidence of an immune reaction. Non-immunologically induced proteinurias will cause concentration of these proteins in the glomeruli, principally in the mesangial regions. In order to have immunologic significance, gamma globulin and β1C should be present in a capillary pattern.

2. Gamma globulin and β1C in a capillary pattern of distribution, while suggestive of an immunologic event, do not necessarily signify a recent or active one. Thus, in NTN, rat GG, β1C, and the heterologous globulins persist in the glomeruli for long periods of time, far beyond the 2 to 3 week period of identifiable active immunologic reactions. In addition to NTN (49, 50), similar observations have also been made in nephritis induced by circulating immune complexes (41).

3. The tissue C′ fixation test is indicative of tissue reactants (aggregated gamma globulin or immune complexes) which are able to fix C′ in tissue sections *in vitro* but which do not necessarily correlate with a similar ability to fix detectable amounts of C′ *in vivo*. Apparently, the conditions *in vitro* are more favorable to fixation of C′ than are the *in vivo* conditions. First, the *in vitro* method may be more sensitive by virtue of greater concentrations of C′ available to the tissue. Secondly, sectioning of tissues may make available for reaction anatomic sites unavailable *in vivo*. Thus, *in vitro* tissue fixation of guinea pig C′ was noted in rats given doses of NTS which were insufficient to produce detectable *in vivo* binding of rat β1C. Chronic nephritic rats also showed *in vitro* tissue C′ fixation and no fixation of circulating guinea pig C′. Other examples of this discrepancy have already been reported by Vogt *et al.* and Lachman *et al.* (11, 51).

CONCLUSION

Rats with nephrotoxic serum nephritis may show fixation of complement (C′) in the glomeruli during two periods. The first period occurs immediately after the injection of the antisera and extends until the host response occurs. The second period is related to the deposit of rat gamma globulin in the glomeruli presumably occurring at the time of the immune response to the heterologous nephrotoxic serum (NTS). This second period terminates about 2 to 3 weeks after injection of NTS, presumably at the time the rat stops making antibodies to the nephrotoxic gamma globulin fixed in the glomeruli. After this period, the glomerular lesion is incapable of fixing detectable amounts of circulating C′ unless the antibody response to the heterologous globulin is reactivated. The immunological events which take place in the initial 2 to 3

week period appear to produce a permanent and irreparable lesion in the glomeruli leading to chronic nephritis.

Rabbit NTS produces fixation of rat C' during both periods. Duck NTS shows no detectable fixation of rat C' until rat gamma globulin fixes in the glomeruli at the time of the host immune response to the heterologous nephrotoxic gamma globulin. Decomplementation of rats injected with rabbit NTS produces an amelioration of the initial period of nephrotoxic serum nephritis.

The fixation of C' in the glomerular lesions was studied with the use of fluorescent antibody methods. The detection of host beta-1C globulin and gamma globulin in the glomerulus pointed to an immune reaction but not necessarily an active one. The tissue C' fixation test using kidney slices and guinea pig C' proved to be a more sensitive method of detecting tissue reactants capable of fixing C', but its relationship to *in vivo* events is not certain.

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EXPLANATION OF PLATES

PLATE 94

FIGS. 1 *a* and 1 *b*. Kidney sections from rat receiving potent rabbit NTS 1 week earlier. Fig. 1 *a*: Section stained with fluorescent anti-rat β1C. Glomerulus shows positive reaction predominantly in a "capillary pattern." Fig. 1 *b*: a similar section stained with fluorescent anti-rat β1C absorbed by antigen-antibody precipitate containing rat C′. Fluorescent staining has been blocked. Fluorescence micrograph × 250.

FIGS. 2 *a* and 2 *b*. Kidney sections from nephritic rats injected with guinea pig serum 3 hours before sacrifice. Sections stained with fluorescent anti–guinea pig β1C. Fig. 2 *a*: a glomerulus from rat receiving rabbit NTS 7 days before. Guinea pig β1C is distributed predominantly in a capillary pattern. Fig. 2 *b*: a glomerulus from a rat receiving rabbit NTS 32 days before. Guinea pig β1C is distributed largely in a mesangial pattern. Fluorescence micrograph × 250.
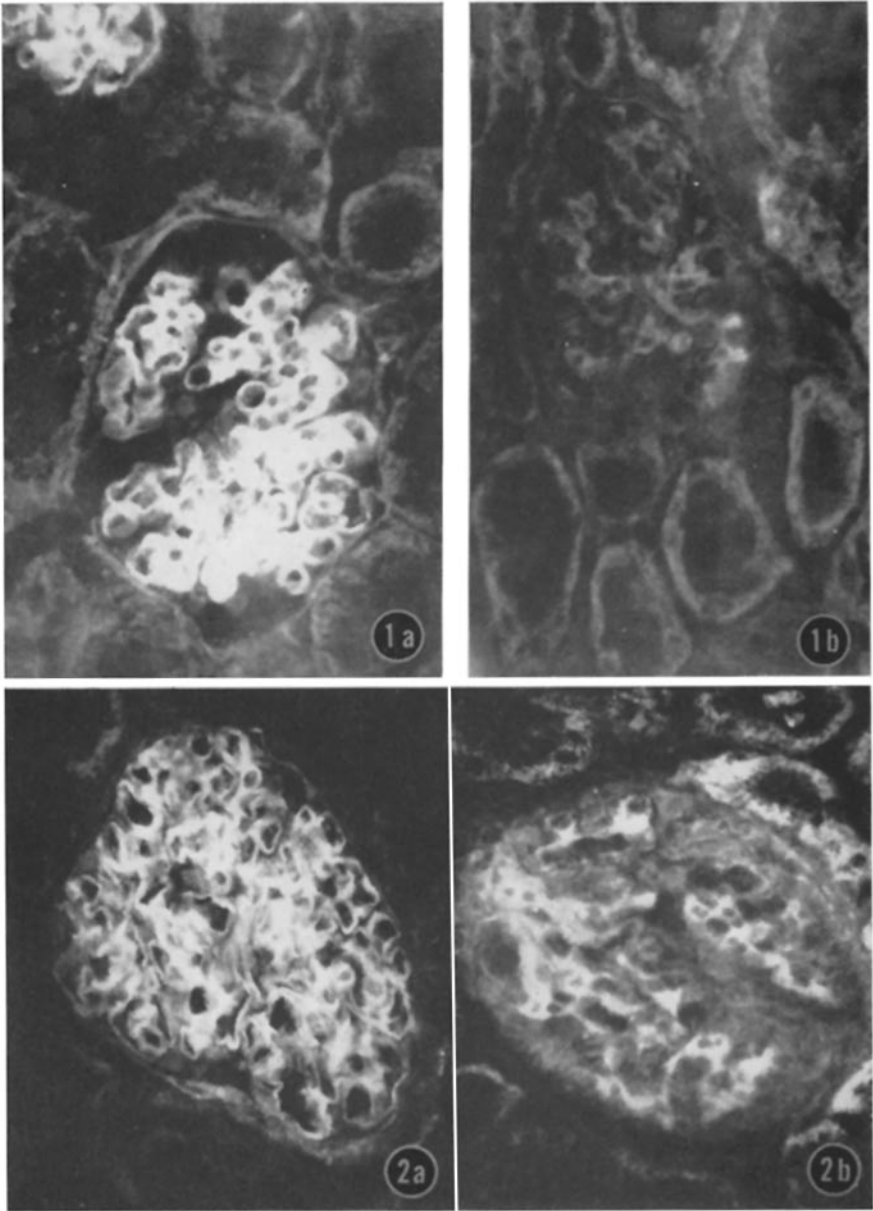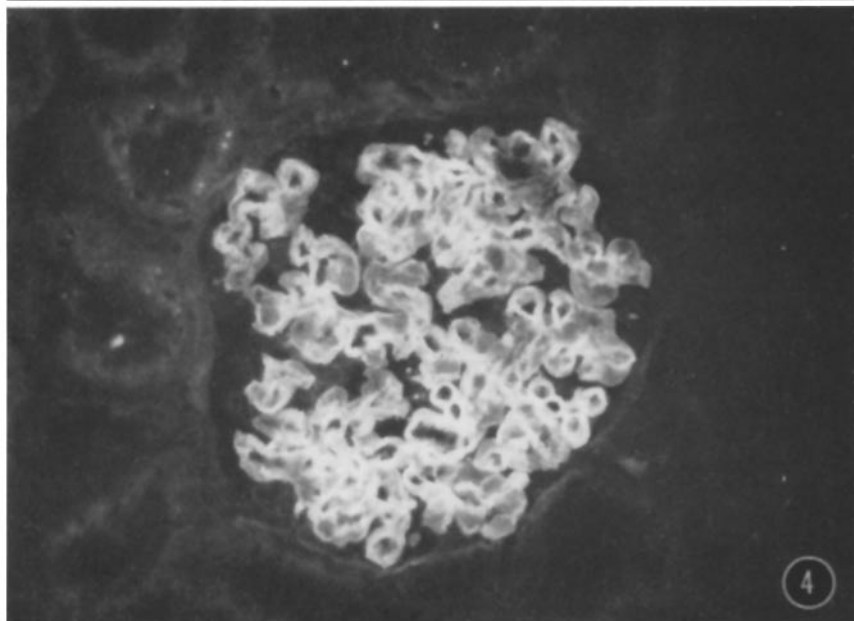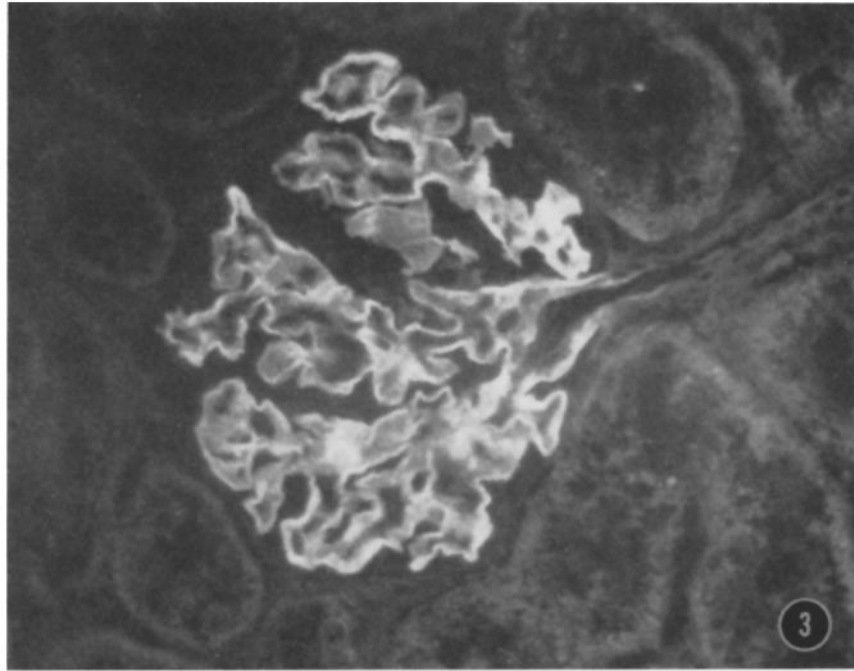
(Unanue and Dixon: Experimental glomerulonephritis. IV)

FIG. 3. Section of kidney from rat injected with rabbit NTS 10 days before, stained with fluorescent anti-rabbit GG. Glomerulus is stained exclusively in a capillary pattern. Fluorescence micrograph × 450.

FIG. 4. Section of kidney from rat receiving rabbit NTS 30 days before and stained with fluorescent anti-rat GG. Glomerulus stained in predominantly capillary pattern. Fluorescence micrograph × 350.
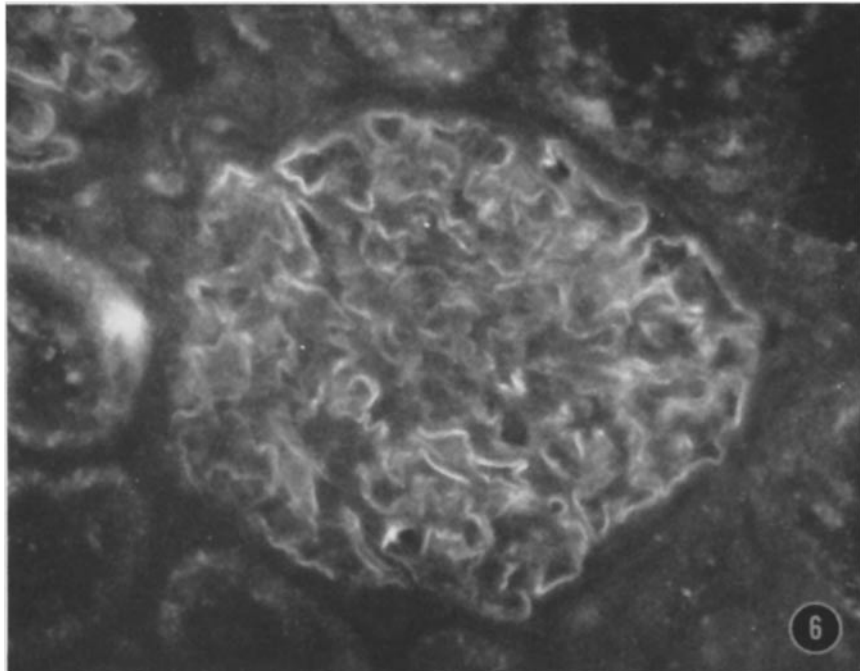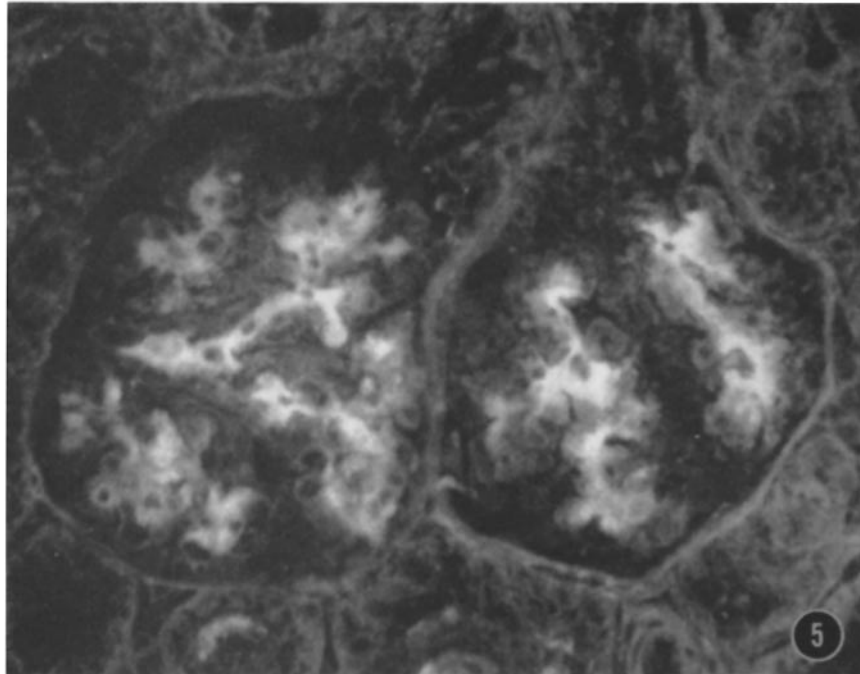
(Unanue and Dixon: Experimental glomerulonephritis. IV)

Plate 96

Fig. 5. Section of kidney from rat made nephritic by aminonucleoside, stained with fluorescent anti-rat GG. The two glomeruli show fluorescence in a mesangial pattern, with sparing of the capillary loops. Fluorescence micrograph × 450.

Fig. 6. Section of kidney from rat receiving duck NTS 11 days before. Section exposed to guinea pig serum *in vitro* (tissue C′ fixation) and stained with fluorescent anti–guinea pig β1C. Glomerulus shows positive capillary pattern. Fluorescence micrograph × 450.

(Unanue and Dixon: Experimental glomerulonephritis. IV)