# THE PATHOGENESIS OF THE RENAL INJURY PRODUCED IN THE DOG BY HEMOGLOBIN OR METHEMOGLOBIN*

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Plates 32 to 34

(Received for publication, June 10, 1947)

Serious impairment of renal function may be found associated with the hemoglobinuria resulting from intravascular hemolysis or the injection of solutions of hemoglobin (1, 2). Similar disturbances of kidney function are observed following the crush injury of skeletal muscle with liberation of myoglobin from the injured muscle and excretion of metmyoglobin in the urine (3). Three general hypotheses have been suggested to explain this type of renal injury: obstruction of the renal tubules by the precipitation of derivatives of hemoglobin or myoglobin in their lumina (1), injury of renal tubule cells by toxic concentrations of these heme pigments (4–6), and diminished renal blood-flow due to vasoconstriction of renal blood vessels (7, 8). Various modifications and elaborations of all of these hypotheses have been proposed and the possibility that all three mechanisms may be involved in varying degrees has been appreciated.

During the course of experiments designed to study methods of treatment of arsine poisoning, the pathogenesis of the renal dysfunction resulting from the intravascular hemolysis produced by arsine was investigated. Experiments were also carried out on dogs in which renal injury was produced by the intravenous injection of solutions of dog hemoglobin or methemoglobin.

Dogs were placed in a static[1] gassing chamber in which arsine was generated by the reaction of magnesium arsenide with water. The concentration of arsine in the chamber air was controlled by the amount of magnesium arsenide introduced into the reaction vessel. A concentration of 1 mg. of arsine per liter of air was most commonly used and the mortality rate of dogs exposed to this concentration for 15 minutes was 79 per cent.

Solutions of dog hemoglobin were prepared by the method of Hamilton and Van Slyke (9). Washed dog red blood cells were hemolyzed by the addition of distilled water, and the stroma proteins were precipitated by acidification of the solution to pH 5.8 with 0.1 N HCl. Following centrifugalization and filtration of the supernatant through filter paper, the clear solution was

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Yale University, July 1, 1944, to December 31, 1945.

[1] In the static type of gassing chamber there is no exchange of air during the period of exposure of the animal to the toxic vapor.

brought back to pH 7.0 by the addition of 0.1 N NaOH and sterilized by filtration through a Seitz filter. Solutions of methemoglobin were prepared by the addition of 4 moles of $NaNO_2$ per mole of hemoglobin. The concentrations of hemoglobin and methemoglobin in the solutions and in the plasma of the experimental animals were determined by the method of Evelyn and Malloy (10).

The effects of intravascular hemolysis or of administration of solutions of hemoglobin and methemoglobin upon kidney function were measured by serial determinations of the concentration of urea nitrogen and creatinine in the plasma, and by determinations of the creatinine clearances. Urea nitrogen was determined by the manometric method of Van Slyke and Kugel (11) and creatinine by the Folin and Wu method (12) adapted for the photoelectric colorimeter.

An adaptation of Gersh's histochemical technique (13) for the study of kidney function was utilized in an attempt to determine whether the renal tubules were functionally obstructed. The details of the method as used are described elsewhere (14). Following the intravenous injection of solutions of sodium ferrocyanide, the distribution of ferrocyanide can be visualized in sections of kidney frozen and dehydrated *in vacuo*. In the dog, ferrocyanide is filtered through the glomerular membrane and is not reabsorbed by the renal tubule cells (15). Thus, in the kidney of the normal dog, ferrocyanide can be demonstrated histochemically in the glomerular space and lumen of the tubule while the tubule cells do not contain ferrocyanide ion. If the tubule cells are injured by the administration of known cytotoxic agents such as mercuric chloride, the damaged cells can be seen to contain ferrocyanide. Mechanical obstruction to the flow of urine through the tubules is indicated by failure to demonstrate ferrocyanide ion in the distal portions of the tubular lumina.

The kidney sections were also examined following fixation and staining, and the number of pigment casts per unit area in the sections of kidney stained with hematoxylin and eosin following fixation in Zenker formol solution was counted. Benzidine and iron-alum-hematoxylin stains were used to demonstrate the presence of hemoglobin or methemoglobin (16, 17).

Studies were made of the nature of the hemoglobin derivative comprising the pigment of the casts. The casts were teased out of frozen sections of unfixed kidney tissue, dissolved in buffer solutions, and the absorption spectrum of the pigment examined by means of a Beckman spectrophotometer. The solubilities of the casts in buffer solutions of varying pH were also determined in a qualitative fashion.

Direct measurements of total renal blood flow were made in anesthetized dogs before and after the intravenous injection of solutions of dog methemoglobin. The left kidney of a dog anesthetized with sodium pentobarbital was brought out through a flank incision without tension on the pedicle and fixed with sutures beneath the skin. Following the intravenous injection of heparin, a T-tube cannula was inserted into the renal vein and tied in place, permitting unrestricted flow of blood through the cannula into the inferior vena cava. To determine the rate of blood flow through the kidney, the segment of renal vein between the cannula and the vena cava was occluded with simultaneous opening of the side arm of the T-tube. The blood flowing out of the side arm for a period of time (measured by stopwatch) was collected and its volume measured. The side arm was then closed, and the constriction of the renal vein released, restoring the flow into the vena cava. Repeated determinations were made and the blood removed was returned to the circulation whenever 30 or 40 cc. had been collected.

## *Methods*

### *Studies of Renal Function and Renal Blood Flow*

In the dog intravascular hemolysis produced by poisoning with arsine may cause impairment of kidney function as indicated by a progressive rise of the

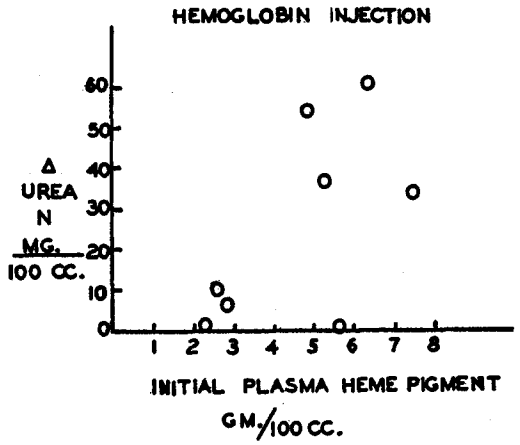
concentration of urea nitrogen in the plasma. The dog was found, however, to be extremely resistant to the injurious effects of hemoglobinemia and hemoglobinuria, and renal injury could be demonstrated only in animals with extreme hemolysis. The findings in two dogs, which survived over 48 hours following exposure to arsine and developed evidences of renal injury are given in Table

TABLE I

*Course of Events in Dogs Exposed to Arsine*

| Dog No. | Packed R. B. C. volume* 24 hrs. post gassing | Plasma hemoglobin 24 hrs. post gassing | Blood urea N 48 hrs. post gassing |
|---|---|---|---|
| | *per cent* | *gm./100 cc.* | *mg./100 cc.* |
| 14–48 | 10 | 3.9 | 122 |
| 14–47 | 35 | 6.7 | 161 |
| 15–34 | 51 | 2.0 | 21 |
| 15–40 | 24 | 4.0 | 24 |

* Expressed as per cent of the pregassing volume.

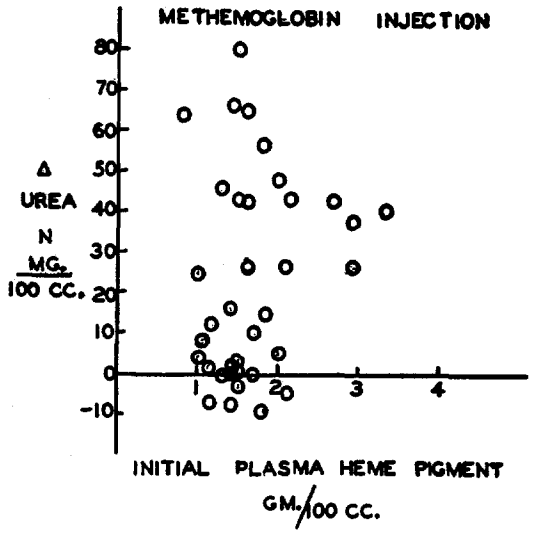



TEXT-FIG. 1. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of hemoglobin solution.

I, in comparison with those of two dogs which survived without evidences of renal damage. The degree of hemolysis of the red cells is indicated by the decrease in the packed red cell volume and by the concentration of hemoglobin in solution in the plasma.

The experiments in which solutions of dog hemoglobin were injected into normal dogs also indicated that disturbance of kidney function could be produced only by injections of large amounts of hemoglobin in concentrated solution. In Text-fig. 1, the initial concentration of hemoglobin in the plasma obtained 3 to 5 minutes following intravenous injection of a solution of hemo-

globin is plotted against the change in concentration of urea nitrogen in the plasma during the first 24 hours following a single injection of hemoglobin. A progressive increase in the plasma urea nitrogen was found in dogs which had received sufficient hemoglobin to raise the plasma hemoglobin concentration to approximately 5 gm. or more per 100 cc. The apparent lack of injurious effect of the lower concentrations of hemoglobin is emphasized by the fact that four of the dogs in which little or no rise in plasma urea nitrogen was found had



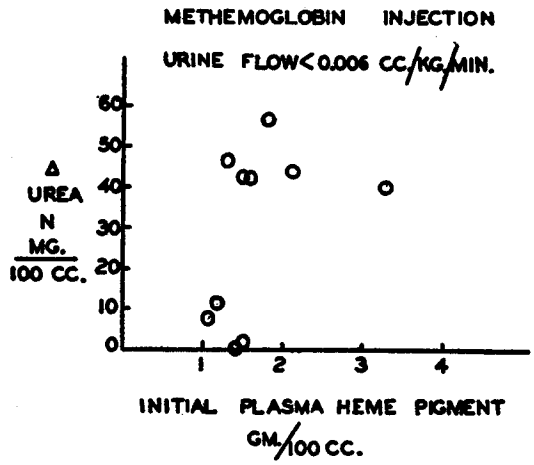

TEXT-FIG. 2. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution.

been made oliguric by deprivation of water and their urine flow had been reduced to 0.0057 cc. per kilogram per minute or less prior to the injection of hemoglobin.

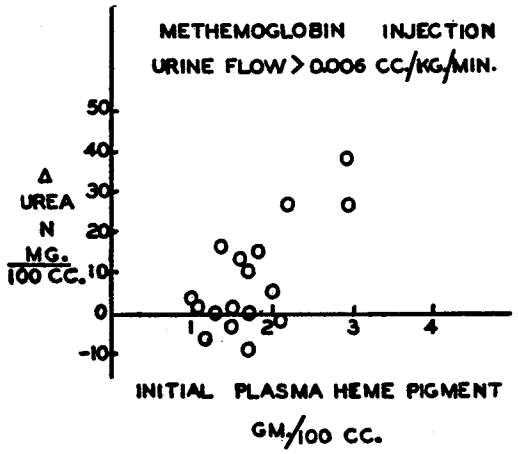
Bing (18) has reported that a severe degree of renal injury could be produced by the injection of methemoglobin solutions into dogs made acidotic by the administration of ammonium chloride. Comparable amounts of hemoglobin injected into acidotic dogs had little effect upon kidney function and the injection of methemoglobin into normal dogs also failed to reduce kidney function. We determined the effects of injection of methemoglobin into normal dogs, dogs made oliguric by deprivation of water, and dogs made acidotic by intragastric administration of 0.1 N hydrochloric acid for several days. The experiments in all of the non-acidotic dogs are summarized in Text-fig. 2 in which the initial concentration of plasma heme pigment (methemoglobin plus hemoglobin)[2] is plotted against the change in concentration of urea nitrogen

[2] The solutions of methemoglobin employed in many instances contained some unoxidized hemoglobin.

in the plasma during the 24 hours following the injection of the methemoglobin solution. The apparent lack of correlation between concentration of heme

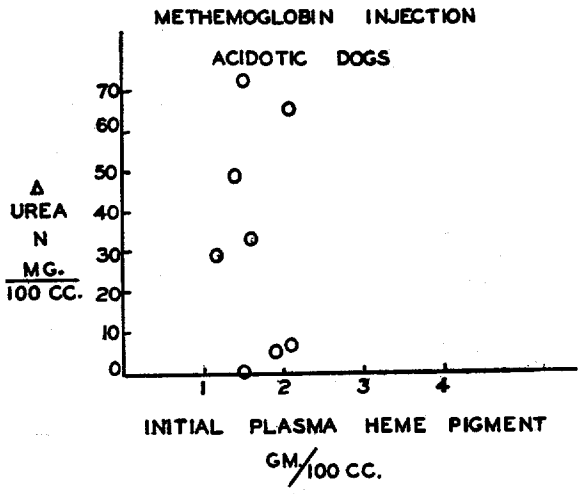



TEXT-FIG. 3. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into oliguric dogs with urine flow of less than 0.006 cc. per kilo per minute at time of injection.




TEXT-FIG. 4. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into dogs with urine flow greater than 0.006 cc. per kilo per minute.

pigment in the plasma and the elevation of the blood urea nitrogen is explained when the results are separated into 2 groups: dogs with urine flow below 0.006 cc. per kilo per minute at the time of injection and those with urine output greater than this value. In Text-fig. 3 the data are given for the dogs deprived




TEXT-FIG. 5. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into acidotic dogs.

TABLE II

*Clearances of Endogenous Creatinine in Oliguric Dogs before and after Injection of Hemoglobin*

| Elapsed time | Urine flow | Plasma creatinine | Creatinine clearance | Plasma hemoglobin |
|---|---|---|---|---|
| *min.* | *cc./min.* | *mg./100 cc.* | *cc./min.* | *gm./100 cc.* |
| Dog 16–28, weight 9.3 kg. | | | | |
| 0–28 | 0.033 | 0.98 | 15.1 | — |
| 28–33 | Intravenous injection of 37 gm. hemoglobin | | | |
| 33 | — | — | — | 5.3 |
| 33–58 | Trace | — | — | — |
| 58–119 | 0.20 | 1.35 | 4.6 | |
| 119–188 | 0.53 | 1.45 | 6.8 | — |
| 1274 | — | — | — | 1.35 |
| 1274–1340 | 0.11 | 2.04 | 7.4 | — |
| Dog 16–57, weight 5.9 kg. | | | | |
| 0–90 | 0.028 | 0.72 | 17.0 | — |
| 96–100 | Intravenous injection of 24 gm. hemoglobin | | | |
| 100 | — | — | — | 4.9 |
| 100–311 | Trace | — | Trace | — |
| 311 | — | 1.58 | — | — |
| 1346–1418 | 0.056 | 3.13 | 0.29 | — |

of water with reduction of urine flow to 0.006 cc. per kilo per minute or less at the time of injection of methemoglobin and in Text-fig. 4 are plotted the results

for dogs with urine flows in excess of this value. In the oliguric dogs severe impairment of renal function is seen following the injection of methemoglobin

TABLE III

*Clearances of Endogeno us Creatinine in Oliguric Dogs before and after Injection of Methemoglobin*

| Elapsed time | Urine flow | Plasma creatinine | Creatinine clearance | Plasma heme pigment |
|---|---|---|---|---|
| *min.* | *cc./min.* | *mg./100 cc.* | *cc./min.* | *gm./100 cc.* |
| Dog 16–45, weight 10.4 kg. | | | | |
| 0–96 | 0.057 | 0.70 | 25.9 | — |
| 100–104 | Intravenous injection of 9.9 gm. methemoglobin | | | |
| 106 | — | — | — | 1.65 |
| 106–145 | Trace | — | — | — |
| 145–232 | 0.037 | 1.07 | 0.5 | — |
| 232–310 | 0.088 | 1.25 | 1.4 | — |
| 310–380 | 0.049 | 1.37 | 1.5 | — |
| Dog 16–29, weight 8.2 kg. | | | | |
| 0–81 | 0.058 | 1.31 | 19.7 | — |
| 81–90 | 0.037 | 1.31 | 15.5 | — |
| | Intravenous injection of 8.2 gm. methemoglobin | | | |
| 99 | — | — | — | 1.82 |
| 99–435 | Trace | — | Trace | — |
| 435 | — | 2.59 | — | — |

TABLE IV

*Clearances of Endogenous Creatinine in Acidotic Dog before and after Injection of Methemoglobin*

| Elapsed time | Plasma $CO_2$ | Urine flow | Urine pH | Plasma creatinine | Creatinine clearance | Plasma heme pigment |
|---|---|---|---|---|---|---|
| *min.* | *mM/liter* | *cc./min.* | | *mg./100 cc.* | *cc./min.* | *gm./100 cc.* |
| Dog 14–19, weight 17.2 kg. | | | | | | |
| 0–89 | 10.0 | 0.37 | 5.3 | 0.74 | 36.8 | |
| | Intravenous injection of 13.9 gm. methemoglobin | | | | | |
| 104 | | | | | | 1.37 |
| 104–140 | | 1.19 | 6.0 | 0.84 | 21.1 | |
| 140–192 | | 0.54 | 6.0 | 0.94 | 10.8 | |
| 192–299 | | 0.52 | — | 1.08 | 9.7 | |
| 299–403 | | 0.48 | — | 1.22 | 8.3 | |

in amounts which result in an initial plasma pigment concentration of approximately 1 gm. per 100 cc. or more, whereas in the dogs with the greater urine output much greater concentrations of plasma methemoglobin are found without severe renal injury.

The results of the experiments in the acidotic dogs are similarly plotted in Text-fig. 5. At first glance it appears that an acidosis of moderate severity ($CO_2$ content of serum 10 to 15 mM per liter) did not intensify the injurious effect of methemoglobin upon kidney function. The rate of urine flow in these dogs at the time of injection of methemoglobin was, however, much greater than in the non-acidotic animals. Although acidosis probably did have some effect in increasing the kidney damage resulting from injection of methemoglobin, in these experments the rate of urine flow was the more important factor.

Creatinine clearances were determined in these dogs before and after the injection of solutions of hemoglobin or methemoglobin. Because of the low rates of urine flow endogenous creatinine clearances were determined, and the individual collection periods were usually 30 minutes or longer. The urine was collected by an inlying catheter, and the bladder was carefully washed at the end of each period and the washings added to the urine sample. Typical experiments are tabulated in Tables II, III, and IV. Immediately following the injection of hemoglobin in a dosage of about 4 gm. per kilo or methemoglobin in a dosage of approximately 1 gm. per kilo into the oliguric dog, urine flow may abruptly cease. After a variable period of time measurable amounts of urine can be collected, but as the results given in Tables II and III indicate, the creatinine clearances are reduced to extremely low levels. In the experiment on the acidotic dog with normal urine flow (Table IV) no period of anuria was observed following the injection of methemoglobin but the creatinine clearance dropped rapidly and progressively. The urine pH rose immediately following the injection of methemoglobin and this phenomenon has been seen in all of the acidotic dogs. In several of the experiments the urine pH rose from 5.2 or 5.3 to approximately 7.0 at the onset of hemoglobinuria. A similar rise in urine pH with onset of hemoglobinuria was found in dogs exposed to arsine.

Because of the abrupt drop in the creatinine clearance seen in many of these experiments the possibility of marked reduction of renal blood flow following the injection of methemoglobin or hemoglobin was considered. Direct measurements of renal blood flow by the technique described above were made in two dogs injected with methemoglobin. The protocols of these experiments are given in Table V. No evidence of reduction of blood flow through the kidneys was found except after prolonged anesthesia and manipulation. An increase of renal blood flow was seen immediately following the injection of methemoglobin solutions, which was probably due to the increase of plasma volume resulting from the injection of a 5 per cent solution of methemoglobin. One of the animals, CK 8, was essentially anuric at a time when total renal blood flow was normal.

TABLE V

*The Effect of Intravenous Injection of Methemoglobin on Renal Blood Flow*

| Elapsed time | Urine output | Renal blood flow |
|---|---|---|
| *min.* | *cc./min.* | *cc./min.* |
| Dog CK 8, weight 6.1 kg. | | |
| 0 | Anesthetized with pentobarbital—renal vein cannulated | |
| 9 | | |
| 23 | | 51.0 |
| 35 | | 52.8 |
| 41 | 0.041 | 47.6 |
| 47 | | 52.1 |
| 52 | | 52.0 |
| 57 | | |
| 57–60 | Intravenous infusion of 114 cc. 5.3 per cent methemoglobin solution | |
| 64 | | 62.8 |
| 66 | | 62.1 |
| 74 | Too little to measure* | 52.3 |
| 79 | | 49.0 |
| 85 | | |
| 103 | | 58.1 |
| 115 | | 55.2 |
| 157 | Too little to measure* | 27.6 |
| 159 | | 24.0 |
| 163 | | 28.8 |
| 165 | Experiment terminated | |
| Dog CK 9, weight 6 kg. | | |
| 0 | Anesthetized with pentobarbital—renal vein cannulated | |
| 10 | | |
| 43 | | 96 |
| 44 | | 140 |
| 45 | | 108 |
| 50 | | 97 |
| 66 | 0.049 | 81.6 |
| 86 | | 125 |
| 93 | | 96 |
| 96 | | 99.4 |
| 99 | | 122 |
| 104 | | |
| 117–120 | Intravenous infusion of 140 cc. of 4.2 per cent methemoglobin solution | |
| 121 | | 173 |

TABLE V—*Continued*

| Elapsed time | Renal blood flow | Urine output |
|---|---|---|
| *min.* | *cc./min.* | *cc./min.* |
| Dog CK 9, weight 6 kg.—*Concluded* | | |
| 122 | | 162 |
| 127 | 0.17‡ | 138 |
| 142 | | 151 |
| 171 | | 84.5 |
| 205 | | 81.3 |
| 256 | | 62.1 |
| 262 | 0.09‡ | 84 |
| 265 | Experiment terminated | |

* Bladder washings contained methemoglobin.

‡ Methemoglobin in urine.

### *Histological Findings*

The kidneys of dogs examined 2 hours or more after the onset of hemoglobinuria due to exposure to arsine or following the injection of solutions of hemoglobin or methemoglobin were found to contain an eosinophilic granular precipitate within the glomerular spaces and the lumina of the tubules. This material stained greenish brown with benzidine or alum-hematoxylin as did the hemoglobin within the red blood cells. By 11 to 24 hours after exposure to arsine or 1½ to 2 hours after the intravenous injection of hemoglobin or methemoglobin, masses of granular yellow-brown refractile material and well formed casts of similar appearance were found within the loops of Henle, the distal convoluted tubules, and collecting tubules (Fig. 1). In the kidneys of animals which showed marked hemoglobinuria, the epithelium of the proximal convoluted tubules contained hyaline eosinophilic droplets which stained greenish brown with benzidine or alum-hematoxylin. This material presumably represented hemoglobin or a derivative which had been absorbed by the epithelial cells from the glomerular filtrate (Fig. 2). Only an occasional necrotic cell was seen in tubules plugged with pigment casts (Fig. 3). Some of the loops of Henle contained eosinophilic casts that did not stain as did hemoglobin but resembled non-hemoglobin protein. A few dilated tubules lined by flattened epithelium could be found among the large number of cast-filled tubules.

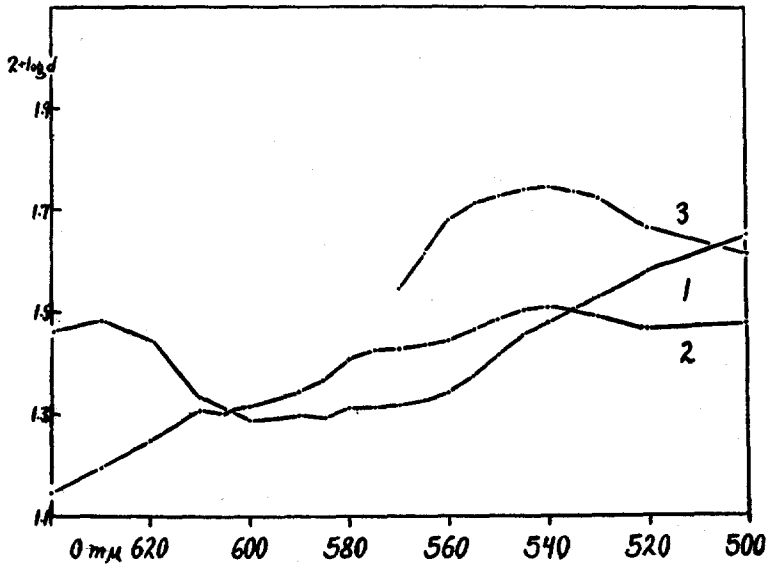
By 36 to 48 hours after the onset of hemoglobinuria, coagulative necrosis of some of the proximal convoluted tubule cells was found in the kidneys of animals in which very extensive intravascular hemolysis had occurred or in those that had received large amounts of methemoglobin intravenously. At times

only a few cells of a tubule were necrotic while the remainder contained in their cytoplasm the eosinophilic droplets described above. Groups of dilated tubules lined by flattened cells were usually present. Regeneration of tubular epithelium was first seen at 48 hours, appearing as a flattened layer of cells surrounding a granular necrotic mass. Intracellular granules containing free iron as indicated by the Prussian blue reaction were seen rarely at this stage but became conspicuous later. The cast material was found to give a positive Prussian blue reaction; occasionally this was limited to the periphery of the cast.

Three or 4 days after the onset of hemoglobinuria, the number of casts was reduced. Radial zones of tubules containing casts alternating with sectors free of casts were often seen (Fig. 4). The tubules of the latter zones frequently were dilated. In other instances few casts were found although there were alternating sectors of dilated and collapsed tubules. The collapsed tubules were lined by tall cuboidal cells containing hemosiderin. This picture was esentially that found in animals examined at later periods except that casts became progressively fewer (Fig. 5).

*Histochemical Determination of Kidney Function with Ferrocyanide.*—The modified Gersh ferrocyanide technique was applied to animals in the groups described above. In the first stage when the tubules were filled with casts ferrocyanide was found within all the glomerular spaces and within the lumina of the proximal convoluted tubules, while the distal portions of the tubular system which were filled with casts contained no ferrocyanide. The few dilated tubules with flattened epithelium which were free of casts contained ferrocyanide within their lumina. No staining of tubule cells was seen (Fig. 6). This distribution of ferrocyanide—in the glomerular spaces and lumina of only the proximal portions of the tubular system—resembled that found in animals in which an acute hydronephrosis had been produced by ligation of the ureters 24 hours before. It is of interest that filtration of fluid through the glomerular capillaries continues despite acute obstruction of the tubules or of the ureter. In the kidneys of dogs studied 3 to 4 days and thereafter following exposure to arsine or injection of hemoglobin, the ferrocyanide was visualized in all of the glomerular spaces and in the lumina of the dilated tubules but not in the collapsed tubules. The absence of ferrocyanide in these collapsed tubules suggested that there was no flow of urine through them although no obstructing casts were seen. The large dilated tubules seen in the late stages (dog 16-45, Fig. 5) contained ferrocyanide and were presumably the only functioning tubule systems.

*Properties and Identification of Pigment of the Casts.*—Thick frozen sections of unfixed kidney were mounted on glass slides without contact with water and then kept in a humid chamber to prevent dehydration. The yellow-brown casts were teased out by micro dissection. They were jelly-like in consistency;




TEXT-FIG. 6. Spectrophotometric absorption curves of dissolved casts from kidney o dog 16-97, examined 4 days after exposure to arsine. Wave length given on abscissa and light transmission in terms of $2 + \log_{10} d$ ($d = \log \frac{I_o}{I}$) on ordinate. By this method of plotting, the shapes of the curves are independent of the concentration of pigment (19). Curve 1, absorption spectrum, pH 5.2, acid methemoglobin. Curve 2, absorption spectrum, pH 9.0, alkaline methemoglobin. Curve 3, absorption spectrum, following addition of 0.1 per cent NaCN to solution at pH 7.7, cyanmethemoglobin.

TABLE VI

*Identification of Pigment Casts*

| Dog No. | Treatment | Interval post exposure or injection | Absorption spectrum of dissolved cast material | Qualitative test for methemoglobin |
|---|---|---|---|---|
| | | *hrs.* | | |
| 16-98 | Arsine | 15-18 | Oxyhemoglobin | Positive |
| 17-07 | " | 20 | Methemoglobin | — |
| 17-22 | " | 21 | — | Positive |
| 17-23 | " | 23 | — | Positive |
| 16-91 | " | 24 | Oxyhemoglobin | Positive |
| 16-93 | " | 48 | — | Positive |
| 16-94 | " | 48 | — | Positive |
| 16-90 | " | 48 | Methemoglobin and oxyhemoglobin | — |
| 16-92 | " | 72 | Methemoglobin | Positive |
| 16-97 | " | 96 | Methemoglobin | Positive |
| 17-16 | Injection of methemoglobin | 2 | — | Positive |

TABLE VII

*Correlation of Number of Casts, Histological Changes in Proximal Convoluted Tubules, and Evidences of Impairment of Renal Function*

| Dog No. | Days post exposure | Plasma | | Average No. casts per field | | Renal tubular "hemoglobin" droplets | Epithelium necrosis |
|---|---|---|---|---|---|---|---|
| | | Urea N | Creatinine | Cortex | Medulla | | |
| | | *mg./100 cc.* | *mg./100 cc.* | | | | |
| **A. Dogs exposed to arsine** | | | | | | | |
| 15-83 | ½ | 36 | — | 12 | 0 | + | 0 |
| 15-38 | 1 | 26 | — | 7 | 20 | + | 0 |
| 15-39 | 1 | 35 | — | 4 | 10 | + | 0 |
| 15-90 | 1 | 47 | — | 35 | 10 | + | 0 |
| 15-02 | 1 | 55 | — | 80 | 35 | + | 0 |
| 15-22 | 1 | 56 | — | 30 | 12 | + | 0 |
| 14-46 | 1 | 59 | — | 40 | 55 | + | 0 |
| 15-84 | 1 | 93 | — | 60 | 80 | + | + |
| 14-96 | 1 | 93 | — | 80 | 90 | + | + |
| 15-13 | 1 | 105 | — | 12 | 65 | + | + |
| 15-51 | 1½ | 24 | — | 30 | 15 | 0 | 0 |
| 15-01 | 1½ | 42 | — | 2 | 30 | + | + |
| 14-79 | 2 | 86 | — | 20 | 0 | + | 0 |
| 16-86 | 2 | 96 | 2.2 | 90 | 10 | + | + |
| 16-94 | 2 | — | 4.6 | 160 | 95 | 0 | + |
| 16-93 | 2 | — | 8.9 | 90 | 200 | 0 | + |
| 15-41 | 2½ | 75 | — | 12 | 0 | 0 | 0 |
| 14-36 | 3 | 25 | — | 6 | 5 | 0 | 0 |
| 14-54 | 3 | 31 | — | 0 | 2 | 0 | 0 |
| 15-04 | 3 | 41 | 1.3 | 10 | 20 | 0 | 0 |
| 16-92 | 3 | 123 | — | 150 | 140 | + | + |
| 16-80 | 3 | 123 | 3.3 | 25 | 35 | 0 | + |
| 16-97 | 4 | 91 | — | 50 | 50 | 0 | + |
| 15-98 | 4 | 132 | — | 50 | 10 | 0 | 0 |
| 16-83 | 4 | 257 | — | 40 | 70 | + | + |
| 17-08 | 4 | — | 7.4 | 200 | 100 | 0 | 0 |
| **B. Dogs injected with methemoglobin** | | | | | | | |
| 16-64 | 1 | — | 1.1 | 10 | 12 | 0 | 0 |
| 16-63 | 1 | — | 4.6 | 18 | 100 | + | + |
| 15-70 | 2 | 8 | — | 5 | 12 | 0 | 0 |
| 15-59 | 2 | 185 | — | 180 | 10 | + | + |
| 16-57 | 3 | — | 3.9 | 120 | 90 | + | + |
| 16-30 | 3 | 92 | — | 5 | 30 | 0 | 0 |
| 16-12 | 4 | 183 | — | 2 | 10 | 0 | 0 |
| 16-41 | 5 | 185 | — | 3 | 85 | 0 | 0 |
| 15-65 | 5 | 253 | — | 3 | 35 | 0 | 0 |
| 15-44 | 5 | 261 | — | 3 | 75 | 0 | + |

they retained their shape when manipulated gently and could be cut up into discrete segments. Their solubilities in buffer solutions of varying pH were determined by dropping the free casts into the buffer solutions or by adding a drop of buffer solution to the frozen section and observing the dissolution of the casts under the microscope. The casts observed in the kidneys of animals 18 hours to 4 days following exposure to arsine or injection of hemoglobin or methemoglobin were dissolved rapidly by solutions of pH 5.2 or below, or 7.6 or above, while they were dissolved slowly by solutions of pH 6.7 to 7.0.

The absorption spectra of the solutions of the pigment casts were determined with a Beckman spectrophotometer. In the specimens obtained from kidneys with large number of casts, the characteristic absorption curves of methemoglobin were found (Text-fig. 6). By suitable change of pH the spectrum of acid or alkaline methemoglobin was observed, and the typical shift to the curve of cyanmethenoglobin was seen when NaCN was added. An occasional preparation from a kidney in which casts were few in number gave the curve of oxyhemoglobin, probably due to the residual red blood cells in the section. Qualitative histochemical tests were made on the casts *in situ* by exposure of the frozen sections to vapors of HCN or addition of a drop of NaCN solution while the casts were observed through the microscope. In all instances in which this was done, the casts developed the characteristic orange-red color of cyanmethemoglobin (Table VI).

*Correlation of Number of Casts and Degree of Impairment of Kidney Function.*—The number of casts per unit area in both the cortex and medulla were determined. Within the field used (2.2 sq. mm.) the total number of tubule cross-sections was found to average approximately 500 in the cortex and 400 in the medulla. The yellow-brown casts only were counted since these were assumed to be the ones capable of obstructing the tubules. In Table VII are given the data on the kidneys of dogs exposed to arsine and those given intravenous injections of methemoglobin and hemoglobin solutions. The cast count is the average of several fields. The data are necessarily approximations but they indicate that the degree of impairment of kidney function as measured by the elevation of plasma urea and creatinine was greatest in those animals in which the casts were most numerous. Conversely, when few casts were found, there was little evidence of impairment of kidney function. In four instances a moderate degree of renal insufficiency was present with only a small number of casts (30 to 40 per field). In these animals, no anatomical basis for the impaired renal function was found.

## DISCUSSION

The experiments reported here indicate that the early impairment of renal function observed in dogs after extensive intravascular hemolysis due to arsine poisoning or after intravenous injection of solutions of methemoglobin and hemoglobin can in large part be explained by obstruction to flow of urine

through the renal tubules. By means of the ferrocyanide histochemical method it was demonstrated that filtration through the glomerular capillaries was continuing since ferrocyanide was found in the glomerular spaces. No evidence was found that the tubule cells were sufficiently injured at this stage to permit extensive back diffusion of the glomerular filtrate since staining of the tubule cells by ferrocyanide was not seen. On the other hand, in animals in which renal injury was produced by mercuric chloride, the tubule cells were found to be permeable to ferrocyanide. The absence of ferrocyanide in the distal portions of the tubular system in the hemoglobinuric animals can be explained on the basis of obstruction to flow of urine through the renal tubules. By means of micro dissection of nephrons Oliver (20) has also concluded that obstruction to the flow of urine through the tubules is the basis for impaired renal function in hemoglobinuria and other states in which protein solutions of high viscosity are found within the tubule. It must be kept in mind that in the dog, impairment of kidney function as the result of hemoglobinemia occurs only with high concentrations of plasma pigment. When methemoglobin concentrations in the plasma of 1 to 2 gm. per 100 cc. were produced, severe progressive renal injury was found only when the urine flow was reduced at the time of the injection of methemoglobin. These conditions would obviously predispose to the development of tubular obstruction because of the high concentration of heme pigment in the urine. Under other conditions in other species of animals the findings might not be the same.

Previous workers have suggested that obstruction of the renal tubules is the important factor in the renal injury produced by hemoglobin or its derivatives and have postulated that the obstruction is due to the precipitation of an insoluble heme pigment in the tubules; *viz.*, hematin (1). In our studies, the casts dissected from the tubules in freshly frozen unfixed sections of kidney were found to be composed chiefly of methemoglobin. Solubility studies of the cast material indicated that the casts were soluble in buffer solutions over a wide range of H ion concentration, but were least soluble at pH 6.7 to 7.0, which is approximately the isoelectric point of hemoglobin and methemoglobin. Obstruction to flow of urine was not due to the precipitation of insoluble hematin in the lumen of the tubule, but apparently resulted from the viscosity of the concentrated solution of hemoglobin or methemoglobin. If the resistance to flow of such a solution through the tubules were greater than the pressure gradient available for propelling the solution through the renal tubules *i.e.* the blood pressure in the glomerular capillaries plus the forces resulting from reabsorption of water in the tubules, cessation of urine flow could be explained. Once the tubules are obstructed by this viscous protein solution it is unlikely that urine flow can be restored by the administration of water or electrolyte solutions.

The greater degree of depression of renal function produced by injections of

methemoglobin than that resulting from administration of oxyhemoglobin and the effect of acidosis in increasing the injurious action of methemoglobin cannot be explained on this basis. The increased toxicity of methemoglobin was thought by Corcoran and Page (21) to be due to the more rapid formation of hematin from methemoglobin than from oxyhemoglobin and the effects of acidosis were considered to be due to the influence of urine pH on the conversion of methemoglobin to hematin. Our studies, however, do not show any evidence of the formation of detectable amounts of hemochromogen or hematin in the lumina of the tubules either after intravascular hemolysis or after the intravenous injection of methemoglobin. The possibility that methemoglobin might be filtered more readily through the glomeruli than hemoglobin and thus be found in greater concentration in the tubular lumen was considered, but comparison of renal clearances of hemoglobin and methemoglobin does not show any consistent differences (22). Histological evidence of necrosis of renal tubule cells was found in these studies as in many of the earlier reports of renal injury due to hemoglobin (4, 5). The maximum degree of necrosis was found 1½ to 2 days after the infusion and unquestionably served as an additional factor which in combination with the increase in resistance of urinary flow due to the viscosity of the tubular contents could account for the persistent impairment of renal function seen in these animals. Hemoglobin or methemoglobin (these pigments stain alike with the benzidine or alum-hematoxylin method) is found within the cells of the proximal convoluted tubules following their filtration through the glomeruli. Hemoglobin is oxidized to methemoglobin in both the plasma and urine, but this oxidation is more complete in the urine particularly when obstruction to urine flow exists. Methemoglobin may act as an oxidant and a possible mode of its toxic action may be the catalysis of the oxidation of sulfhydryl groups. There is evidence that the renal tubular epithelium is extremely susceptible to agents which combine with or oxidize sulfhydryl groups (23).

Renal ischemia has been considered as a possible cause of the depression of renal function in conditions associated with hemoglobinuria. A decrease of kidney volume has been observed immediately following injection of hemoglobin solutions and interpreted as an indication of reduction of renal blood flow (7, 8). Our direct measurements of renal blood flow have not confirmed this interpretation. The reduction of diodrast clearance seen at a later stage of renal injury following injection of methemoglobin (18) may be due to the presence of many non-functioning nephrons rather than to diminution of total renal blood flow.

## SUMMARY

Severe and persistent impairment of kidney function has been produced in dogs by intravascular hemolysis due to arsine, or by the intravenous injection of solutions of dog hemoglobin and methemoglobin.

The kidneys of these animals have been examined by the usual histological methods and also by means of the ferrocyanide histochemical method to determine the pathogenesis of the renal injury. These observations indicate that obstruction to flow of urine through the renal tubules is an important factor in the early reduction of kidney function. The material filling the lumina of the renal tubules was found to be chiefly methemoglobin in concentrated solution of gel-like consistency. No evidence of formation of a pigment insoluble at the pH of the urine such as hemochromogen or hematin was found. The cessation of urine flow is most readily explained by the increased viscosity of the tubule contents.

The intravenous administration of methemoglobin was found to produce more severe renal injury than the injection of equal amounts of oxyhemoglobin. Necrosis of the proximal convoluted tubule cells was present as a late lesion in animals injected with methemoglobin, large amounts of hemoglobin, or following extensive intravascular hemolysis. Such injury is probably a contributing factor in the persistent severe depression of renal function seen in these animals. Following disappearance of most of the intratubular pigment, a large number of collapsed tubules lined by hemosiderin-filled cells were found. The ferrocyanide histochemical studies indicated that these represented non-functioning nephrons although no obstructing intratubular material was present.

Direct measurements in two animals failed to reveal any reduction of renal blood flow following the injection of methemoglobin in amounts sufficient to produce renal injury.

## BIBLIOGRAPHY

1. Baker, S. L., and Dodds, E. C., *Brit. J. Exp. Path.*, 1925, **6,** 247.
2. Foy, H., Altmann, A., Barnes, H. D., and Kondi, A., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1943, **36,** 197.
3. Bywaters, E. G. L., and Beall, D., *Brit. Med. J.*, 1941, **1,** 427.
4. Bordley, J., *Arch. Int. Med.*, 1931, **47,** 288.
5. DeGowin, E. L., Warner, E. C., and Randall, W. L., *Arch. Int. Med.*, 1938, **61,** 609.
6. Ayer, G. D., and Gauld, A. G., *Arch. Path.*, 1942, **33,** 513.
7. Mason, J. B., and Mann, F. G., *Am. J. Physiol.*, 1931, **98,** 181.
8. Hesse, E., and Filatow, A., *Z. ges. exp. Med.*, 1933, **86,** 211.
9. Hamilton, P. B., and Van Slyke, D. D., personal communication.
10. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126,** 655.
11. Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102,** 489.
12. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38,** 81.
13. Gersh, I., and Stieglitz, E. J., *Anat. Rec.*, 1933–34, **58,** 349.
14. Harrison, H. E., and Bunting, H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63,** 120.
15. Van Slyke, D. D., Hiller, A., and Miller, B. F., *Am. J. Physiol.*, 1935, **113,** 611.
16. Ralph, P. H., *Stain Technol.*, 1941, **16,** 105.
17. Dunn, R. C., and Thompson, E. C., *Arch. Path.*, 1945, **39,** 49.

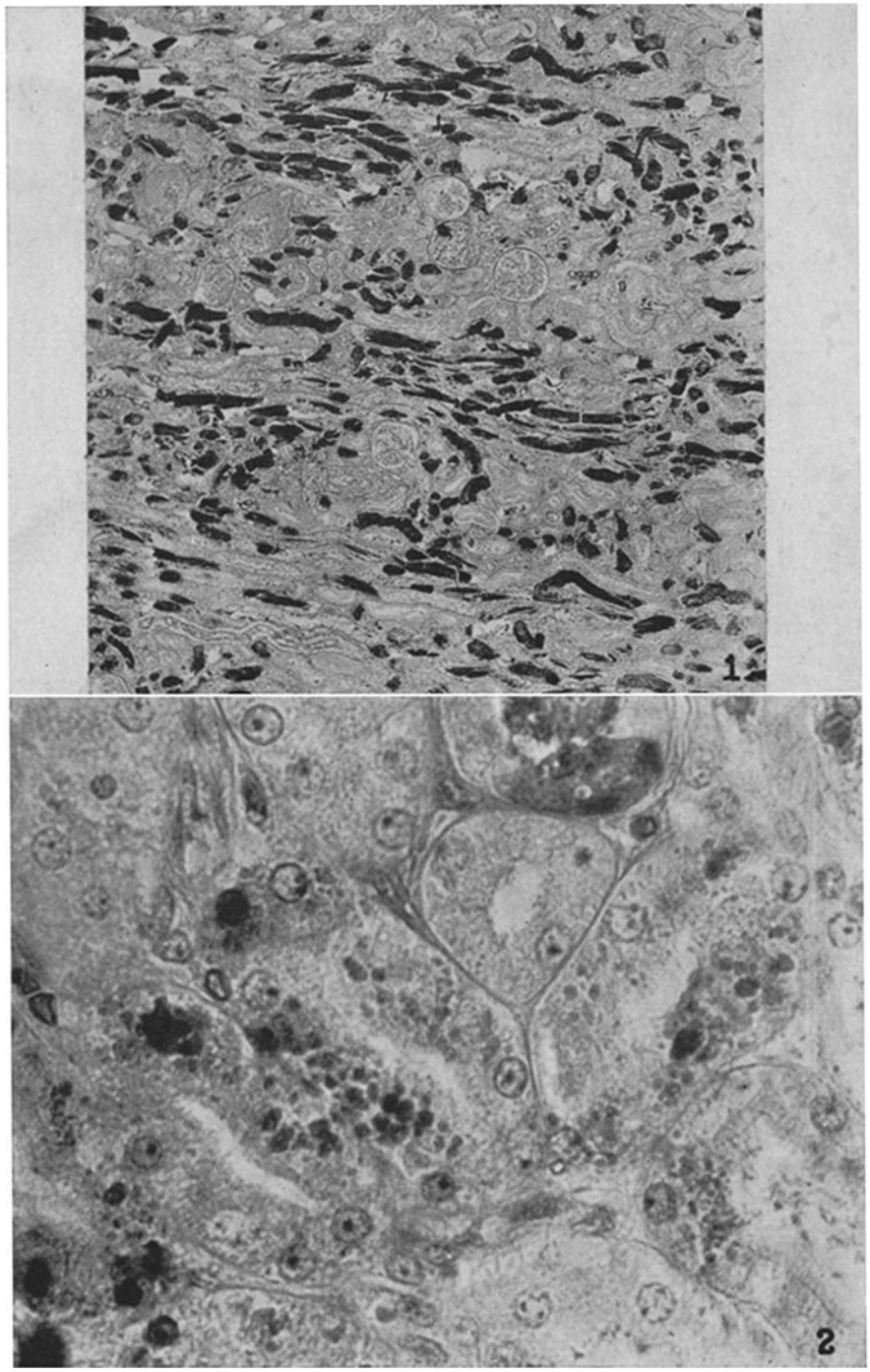
18. Bing, R. J., *Bull. Johns Hopkins Hosp.*, 1944, **74**, 161.
19. Heilmeyer, L., Spectrophotometry in Medicine, translated by Jordan, A., and Tippell, T. L., London, A. Hilger Ltd., 1943.
20. Oliver, J., *Harvey Lectures*, 1944–45, **40**, 102.
21. Corcoran, A. C., and Page, I. H., *Texas Rep. Biol. and Med.*, 1945, **3**, 528.
22. Harrison, H. E., and Ordway, N. K., unpublished observations.
23. Gilman, A., Philips, F. S., Koelle, E., Allen, R. P., and St. John, E., *Am. J. Physiol.*, 1946, **147**, 115.

## EXPLANATION OF PLATES

### PLATE 32

FIG. 1. Dog 14-63. Died approximately 24 hours after exposure to arsine. Extensive deposition of hemoglobin or its derivatives within the tubules, chiefly distal convoluted and collecting tubules. Iron-alum-hematoxylin. × 52.

FIG. 2. Dog 14-79. Died 2 days after exposure to arsine. Plasma urea N 86 mg. per 100 cc. "Hemoglobin" droplets within epithelium of the proximal convoluted tubules. Iron-alum-hematoxylin. × 575.



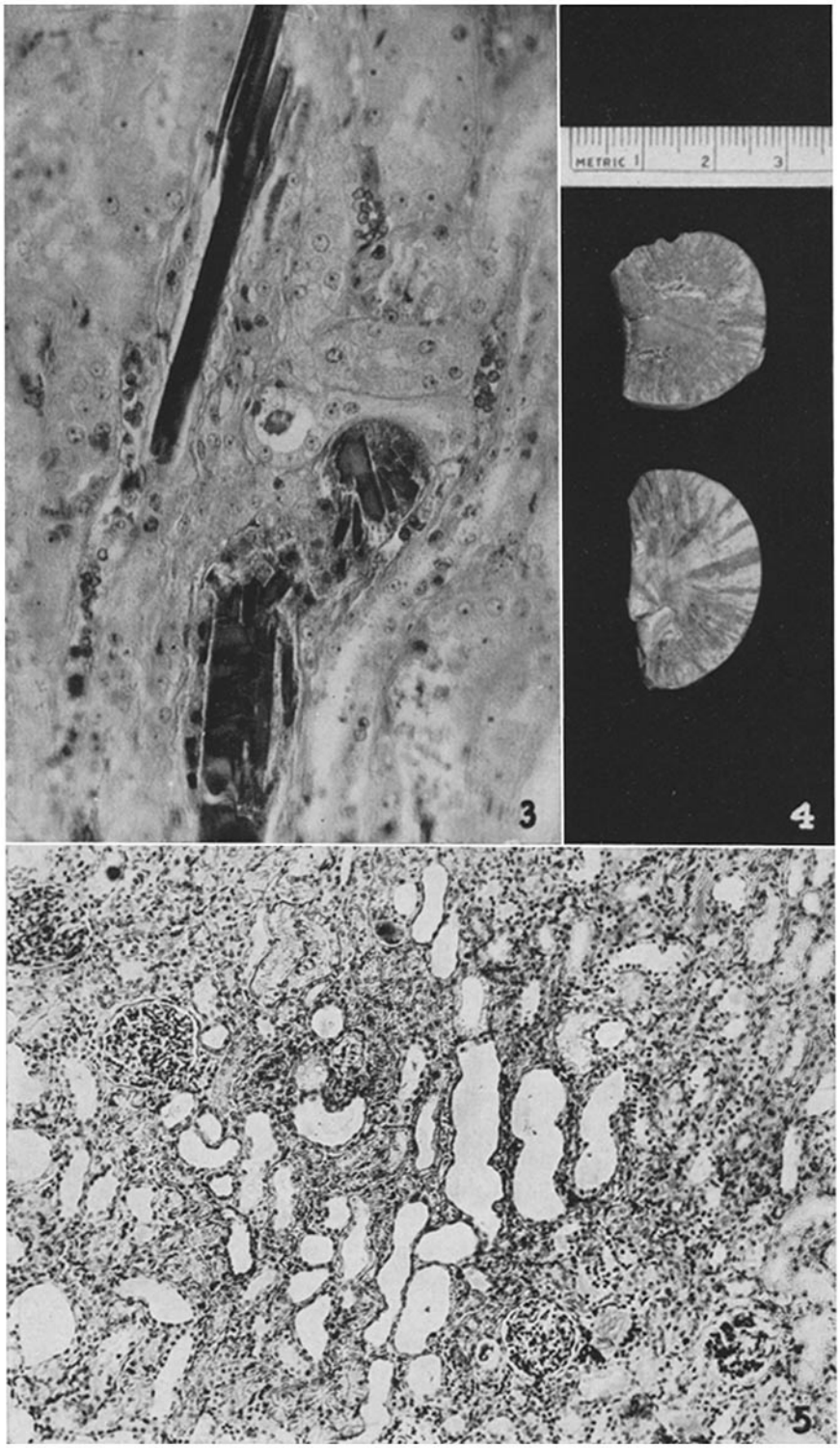

(Harrison *et al.*: Pathogenesis of renal injury in dog)

PLATE 33

FIG. 3. Dog. 14-79. Pyknosis of some of the cells of ascending limb and distal convoluted tubules adjacent to pigment casts within the lumen. Hematoxylin and eosin. × 355.

FIG. 4. Dog 16–97. Examined 4 days after exposure to arsine. Blood NPN 91 mg. per 100 cc. Transverse and longitudinal sections of the kidneys showing the radial arrangement of the zones of persistent pigment casts in cortex and medulla.

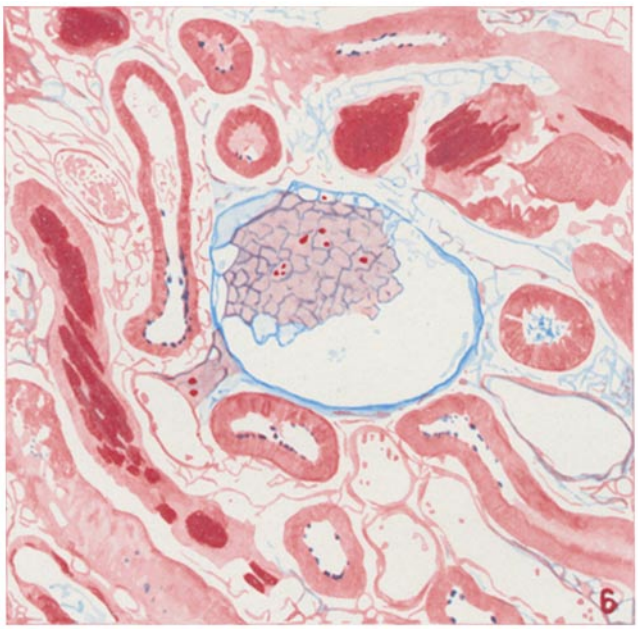
FIG. 5. Dog 16-45. Sacrificed 10 days after injection of methemoglobin solution. Plasma urea N 139 mg. per 100 cc. Plasma creatinine 6.2 mg. per 100 cc. Irregular dilatation of some of the tubules with zones of collapsed tubules. Hematoxylin and eosin. × 100.




(Harrison *et al.*: Pathogenesis of renal injury in dog)

PLATE 34

FIG. 6. Dog 16-63. Sacrificed 1 day after injection of methemoglobin. Plasma creatinine 4.6 mg. per 100 cc. Section of kidney prepared by modified Gersh method (14). The drawing shows the blue precipitate of ferric ferrocyanide in Bowman's space and the lumina of the proximal convoluted tubules but not in the distal convoluted tubules that contain pigment casts. The tubular epithelium is not stained. The presence of ferrocyanide in the lumen of some of the blood vessels and lymphatics is noted.




(Harrison *et al.*: Pathogenesis of renal injury in dog)