THE RENAL LESION ASSOCIATED WITH HEMOGLOBINEMIA

II. Its Structural Characteristics in the Rat*

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PLATES 61 TO 65

A method has been established for the production of acute, reversible, renal failure in the rat by the intravenous injection of relatively small amounts of hemoglobin (1). The functional evolution of the lesion, from its time of onset to virtual recovery, has been characterized by the measurement of the inulin clearance rate (1). Histological studies at each stage of the lesion have also been performed, permitting a correlation of the morphological and functional alterations associated with this variety of acute renal failure. This correlation has led to the formulation of certain hypotheses regarding the pathogenesis of this lesion which are subject to further critical testing.

Materials and Methods

The means of production of the renal lesion and of its functional evaluation have been described in detail in a separate report from this laboratory (1).

For the determination of kidney weight and water content, the kidney was removed quickly from the anesthetized animal, trimmed of perirenal fat, placed in a tared vessel, and weighed on an analytical balance. It was subsequently dried in an oven for 48 hr and reweighed.

Kidneys for histological study were quickly removed from the anesthetized animal. Sections were fixed in 20% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin, or with Lepehne stain for hemoglobin (2). Small pieces of cortex, outer medulla, and papilla were cut into 1 mm blocks in a drop of cold 1% veronal acetate buffered OsO_4 containing 0.2 M sucrose. The tissue was then fixed in the OsO_4 fixative for 1½ hr at 4°C, rapidly dehydrated in cold graded ethyl alcohols, and embedded in Epon (3). One micron sections were stained with toluidine blue and examined with the light microscope. Thin sections for electron microscopy were stained with Karnovsky lead stain (4) and examined with a Siemens Elmiskop I, Siemens and Halske, Berlin, Germany.

RESULTS

Changes in Kidney Weight and Water Content.—Table I summarizes the changes in kidney weight and content of water and solids in rats 24 to 72 hr

after hemoglobin injection. These have been compared with values from rats similarly treated except that they were injected with 1.0 ml isotonic saline, rather than hemoglobin, 24 hr prior to sacrifice. The data reveal that the content of both water and solids is increased significantly in the hemoglobin-injected rats, although the ratio of water to solids is normal in rats 24 hr after injection. The increase in water content is more striking 72 hr after hemoglobin administration and is associated with a high ratio of water to solids in the kidney. The latter is consistent with the marked tubular dilatation noted histologically 72 hr after injection (see below).

Histological Changes during the Initial Time Period, 30 min to 6 hr after Hemo-

TABLE I

Kidney Weight and Content of Water and Solids, 24 and 72 hr after Hemoglobin Injection
A comparison with saline-injected control rats.

	No. of rats	Kidney			
		Total weight	Water content	Dry weight	Ratio
		g/100 g body wt	g/100 g body wt	g/100 g body wt	water/dry wt
Control..................	14	0.393	0.310	0.083	3.84
Hemoglobin, 24 hr........	8	0.477*	0.376*	0.101*	3.71
Hemoglobin, 72 hr........	9	0.557*	0.456*	0.101*	4.51*

* Significantly different from control group (P = <0.05).

At 72 hr, the kidneys of hemoglobin-injected rats have a significantly higher (P = <0.05) total weight, water content, and ratio of water to dry weight than the 24 hr hemoglobin-injected group.

All values are for one kidney. Data were included only from hemoglobin-injected rats with a significant reduction in inulin clearance: at 24 hr, less than 0.2 ml/min/100 g; at 72 hr, less than 0.5 ml/min/100 g.

globin Injection.—A prominent finding in the kidney during the initial hours after hemoglobin administration is the presence of hemoglobin-staining material in the vessels of the outer medullary zone, most apparent in the bundles of vessels comprising the vasa recta (Figs. 1 and 2). That this does not merely represent hemoglobin present in the plasma at the time the kidneys were removed is demonstrated by the following observations: (*a*) this material is not present in the kidneys of rats which do not manifest a functional defect following hemoglobin injection; and (*b*) the hemoglobin staining of the vascular contents persists in the affected kidneys examined at 6 and 24 hr after injection, despite the fact that at 6 hr only a minimal concentration of hemoglobin persists in the plasma and at 24 hr none is detectable. In addition to involvement of the vasa recta, hemoglobin-staining material is also present in the peritubular spaces throughout the outer medulla, admixed with erythrocytes and thus presumably contained within peritubular capillaries. A similar involvement of

peritubular capillaries in the cortex is suggested in certain sections, but this is less well defined than the involvement of the outer medulla.

Hemoglobin is present within the lumen of many tubules in the early stages of the lesion. It is most prominent in the thick ascending limbs of the loop of Henle, where it stains with greatest intensity (Fig. 3). Occasional distal convolutions are similarly involved. During the first few hours after injection hemoglobin may also be present within the thin limbs of the loops and in the collecting ducts. In the latter area it stains lightly and does not present the dark, compact appearance seen in the thick ascending limbs and occasional distal convolutions. A few proximal convolutions may also contain hemoglobin within the lumen. In kidneys which are not severely affected functionally by the injected hemoglobin, material which stains lightly for hemoglobin may be seen in collecting ducts, distal convolutions, and loops of Henle during the period of intense hemoglobinuria. Nowhere does it present the deeply staining appearance observed in the kidney with markedly reduced function.

Certain cellular changes are evident in the initial hours after hemoglobin injection. As early as 30 min after injection some proximal tubules contain sloughed cells and cellular debris within the lumen (Fig. 4). The lining epithelium of these tubules is flattened and the subcellular architecture is obscured.

Cellular changes are also seen in the thick ascending limbs of Henle and consist of isolated cells, the cytoplasm of which stains deeply with toluidine blue (Fig. 3), while others contain darkly stained mitochondria (Fig. 5). A few ascending limb cells show cytoplasmic vacuolization (Fig. 6).

Histological Changes at 24 hr after Hemoglobin Injection.—It has previously been established (1) that the reduction in renal function observed acutely after hemoglobin injection persists at 24 hr. Histological examination of the affected kidney at this time reveals changes which are generally similar to those seen during the initial several hours of the lesion. Hemoglobin-staining material persists within the vasa recta (Fig. 7), although it is usually less dense in appearance than in sections taken during the first several hours. Occasional vasa recta show an accumulation of mononuclear cells, apparently within the lumen (Fig. 8), a finding previously reported in cases of clinical acute renal failure (5).

Intratubular pigment is mainly localized to the thick ascending limbs, where it stains intensely (Figs. 8 and 9). Hemoglobin is also present in occasional distal convolutions, however, cortical involvement is often minimal in kidneys with severe functional impairment. No pigment casts are present within the structures of the inner medulla or papilla. The collecting ducts are singularly free of hemoglobin.

Patchy proximal tubular involvement persists at this stage of the lesion, and the cellular changes are similar to those described in kidneys 30 min after hemoglobin injection (Fig. 10). However, a few distal convolutions now also

show dilatation with flattening of the lining epithelium (Fig. 10). The epithelium of those ascending limbs which contain hemoglobin casts is flattened, and some cells contain darkly staining, altered mitochondria (Fig. 9). Other ascending limbs are dilated, contain sloughed cells, and are lined by focally fragmented and vacuolated epithelial cells (Fig. 9).

Histological Changes 72 hr after Hemoglobin Injection.—Between 24 and 72 hr after injection, marked functional improvement occurs in the involved kidney (1). The kidneys have consequently been examined at the latter time in order to determine whether there are morphological concomitants of this functional improvement. The appearance of the kidneys at 72 hr differs in several respects from that at 24 hr or an earlier period in the evolution of the lesion. Involvement of the vasa recta has largely disappeared. As shown in Fig. 11 the majority of these vessels now appear patent, showing the empty appearance character-istic of these structures in the normal kidney. Some, however, contain a ho-mogeneous appearing material which stains lightly for hemoglobin. A second notable change from earlier sections is the prominent degree of tubular dilata-tion. This is particularly marked in the cortex, in which proximal tubules are involved (Fig. 12), but occasional collecting ducts are also significantly dilated. No interstitial edema is apparent at this or any other stage of the renal lesion. Microdissection studies have confirmed the presence of dilatation of numerous proximal tubules.

Hemoglobin casts persist at 72 hr, located predominantly in the thick as-cending limbs. They appear to be less numerous than at 24 hr but the absence of a method for quantifying these and the lack of serial sections on the same rat preclude definite conclusions on this point.

Histological Changes 7 days after Hemoglobin Injection.—In the outer medulla, hemoglobin casts remain in a few scattered thick ascending limbs of Henle, while others are surrounded by increased numbers of fibroblasts and collec-tions of mononuclear cells. Many proximal convoluted tubules are dilated and lined by neatly arranged epithelial cells with intact brush borders and eosino-philic cytoplasm. A few isolated clusters of proximal convoluted tubules are lined by distorted, vacuolated cells. The glomeruli are normal.

DISCUSSION

A characteristic finding in the kidneys of rats injected with hemoglobin and subsequently manifesting severe functional impairment is the presence of hemo-globin within the lumen of small renal vessels, particularly in the outer medul-lary zone. The persistence of pigment within these vessels many hours after it has disappeared from the plasma is direct evidence that flow through these vascular channels has ceased. There are several factors which might predispose to aggregation of hemoglobin in the vessels of the outer medulla: (a) osmotic abstraction of water from the plasma in the vasa recta, increasing the plasma

concentration of relatively poorly diffusable molecules such as hemoglobin and albumin (6); (b) a low medullary blood flow associated with the elaboration of a highly concentrated urine (7); and (c) renal vasoconstriction resulting from ether anesthesia in the dehydrated animal. The association of this structural change with a depressed inulin clearance rate in the early stages of this lesion suggests that intravascular aggregation of hemoglobin is a primary factor in the pathogenesis of the renal functional defect. This view is reinforced by the observation that the functional improvement occurring at 72 hr after hemoglobin injection is accompanied by a return towards normal in the appearance of the vasa recta. Alternatively it might be proposed that some other factor or factors, associated with hemoglobin injection under these experimental conditions, imposes a state of prolonged renal ischemia and that the presence of hemoglobin within vessels is fortuitous; it has been trapped in that location because of the concurrence of a high plasma hemoglobin concentration with the onset of renal ischemia. The present data do not permit a distinction between these alternative interpretations of this morphological finding.

Whereas the foregoing observations do not provide conclusive information regarding the pathogenesis of this renal lesion, they do permit the formulation of a working hypothesis which can be subjected to further testing. For this purpose it is postulated that renal ischemia, perhaps patchy and circumscribed in nature, is a primary determinant of this renal lesion. Additional support for this hypothesis is provided by the observation that tubular epithelial changes, suggestive of renal ischemia, are invariably present in this lesion. Ischemic injury to the thick ascending limbs may be explained by obstruction of their nutrient vessels with hemoglobin. It is anticipated that the medullary segment of the nephron which is the most active metabolically would be most susceptible to ischemic injury. Structurally, as evidenced by its numerous mitochondria, and functionally, in view of its capacity to transport sodium against a high gradient, the thick ascending limb appears to meet this criterion. Involvement of proximal convoluted tubules is less readily explained by the present data. It may be postulated that obstruction to flow in the vasa recta, since these are derived from glomerular efferent arterioles (8), would reduce blood flow in the corresponding proximal convoluted tubules. However, anatomic studies suggest that only juxtamedullary glomeruli are involved in the formation of the vasa recta (8) and consequently this mechanism would not account for the involvement of more superficial cortical tubules observed in the present lesion. The possibility that aggregation of hemoglobin occurs in cortical capillaries is suggested by the histological studies, but has not been unequivocally demonstrated. If present, this involvement could account for a patchy cortical ischemia. Finally, cortical ischemia in this lesion may be the product of other unknown factors associated with hemoglobin injection under these experimental conditions.

An alternative explanation for the tubular epithelial changes associated with this lesion is that filtered hemoglobin or hemoglobin cast formation produce cellular injury. The patchy nature of the proximal tubular lesion does not suggest the direct action of a nephrotoxic agent, since characteristically such an agent involves all proximal tubules to a relatively uniform degree (9). Furthermore high rates of hemoglobin excretion have been observed in the present study, without the occurrence of histological changes in proximal tubular cells. The association of hemoglobin cast formation with cellular changes in the thick ascending limbs suggests the possibility of an etiological relationship. Menefee and his coworkers (10) have produced acute renal failure in rats by the injection of globin and have noted cast formation and cellular changes in the ascending limbs similar to those observed in the present study. They have attributed the cellular injury to a direct toxic effect of globin which has been absorbed by the intact cells. The present data suggest that the cellular damage is secondary to ischemia and raise the possibility that entrance of filtered protein is secondary to a resultant increase in permeability at the luminal membrane. In favor of this latter view is evidence that protein is not absorbed by the intact cells of the thick ascending limb (11), suggesting that such macromolecules gain access to the intracellular compartment only after cellular injury has occurred. It has been considered that cast formation in the ascending limb may in fact be induced by ischemic cellular injury. Alternatively, these may be independent phenomena and precipitation of hemoglobin at this site may be the result solely of the high intratubular concentration of protein resulting from abstraction of water in the proximal tubules and the thin limb of the loop (12), and the presence of tubular fluid of low ionic strength (13) which would diminish the solubility of hemoglobin.

In contrast to the present hypothesis that renal ischemia is a primary initiating event in the renal lesion associated with hemoglobinemia, Goldberg (14) demonstrated that renal blood flow remained essentially unchanged during the acute oliguric state following hemoglobin injection to dogs. He postulated that tubular obstruction by hemoglobin casts was responsible for the resulting defect in renal excretory function. A possible explanation for this apparent discrepancy is the difference in experimental approach. The dogs used in Goldberg's study were normally hydrated and anesthetized with pentobarbital and consequently manifested normal renal blood flow and creatinine clearance just prior to hemoglobin administration. In the rat, employing the dosage of hemoglobin used in the present study, 40 mg/100 g, a functional defect is not produced under these experimental conditions (1). Although a relatively small amount of hemoglobin was used by Goldberg, 20 to 30 mg/100 g, this was infused into the aorta just proximal to the renal arteries so that presumably a very high concentration was transiently achieved in the renal artery and glomerular filtrate. These considerations suggest that two distinctly

different types of renal lesions may be produced by hemoglobinemia, one due solely to distal tubular blockage resulting from a high intraluminal concentration of hemoglobin and not associated with renal ischemia, and the other, as in the present study, in which renal ischemia is a primary etiological factor. The lack of serial functional measurements in the investigation of Goldberg precludes a comparison of the evolution of the lesion in these two studies.

The present data do not suggest that tubular obstruction is an important determinant of renal functional impairment in the early stages of this renal lesion. Many nephrons do contain casts of precipitated hemoglobin, but the lack of significant dilatation proximal to the site of obstruction suggests that these nephrons are essentially nonfunctional. This interpretation is consonant with the micropuncture studies of Arce, Wilson, and Oken (15) on rats in which myohemoglobinuric acute renal failure was produced by the intramuscular injection of glycerol. They observed a marked decrease in tubular flow and inulin clearance rate in individual proximal tubules. These functional defects could not be explained by back pressure from tubular obstruction, since intraluminal hydrostatic pressure was not elevated, and the authors consequently concluded that a primary reduction in filtration rate was responsible for the observed functional impairment. The present study does however reveal the presence of many markedly dilated proximal tubules in the affected kidneys at 72 hr after hemoglobin injection. This suggests a return of filtration in obstructed tubules, presumably also affecting patent tubules, since the measured inulin clearance rate has increased markedly at this time. This observation suggests that tubular obstruction may partially or wholly explain the persistent reduction in inulin clearance observed at 72 hr and beyond in this lesion (1). The suggestion that separate pathological processes may be involved in the evolution of this renal lesion is consonant with the biphasic character of the recovery of renal function in these animals (1).

SUMMARY

Histological studies have been performed on experimental acute renal failure induced by intravenous injection of hemoglobin in rats. These have been correlated with alterations in renal excretory function, assessed by the measurement of inulin clearance, at various stages of the lesion. The most prominent morphological changes during the first 24 hr after hemoglobin injection, when inulin clearance is most markedly suppressed, are: the presence of hemoglobin within the lumen of small intrarenal vessels, particularly the vasa recta; hemoglobin cast formation involving predominantly the thick ascending limbs of the loops of Henle; and evidence of injury of the epithelium of the proximal tubules and thick ascending limbs. Notably absent during this stage of the lesion are marked tubular dilatation, interstitial edema, and cast formation in the distal collecting ducts. The considerable recovery of function which oc-

curs at 72 hr is accompanied by a marked reduction in involvement of the vasa recta. Standard sections and microdissection reveal many markedly dilated proximal tubules at this stage of the lesion, suggesting obstruction of filtering nephrons.

These data have led to a tentative hypothesis regarding the pathogenesis of renal failure in this experimental lesion. It is suggested that renal ischemia and failure of glomerular filtration are the primary factors responsible for the early and severe impairment of renal function, and that these are related to intravascular aggregation of hemoglobin pigment. As this defect recedes, tubular obstruction by hemoglobin casts prevents restitution of excretory function in a variable fraction of the nephrons. The latter accounts for the relatively prolonged, moderate reduction in inulin clearance associated with the late stages of this lesion. These hypotheses form the basis for a continuing study of this renal lesion.

BIBLIOGRAPHY

1. Jaenike, J. R., The renal lesion associated with hemoglobinemia. I. Its production and functional evolution in the rat, *J. Exp. Med.*, 1966, **123**, 523.
2. Manual of Histologic and Special Staining Technics, New York, McGraw-Hill Book Co., 1960.
3. Luft, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
4. Karnovsky, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.
5. Finckh, E. S., Jeremy, D., and Whyte, H. M., Structural renal damage and its relation to clinical features in acute oliguric renal failure, *Quart. J. Med.*, 1962, **31**, 429.
6. Lassiter, W. E., Gottschalk, C. W., and Mylle, M., Albumin concentration and osmolality of vasa recta plasma in hamster papilla, *Clin. Research*, 1962, **10**, 66 (abstract).
7. Thurau, K., Deetjen, P., and Kramer, K., Hämodynamik des Nierenmarks. II Mitteilung. Wechselbeiziehung zwischen vascularem und tubularem Gegen-stromsystem bei arteriellen Drucksteigerungen, Wasserdiurese und osmotischer Diurese, *Arch. ges. Physiol.*, 1960, **270**, 270.
8. Fourman, J., and Moffatt, D. B., Observations on the fine blood vessels of the kidney, *Symp. Zool. Soc. London*, 1964, No. 11, 57.
9. Oliver, J., MacDowell, M., and Tracy, A., The pathogenesis of acute renal failure associated with traumatic and toxic injury. Renal ischemia, nephrotoxic damage and the ischemuric episode, *J. Clin. Inv.*, 1951, **30**, 1305.
10. Menefee, M. G., Mueller, C. B., Miller, T. B., Myers, J. K., and Bell, A. L., Experimental studies in acute renal failure. II. Fine structure changes in tubules associated with renal failure induced by globin, *J. Exp. Med.*, 1964, **120**, 1139.
11. Oliver, J., and MacDowell, M., Cellular mechanisms of protein metabolism in the nephron. VII. The characteristics and significance of the protein absorption

droplets (hyaline droplets) in epidemic hemorrhagic fever and other renal diseases, *J. Exp. Med.*, 1958, **107**, 731.

12. Lassiter, W. E., Gottschalk, C. W., and Mylle, M., Micropuncture study of net transtubular movement of water and urea in nondiuretic mammalian kidney, *Am. J. Physiol.*, 1961, **200**, 1139.

13. Gottschalk, C. W., and Mylle, M., Micropuncture study of the mammalian concentrating mechanism: evidence for the countercurrent hypothesis, *Am. J. Physiol.*, 1959, **196**, 927.

14. Goldberg, M., Studies of the acute renal effects of hemolyzed red blood cells in dogs including estimations of renal blood flow with krypton[85], *J. Clin. Inv.*, 1962, **41**, 2112.

15. Arce, M. L., Wilson, D. R., and Oken, D. E., Micropuncture study of myohemoglobinuric acute renal failure in the rat, *Clin. Research*, 1965, **13**, 300 (abstract).

EXPLANATION OF PLATES

PLATE 61

FIG. 1. Rat kidney 6 hr after hemoglobin injection showing hemoglobin precipitation in vasa recta (VR) of outer medulla. Lepehne stain. × 120.

FIG. 2. Same kidney as in Fig. 1 showing red blood cells (→) admixed with hemoglobin in vasa recta. Several dilated pars recta (PR) are shown. Hemoglobin casts are present in some ascending limbs of Henle (AL). Lepehne stain. × 300.

FIG. 3. Outer medulla of rat kidney 1 hr after hemoglobin injection showing ascending limbs of Henle (AL) containing intraluminal hemoglobin. Many of the cells contain darkly staining mitochondria (→). Epon embedded, toludine blue–stained thin section. × 480.

FIG. 4. Cortex of rat kidney, ½ hr after hemoglobin injection. Several proximal tubules (PT) contain sloughed cells within the lumen while other proximal tubules (PT′) appear normal. The glomerulus (G) shows no morphological abnormality. Epon embedded, toluidine blue–stained thin section. × 300.
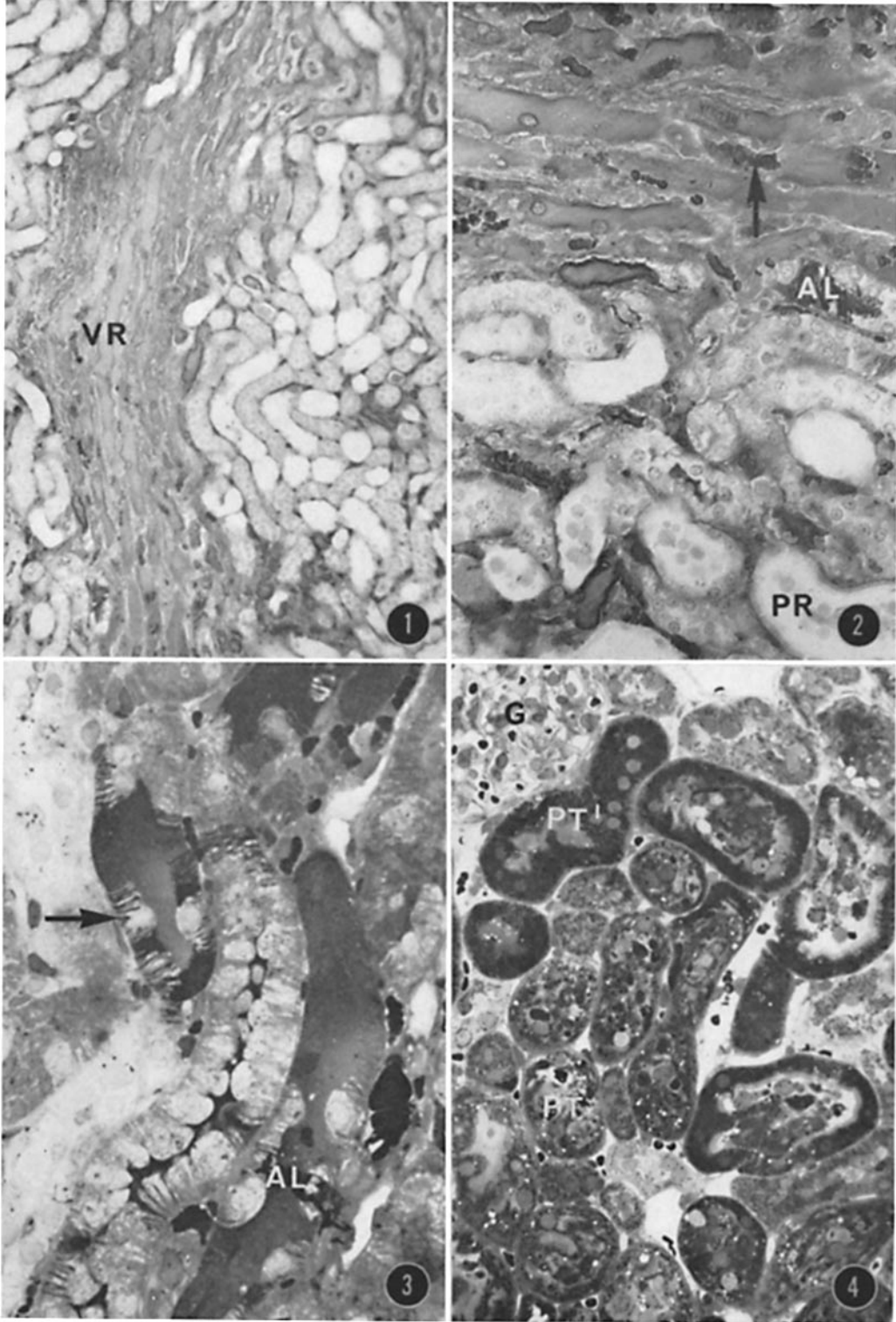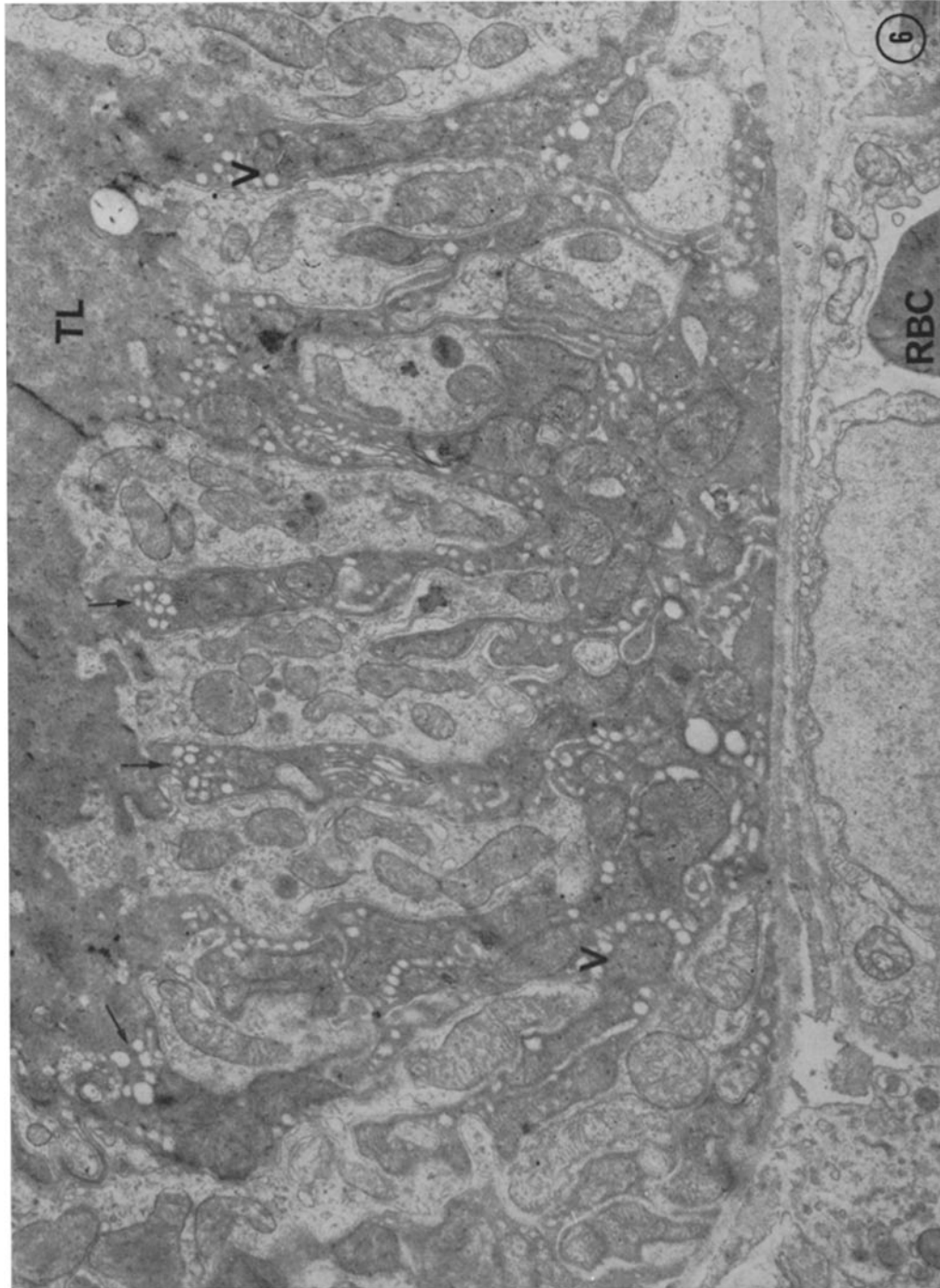
PLATE 62

FIG. 5. Electron micrograph of ascending limb of Henle in rat kidney $\frac{1}{2}$ hr after hemoglobin injection showing darkly staining, somewhat compressed mitochondria (M 1) adjacent to morphologically normal appearing mitochondria (M 2). The basement membrane is indicated by arrows and the adjacent vessel (V) contains a red blood cell (RBC). × 30,000.
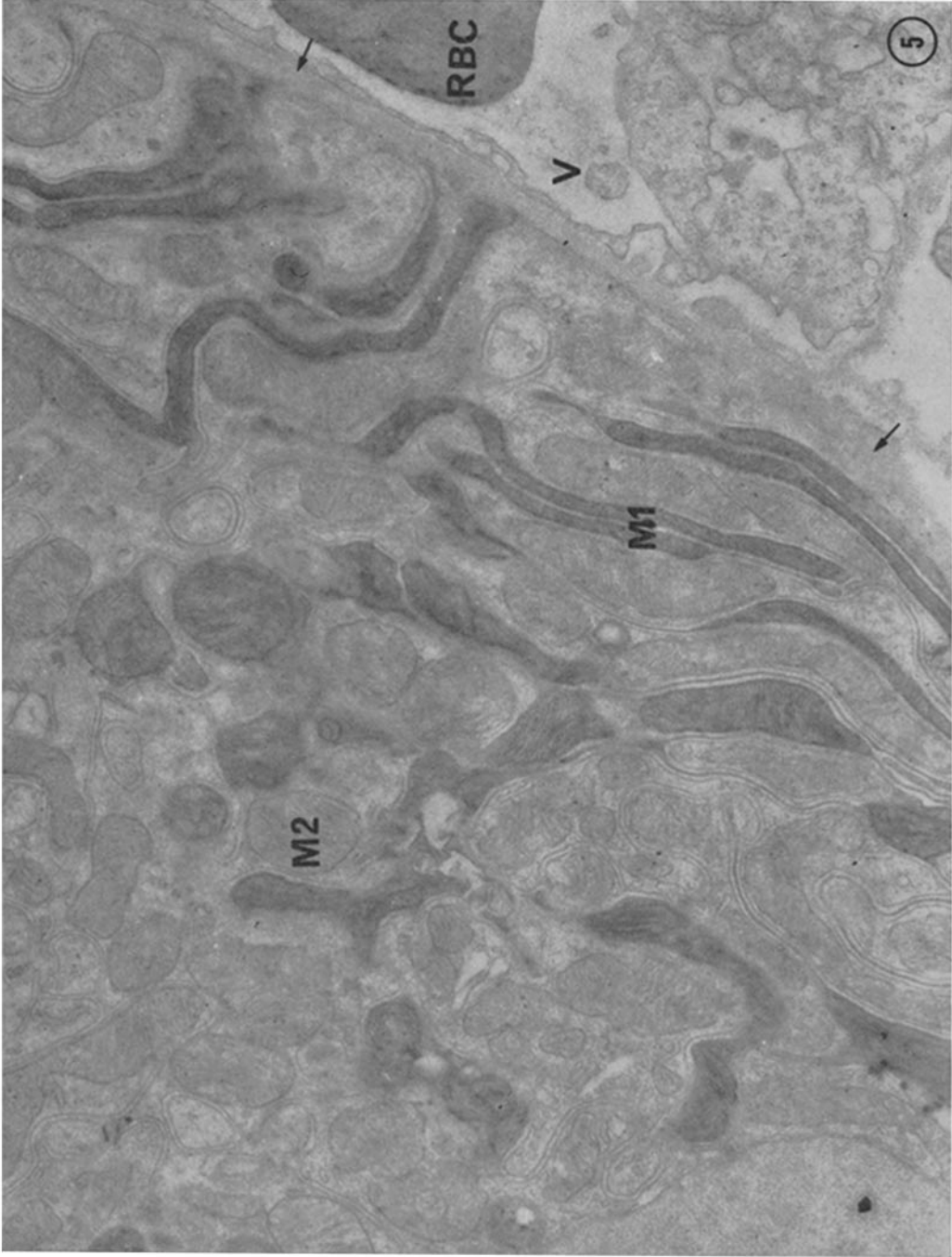
(Jaenike and Schneeberger: Renal lesion with hemoglobinemia. II)

PLATE 63

FIG. 6. Electron micrograph of an ascending limb of Henle from the same kidney as shown in Fig. 5, ½ hr after hemoglobin injection showing cytoplasmic vacuolization (V). In multiple foci the apical cytoplasmic border (→) cannot be seen, and the hemoglobin within the tubular lumen (TL) appears to be continuous with the cytoplasm. A vessel containing a red blood cell (RBC) is seen adjacent to the tubular basement membrane. × 14,000.

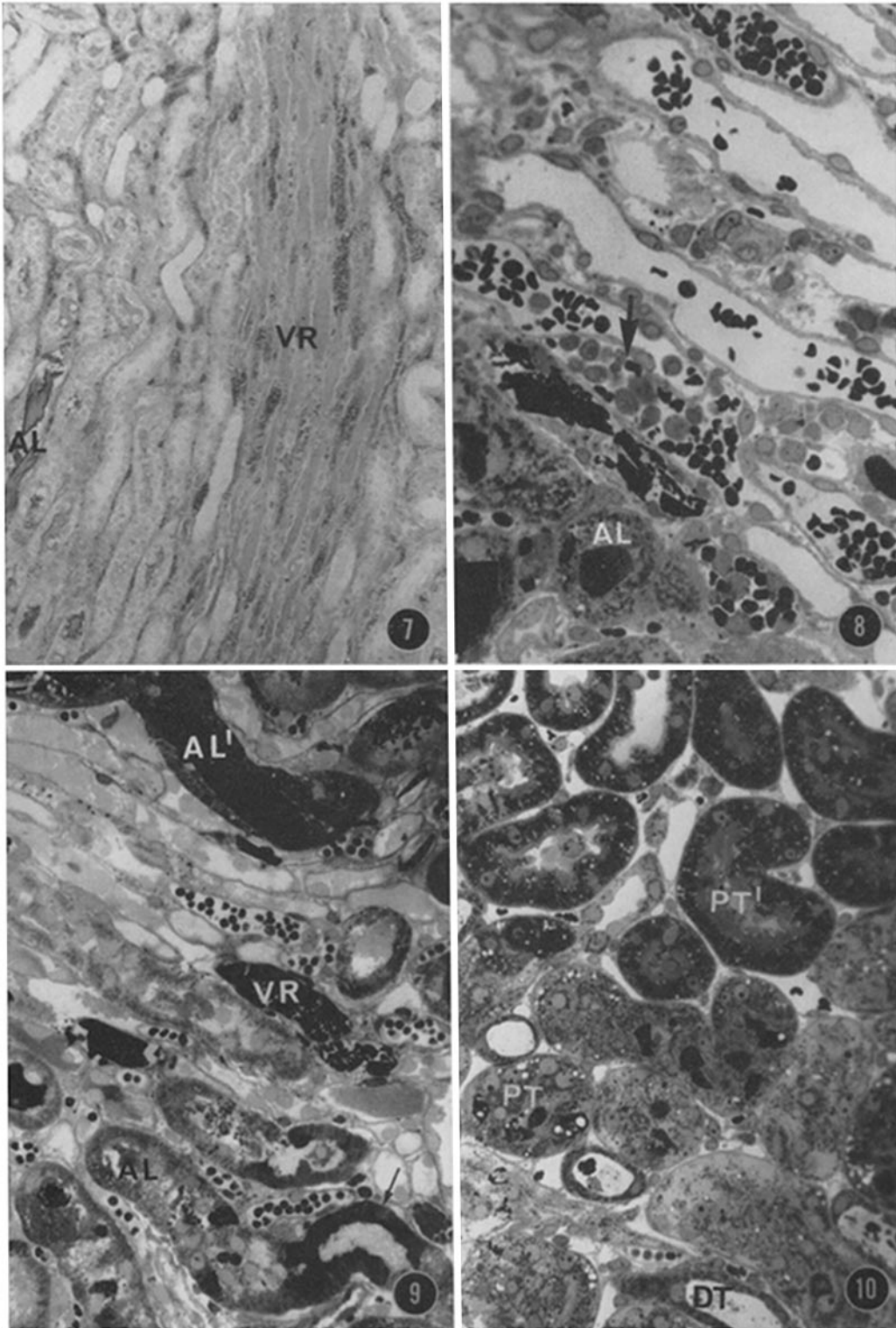(Jaenike and Schneeberger: Renal lesion with hemoglobinemia. II)

PLATE 64

FIG. 7. Rat kidney 24 hr after hemoglobin injection, the vasa recta (VR) still contain hemoglobin and admixed red blood cells. Hemoglobin casts are present in some thick ascending limbs of Henle (AL). Lepehne stain. × 120.

FIG. 8. Outer medulla 24 hr after hemoglobin injection. Several vasa recta contain mononuclear cells (→) and red blood cells. Hemoglobin casts are present in some thick ascending limbs of Henle (AL). Epon embedded, toluidine blue–stained thin section. × 480.

FIG. 9. Outer medulla 24 hr after hemoglobin injection. Many of the thick ascending limbs (AL) are dilated and the epithelium is fragmented. A few of the cells stain darkly (→). Hemoglobin precipitates are present in vasa recta (VR) while some thick ascending limbs (AL′) contain hemoglobin casts. Epon embedded, toluidine blue–stained thin section. × 480.

FIG. 10. Renal cortex 24 hr after hemoglobin injection. Several of the proximal convoluted tubules (PT) contain sloughed cells. Normal appearing proximal convoluted tubules (PT′) are still present. A few dilated distal convoluted tubules (DT) are also seen. Epon embedded, toluidine blue–stained thin section. × 300.
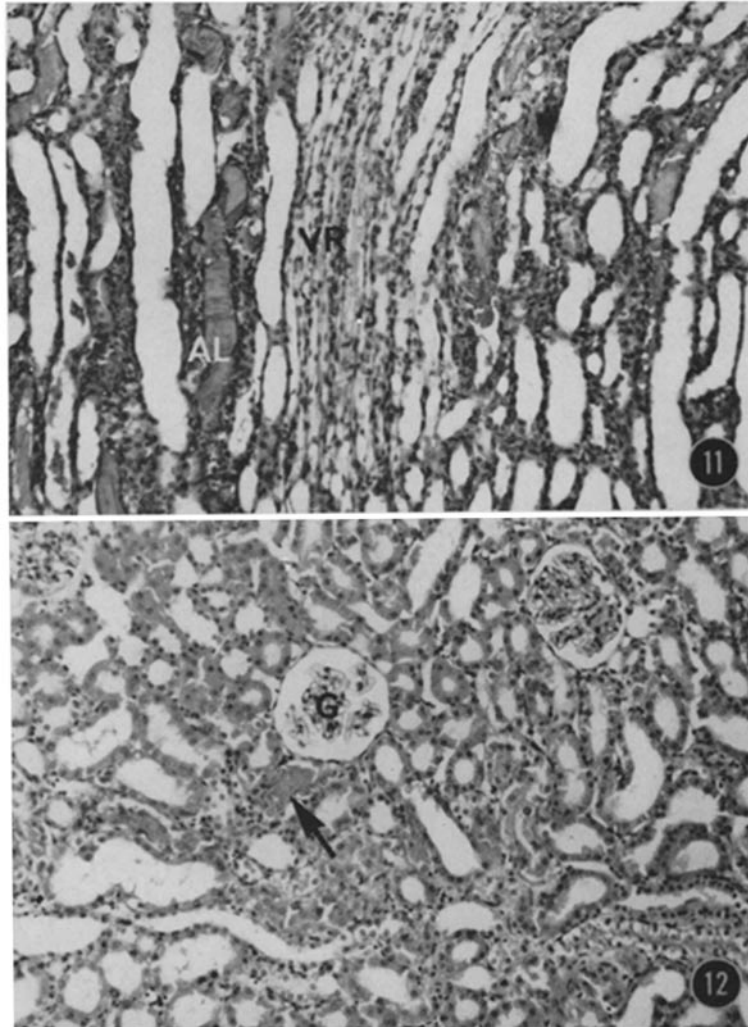
(Jaenike and Schneeberger: Renal lesion with hemoglobinemia. II)

PLATE 65

FIG. 11. Outer medulla of kidney 72 hr after hemoglobin injection showing vasa recta (VR) which are largely free of hemoglobin precipitates. A few tubular casts remain in thick ascending limbs of Henle (AL). Many dilated tubules are present. Hematoxylin and eosin. × 120.

FIG. 12. Cortex of kidney 72 hr after hemoglobin injection. Many dilated proximal and distal convoluted tubules are present. A rare cast (→) remains and the glomeruli (G) appear intact. Hematoxylin and eosin. × 120.

(Jaenike and Schneeberger: Renal lesion with hemoglobinemia. II)