THE PERMEABILITY OF THE RENAL GLOMERULI OF SEVERAL MAMMALIAN SPECIES TO LABELLED PROTEINS *

HANS SMETANA, M.D.
(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York 32, N.Y.)

While studying the origin of colloid droplets in urodeles, it was found that intravenous injection of proteins, labelled with diazotized dyes, was followed by the appearance of tiny granules, colored by these dyes, in the lining cells of the convoluted tubules of both the "closed" and "open" nephrons.[1] Because of this unanticipated finding, a systematic study was made to investigate the permeability of the renal glomerular filter of several mammalian species to labelled proteins.

MATERIALS AND METHODS

The various proteins to be used were coupled with the disodium salt of 2-naphtol-3 : 6 disulfonic acid (R salt) according to procedures described by Kabat and Heidelberger.[2] Nitrogen determinations were made with the micro-Kjeldahl method in duplicate. Three times re-crystallized egg albumin was prepared according to Heidelberger.[3] Purified solutions of serum albumin and serum globulin were prepared in the usual manner.

The preparations used in the experiments were as follows:

Neopeptone (Difco Laboratories, Detroit, Michigan)-R salt
Egg albumin (thrice re-crystallized)-R salt
Serum albumin (cat, dog, mouse)-R salt
Serum globulin (cat, dog, mouse)-R salt
Diazotized R salt alone, 0.12 per cent.

The different dilutions of these protein-dye compounds are given in the reports of the respective experiments.

PROCEDURES

White mice, white rats, albino guinea-pigs, albino rabbits, and mongrel dogs were injected intravenously with varying amounts of the above-mentioned protein-R salt preparations and were allowed to live for periods of time ranging from 30 minutes to 28 days after the injection. During this time, they were fed on the regular stock diets used for these various species.

After autopsy, the organs were fixed immediately in Zenker's fluid and in a 4 per cent solution of formaldehyde. The paraffin sections were examined either unstained but cleared, or stained with hematoxy-

* Received for publication, April 2, 1946.

lin only, or stained with hematoxylin and light green in order to provide a color contrast. The protein-R salt compounds present in the tissues remained unaltered for years and could easily be identified, even in minute amounts, by their intense bright red color.

The Distribution of Protein-R Salt Compounds in the Organs after Intravenous Injection

Mouse Series 1

Groups of 5 mice, each weighing 30 gm., were injected with 0.5 cc. of protein-R salt preparations and the animals were sacrificed 30 minutes, 1 hour, 2 hours, 3½ hours, and 24 hours after a single injection.

(a) Peptone-R salt, 0.2 mg. of N per cc. (21.7 mg. of protein per kg. of body weight). The findings in the organs were as follows: The Kupffer cells showed a faint reddish tint in animals killed 1 hour after the injection; definite red granules were present in these cells 3½ to 24 hours after the injection, and similar granules were found in a few reticulo-endothelial cells of the spleen. The urine of all animals had a definite reddish tint. Sections of the kidneys showed no trace of red matter.

A repetition of the experiment using a concentration of 0.3 mg. of N per cc. (27 mg. of protein per kg. of body weight) gave similar results. Peptone preparations proved to be toxic to mice so that injections had to be given slowly and in small portions.

(b) Egg albumin-R salt, 1.1 mg. of N per cc. (114.5 mg. of protein per kg. of body weight). The Kupffer cells and some of the reticulo-endothelial cells in the spleen and in various other viscera showed granules and small clumps of bright red matter 30 minutes after the injection. The lining cells of the proximal portion of the renal proximal convoluted tubules, including some of the lining cells of the spaces of Bowman, contained tiny bright red granules situated within the cytoplasm (see Figs. 1 and 2). These granules became more definite and abundant the longer the animals lived after the injection, and they appeared to be present in all of the lining cells of the proximal convoluted tubules. They were never observed in any other portion of the renal tubular system.

(c) Serum albumin (cat)-R salt, 1 mg. of N per cc. (104 mg. of protein per kg. of body weight). The results of these experiments were similar to those observed in (b), except that granules in the lining cells of the convoluted tubules were first observed in animals sacrificed 3½ hours after the injection. They were more numerous and definite after 24 hours.

(d) Serum globulin (cat)-R salt, 2 mg. of N per cc. (208 mg.

of protein per kg. of body weight). A few red granules were present in the Kupffer cells only 30 minutes after the injection; 1 hour after the injection similar granules were seen also in the reticulo-endothelial cells of various viscera and tiny red dots made their appearance in the lining cells of the proximal convoluted tubules of the kidneys. These were more numerous and definite in mice which had been allowed to live a longer period of time after the injection.

Summary of Results of Mouse Series 1. Intravenous injection of protein-R salt compounds was followed by the appearance of labelled materials in the reticulo-endothelial cells of the viscera and within the lining cells of the proximal convoluted tubules of the kidneys where they appeared from ½ to 3½ hours after the injection.

Mouse Series 2

Groups of 8 mice each were injected intravenously on 2 successive days with 0.5 cc. of various protein-R salt compounds and pairs of animals of each group were sacrificed 2, 4, 13, and 28 days after the first injection.

(a) Egg albumin-R salt, 1.1 mg. of N per cc. (229 mg. of protein per kg. of body weight). In sections from animals killed 2 days after the injection, all of the lining cells of the proximal convoluted tubules contained abundant cytoplasmic red granules. The Kupffer cells, reticulo-endothelial cells, and interstitial cells of the viscera were laden with clumps and granules of red matter. Similar changes were present in the sections from mice 4 days after the administration of protein-R salt compounds. Thirteen days after the injection, only occasional clumps of granules were left in the lining cells of the convoluted tubules while masses of red matter were present in the Kupffer cells and reticulo-endothelial cells. In animals which lived 28 days after the injection, scarce small granules remained in the lining cells of the tubules while interstitial cells, Kupffer cells, and reticulo-endothelial cells contained ample red material. There was sloughing and degeneration of lining cells of the convoluted tubules so that detached cells, containing red granules, could often be seen within the lumina.

(b) Serum albumin (dog)-R salt, 0.72 mg. of N per cc. (150 mg. of protein per kg. of body weight). The findings were similar to those in (a) except that practically no granules were found in the lining cells of the renal tubules 13 and 28 days after the injection.

(c) Serum globulin (dog)-R salt, 1.17 mg. of N per cc. (244 mg. of protein per kg. of body weight). The findings were similar to those described above. Thirteen days after the injection, only a few of the

lining cells of the convoluted tubules showed groups of granules, which were rather coarse. Most of the interstitial cells exhibited phagocytized red matter and the reticulo-endothelial cells contained masses of red material. Twenty-eight days after the injection no more granules could be found in the lining cells of the renal tubules.

Summary of Results of Mouse Series 2. The number of granules within the lining cells of the renal convoluted tubules decreased steadily the longer the animals were allowed to live after the injection of protein-R salt compounds. There was desquamation of these lining cells so that only scarce red dots remained 28 days after the injection of egg albumin-R salt and serum albumin-R salt; none were seen in mice 28 days after the injection of serum globulin-R salt. The Kupffer cells, reticulo-endothelial cells, and interstitial cells of the viscera retained the material very well.

Mouse Series 3

Two groups of 6 mice each were injected intravenously one to five times on successive days with 0.3 cc. of homologous mouse serum protein preparations coupled with R salt. Two animals of each group were sacrificed 24 hours after the first injection and one animal of each group was killed 24 hours after each following injection. In addition, 2 mice were injected intravenously with diazotized R salt alone and were sacrificed 24 hours afterwards.

(a) Serum Albumin (Mouse)-R Salt, 0.5 mg. of N per cc.

(1) Twenty-four hours after the administration of 31.3 mg. of protein-R salt per kg. of body weight, some of the Kupffer cells showed a faint reddish tint and capillaries of the lungs contained reddish casts. No other changes were recognized.

(2) After intravenous administration of 63 mg. of serum albumin-R salt per kg. of body weight, abundant bright red granules were visible in the lining cells of the proximal convoluted tubules in addition to those present in the reticulo-endothelial cells and interstitial cells of the viscera.

(3) However, 24 hours after the injection of 68 mg. of serum albumin-R salt per kg. of body weight, the results were similar to those described in (a) (1). No granules were seen in the renal tubules.

(4) The results seen after the injection of 94, 125, and 156 mg. of serum albumin-R salt per kg. of body weight were similar to those described under (a) (2). The number of granules present in the lining cells of the tubules was roughly proportional to the amount of material administered (Figs. 1 and 2).

(b) Serum Globulin (Mouse)-R Salt, 0.56 mg. of N per cc.

(1) After the injection of 35.4 mg. of protein-R salt per kg. of body weight, only the Kupffer cells showed a faint reddish tint. No granules were seen in the kidneys.

(2) Injection of 50 mg. of serum globulin-R salt per kg. of body weight was followed by the appearance of a few faintly reddish granules in the lining cells of the renal proximal convoluted tubules; the Kupffer cells contained faintly stained reddish masses.

(3) After injection of 71 mg. of protein-R salt per kg. of body weight, the granules in the kidneys were definite but scarce, while those in the reticulo-endothelial cells were numerous.

(4) After intravenous administration of 106, 142.2, and 174 mg. of homologous serum globulin per kg. of body weight, abundant granules were seen in the tubular lining cells as well as in the reticulo-endothelial cells and interstitial cells of various viscera.

(c) Diazotized R Salt Alone, 0.12 per cent (12 mg. of R Salt per kg. of Body Weight)

Very few tiny red granules were present in the lining cells of the proximal convoluted tubules while numerous granules were apparent in the reticulo-endothelial cells and in some of the interstitial cells of the viscera.

Summary of Results of Mouse Series 3. Intravenous injections of protein preparations from homologous species coupled with R salt were followed by the appearance of phagocytized materials in the reticulo-endothelial cells of the viscera as well as in the lining cells of the renal proximal convoluted tubules. The number of granules depended on the amount of material administered. Injections of diazotized R salt alone produced similar results.

Rat Series

Three groups of 2 rats each, weighing, on the average, 150 gm., were injected intravenously with 3 cc. of protein-R salt compounds and the animals were sacrificed 24 hours later.

(a) Egg albumin-R salt, 1.69 mg. of N per cc. (211.3 mg. of protein per kg. of body weight). Tiny bright red granules were present in the lining cells of the proximal convoluted renal tubules, and the reticulo-endothelial cells of the viscera contained phagocytized red matter.

(b) Serum albumin (dog)-R salt, 1.83 mg. of N per cc. (229 mg. of protein per kg. of body weight). The results of these experiments were similar to those of (a).

(c) Serum-globulin (dog)-R salt, 0.95 mg. of N per cc. (119 mg.

of protein per kg. of body weight). The results of these experiments were similar to those described above, but occasional pale red granules were seen in a few lining cells of the proximal convoluted tubules.

Summary of Results of Rat Series. After intravenous injection of protein-R salt compounds into rats, phagocytized red matter was seen in the reticulo-endothelial cells of the viscera and tiny red granules were present in the lining cells of the proximal convoluted tubules.

Guinea-Pig Series

Three groups of 2 guinea-pigs each, weighing, on the average, 250 gm., were injected intravenously with from 2.5 to 4 cc. of protein-R salt compounds and the animals were sacrificed 4 hours after the injection; 2 animals were injected with diazotized R salt alone.

(a) Egg albumin-R salt, 1.69 mg. of N per cc. (102.5 mg. of protein per kg. of body weight). Some of the Kupffer cells in the liver showed a faint red coloration. There was no trace of the labelled protein in any of the other viscera.

(b) Serum albumin (dog)-R salt, 1.83 mg. of N per cc. (183 mg. of protein per kg. of body weight). The results were similar to those of (a).

(c) Serum globulin (dog)-R salt, 0.95 mg. of N per cc. (71.3 mg. of protein per kg. of body weight). The results of these experiments were similar to those of (a).

(d) Diazotized R salt alone, 0.12 per cent (14.4 mg. of R salt per kg. of body weight). No colored particles were seen in any of the viscera. R salt alone, given intravenously, proved to be quite toxic to guinea-pigs; therefore, injections had to be given slowly and in small portions.

Summary of Results of Guinea-Pig Series. With the exception of Kupffer cells, which showed a slight reddish coloration, there was no evidence of the presence of labelled proteins in any of the viscera after intravenous injection of protein-R salt compounds.

Rabbit Series

(a) One albino rabbit, weighing about 2000 gm., was injected 16 times with 1 cc. each of an egg albumin-R salt preparation containing 0.8 mg. of N per cc. A total amount of 40 mg. of protein per kg. of body weight was given and the animal was sacrificed 1 month after the first injection and 5 days after the last administration. In microscopic sections of the viscera, red granules were present in the reticulo-endothelial cells of the viscera but not elsewhere.

(b) One albino rabbit, weighing approximately 2000 gm., was

injected 16 times with 1 cc. each of a solution of diazotized R salt alone. A total amount of 9.6 mg. of R salt per kg. of body weight was given. The result was similar to that described above.

Summary of Results of Rabbit Series. There was no evidence of the presence of labelled materials in the lining cells of the renal tubules after intravenous injection of egg albumin-R salt or of diazotized R salt alone. The reticulo-endothelial cells of the viscera, however, contained phagocytized red particles.

Dog Series 1

(a) A healthy dog, weighing 10.2 kg., was injected intravenously with 65 cc. of a solution of egg albumin-R salt, containing 1.69 mg. of N per cc. A total amount of 67.3 mg. of protein per kg. of body weight was given and the animal was sacrificed 24 hours afterwards. Microscopic sections showed tiny red granules in the lining cells of the renal proximal convoluted tubules and in the reticulo-endothelial cells of the viscera.

(b) A healthy dog, weighing 12.8 kg., was injected intravenously with 120 cc. of diazotized R salt alone. A total of 11.3 mg. of R salt per kg. of body weight was given and the animal was sacrificed 24 hours later. Sections of the organs showed granular red matter in the reticulo-endothelial cells of the viscera but not elsewhere.

Summary of Dog Series 1. After intravenous injection of egg albumin-R salt, colored particles appeared in the reticulo-endothelial cells of the viscera and in the lining cells of the proximal convoluted tubules. Although red granules were present in the reticulo-endothelial cells after intravenous injection of diazotized R salt alone, the renal tubules showed no evidence of the presence of colored matter.

Dog Series 2

In order to study the renal excretion of R salt after damage to the kidneys, 3 dogs received 4.0, 6.0, and 8.0 mg., respectively, of a solution of uranium nitrate subcutaneously [4] before intravenous injections of diazotized R salt. After the applications of uranium nitrate the urine contained ample albumin.

(a) A healthy dog, weighing 11 kg., was injected subcutaneously with a solution of uranium nitrate; a total amount of 4 mg. of uranium nitrate per kg. of body weight was given. Fifty cc. of a 0.12 per cent solution of diazotized R salt were given intravenously 43 hours after the administration of uranium nitrate and, similarly, 100 cc. of R salt were injected 53 hours later. A total amount of 16.4 mg. of R salt per kg. of body weight was injected, and the animal was sacrificed 4 hours

after the last, and 54 hours after the first, injection of the R salt. At autopsy, the urine showed a faint reddish tint. Microscopic sections of the organs disclosed granular red matter in the reticulo-endothelial cells of the viscera. There was extensive necrosis of the lining cells of the convoluted tubules and within these cells were seen faintly red-stained masses of irregular sizes, but no well formed granules.

(b) A healthy dog, weighing 9.5 kg., was given 6 mg. of uranium nitrate per kg. of body weight subcutaneously. Three days afterwards 100 cc. of diazotized R salt were injected intravenously and 150 cc. were given again the following day. A total amount of 31.6 mg. of R salt per kg. of body weight was administered. The animal died 15 minutes after the last injection. At autopsy, the liver was large and dark red; the gallbladder was distended by abundant bile; the urinary bladder contained urine showing a faint red color. Microscopic sections showed changes similar to those described under (a). The lumina of the convoluted tubules were distended by protein casts exhibiting a faint reddish hue.

(c) A healthy dog, weighing 10.5 kg., was injected with 8 mg. of uranium nitrate subcutaneously, and 100 cc. of diazotized R salt were given 3 days later and again the following day. A total amount of 22.8 mg. of R salt was administered and the animal was sacrificed 5 hours after the last injection. Microscopic studies of the viscera showed results similar to those described above.

Summary of Results of Dog Series 2. After damage to the kidneys by uranium nitrate followed by intravenous injection of diazotized R salt, ill defined, faintly red-stained masses were found in some of the necrotic lining cells of the renal convoluted tubules; however, no well formed granules were seen. Colored particles were present in the reticulo-endothelial cells of the viscera.

Discussion

The presence of labelled protein particles within the lining cells of the renal convoluted tubules after intravenous injection of various protein-dye compounds indicated that such substances were able to pass the glomerular filter. This conception hinges on the validity of the assumption that the diazotized dyes are inseparably coupled with the protein molecule serving as a label by which the presence of the respective protein can be recognized. This was proved chemically by Kabat and Heidelberger [2] and by Smetana and Johnson [1] in electrophoretic experiments.

To what extent the proteins are denatured by coupling them to the R salt is not known. In anaphylactic experiments with guinea-pigs,

using native proteins for sensitization, and proteins coupled with R salt for the final injection or vice versa, no differences were observed; likewise qualitative as well as quantitative precipitin reactions with either coupled antisera against native antigen or vice versa gave results identical to those with controls.[5]

The amount of protein passing through the normal glomerular filter is probably too small to be detected by ordinary laboratory methods, while even minute amounts of protein-R salt preparations can be visualized microscopically. The filtering membrane apparently does not differentiate between foreign or homologous species proteins. No studies were made with homologous serum proteins coupled with R salt; however, it is assumed that diazotized R salt injected into the blood stream combines with the serum proteins, thereby forming labelled homologous serum protein compounds. In mice, these compounds did pass through the glomerular filter, as the presence of tiny red granules within some of the lining cells of renal convoluted tubules indicates.

The passage of protein substances of relatively large molecular sizes, such as serum albumin and serum globulin, through the normal glomerular filter is rather surprising and changes to some extent the physiologic conception of the function of the filtering membrane of the renal glomerulus. However, it has been shown that even under normal condition some protein is regularly escaping into the glomerular filtrate from which it is absorbed by the lining cells of the proximal convoluted tubules.[6]

The appearance of labelled protein particles within the lining cells of the proximal convoluted tubules strongly suggests that they are absorbed by these cells from the glomerular urine. Although secretion of these substances by the lining cells has to be considered, this seems unlikely because the granules first make their appearance in the lining cells of the funnels of the convoluted tubules or even in the lining cells of the spaces of Bowman before they can be seen in the supraglomerular loops, but are never found in cells of any other portion of the renal tubular system. If they were excreted by the lining cells of the tubules, one would expect colored material in the urine to appear for some time after the injection, which is not the case. It is realized, however, that only direct observation of the nephron during an acute experiment can settle this problem definitely.

The fate of the granules within the lining cells of the tubules is linked with the length of life of the cells in which they are situated: their number diminishes, due to desquamation of lining cells, until none or very few are left. In mice this takes about 1 month after the last

administration of protein-R salt compounds. The phagocytized particles of labelled protein compounds in the Kupffer cells, reticulo-endothelial cells, and in the phagocytic interstitial cells of various viscera remain indefinitely.

The failure to demonstrate labelled protein granules within the lining cells of renal tubules in experiments with guinea-pigs and rabbits perhaps indicates species differences. However, when the amount of injected protein-dye compounds per kg. of body weight is computed, it appears likely that too little material was given to rabbits, so that the results obtained in this series are inconclusive (Table I). Similarly,

TABLE I

Tabulation of Results to Show Quantitative Level at Which Granules Appeared in the Renal Tubular Epithelium

Animals	Mg. of substrate per kg. of body weight				
	R salt	Peptone	Egg-albumin	Serum-albumin	Serum-globulin
Mouse series	12.0	21.7	114.5	31.3	35.4
Mouse series		27.0	229.0	63.0	50.0
Mouse series				68.0	70.8
Mouse series				93.8*	106.0*
Rat series			211.3	229.0	119.0
Guinea-pig series	14.4		102.5	183.0	71.3
Rabbit series	9.6		40.0		
Dog series	11.3		67.3		

Underscored figures indicate granules in renal tubules; figures not underscored indicate no granules in renal tubules.

* Amounts larger than these always gave positive results.

the results obtained in mice with peptone-R salt preparations are inconclusive; due to the toxicity of this preparation, larger amounts were not tolerated.

In general, it can be stated that the greater the amount of protein-R salt given, the more extensive were the deposits found in the lining cells of the renal convoluted tubules as well as in the reticulo-endothelial cells of the various viscera. A minimal amount of about 60 mg. of protein per kg. of body weight has to be administered before granules appear in the lining cells of the kidney tubules (Table I).

After intravenous injection of diazotized R salt alone into dogs following damage of the kidneys due to uranium nitrate, small amounts of this dye passed through the glomerular filter and faintly stained masses of red substance were seen in some of the necrotic lining cells of the convoluted tubules. This is in contrast to the brilliantly stained, well defined granules which appear in the tubular cells of normal animals injected with protein-R salt compounds. It is suggested that

the formation of these granules is an expression of a normally functioning cell and this has perhaps a bearing on the interpretation of colloid droplets as being protein particles stored in functioning lining cells of tubules after re-absorption from tubular lumina of protein substances which have passed through the glomerular filter.

Conclusions

1. Preparations of egg albumin, and of serum albumin and serum globulin of heterologous and homologous species, labelled by R salt, pass the glomerular filter of normal mice, rats, and dogs after intravenous injection; particles of these substances are re-absorbed by the lining cells of the proximal convoluted tubules after passage through the glomerular filter and are stored in these cells in the form of tiny granules.

2. The number of granules present in the lining cells of the tubules is roughly proportional to the amounts of protein-dye compounds administered.

3. The particles of protein-dye compound remain in the lining cells of the tubules until these cells are desquamated.

REFERENCES

1. Smetana, H., and Johnson, F. R. The origin of colloid and lipoid droplets in the epithelial cells of the renal tubules. *Am. J. Path.*, 1942, **18**, 1029–1049.
2. Kabat, E. A., and Heidelberger, M. A quantitative theory of the precipitin reaction. V. The reaction between crystalline horse serum albumin and antibody formed in the rabbit. *J. Exper. Med.*, 1937, **66**, 229–250.
3. Heidelberger, M. Advanced Laboratory Manual of Organic Chemistry. Chemical Catalog Co., New York, 1923, p. 83.
4. MacNider, W. de B. Urine formation during the acute and chronic nephritis induced by uranium nitrate. A consideration of the functional value of the proximal convoluted tubule. *Harvey Lectures*, 1928–29, **24**, 82–105.
5. Smetana, H. Unpublished data.
6. Oliver, J. New directions in renal morphology: a method, its results and its future. *Harvey Lectures*, 1944–45, **40**, 102–155.

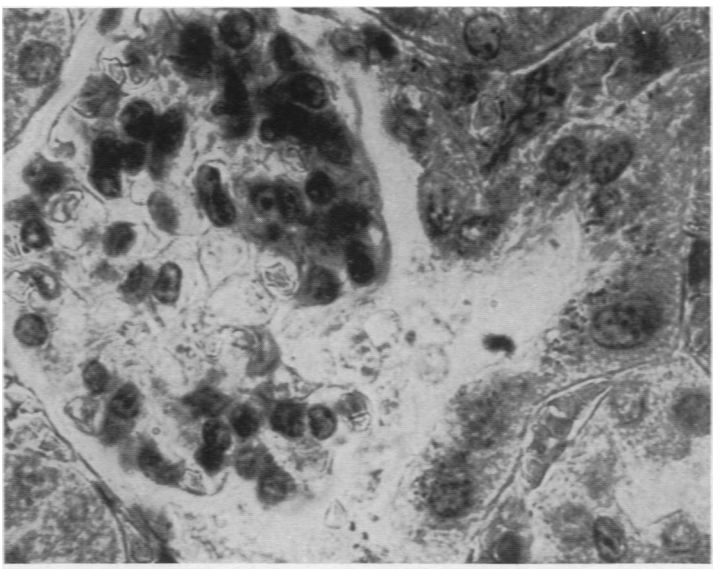
[*Illustrations follow*]

DESCRIPTION OF PLATE

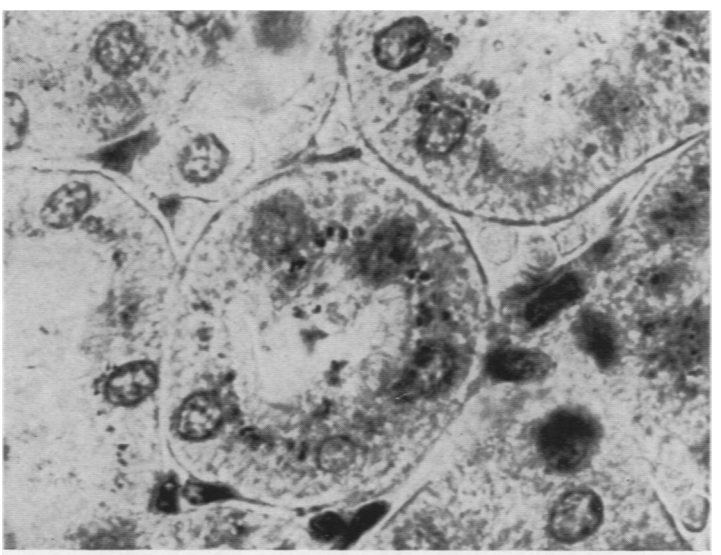
PLATE 44

FIG. 1. Mouse series 3 (five intravenous injections on successive days of homologous mouse serum albumin-R salt, 0.5 mg. of N per cc., totalling 156 mg. of protein per kg. of body weight). Section of kidney, showing a glomerulus, the space of Bowman, and the funnel of the proximal convoluted tubule. The tiny black dots within the cytoplasm of the lining cells of the convoluted tubule and in some of the cells lining the space of Bowman represent brilliantly stained red granules of the serum albumin-R salt. × 1050.

FIG. 2. Section of kidney from the same animal as shown in Figure 1. The small black dots in the cytoplasm of the lining cells of the proximal convoluted tubules represent the intensely stained red granules of the serum albumin-R salt. × 1050.



1



2