A FUNCTIONAL AND ANATOMICAL STUDY OF THE EXCRETION OF HEMOGLOBIN BY THE KIDNEY.

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PLATE 8.

Deductions as to the function of the kidney may be drawn from evidence acquired either from morphological or physiological study of the organ. If the properties of an excreted substance are such that it can be demonstrated *in situ* in the organ as well as estimated quantitatively in the urine, then these two methods of investigation may be combined in a single experiment. Such an opportunity is found in the excretion of hemoglobin.

The mechanism of hemoglobinuria has been studied by many investigators. We shall consider here only those whose work summarizes the general problem or bears directly on our observations.

In 1896, Ribbert (1), by microscopic examination of kidneys of dogs in which hemoglobinuria had been produced by the intravenous injection of water, found deposits of hemoglobin in the capsular space of the glomeruli and in the lumen of the convoluted tubules connected with such glomeruli. He therefore concluded that the hemoglobin was excreted by the glomerulus. Adami (2) in more elaborate experiments observed the same deposits of hemoglobin in Bowman's space and so accepted Ribbert's conclusions.

Objection to such an interpretation was first made by Silbermann (3), who in experiments of a similar type could find only a few glomeruli that showed deposits of hemoglobin. He suggested that the hemoglobin was excreted by the cells of the tubules and that the occasional glomerular deposits were the result of a backing up of urine containing hemoglobin from the tubule.

In these studies the demonstration of hemoglobin in the sections of kidney depended on the occurrence of the substance in sufficient amount for its recognition by its physical characteristics, in particular its yellow color. An elective stain for the substance in weaker concentrations was devised by Miller (4). This method was based on Benda's modification of Weigert's method of staining the medullated fibers of the nervous system. The cells of the convoluted tubules and

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the ascending limbs of Henle's loop were found to react strongly with this method, while no positive reaction was found in the glomerular space. Miller therefore concluded that the principal source of hemoglobin was the secretory cells of the tubule. A criticism followed by Ribbert (5) and a reply by Miller (6), neither, however, adding any essentials to their original articles. Baehr (7) has attempted a reconciliation of the opposing views. He suggests that Ribbert is correct in the assumption that the hemoglobin filters through the glomerulus and that the intracellular hemoglobin described by Miller is the result of a storage of the substance. An analogy is thus suggested between the excretion and storage of carmine as described by Suzuki (8).

Finally, it must be noted that many investigators have studied the excretion of hemoglobin following various hemolytic poisons, and have pointed out the effects that damage to certain parts of the kidney may have on the manner of this excretion. We are interested here only in the method of excretion by the normal kidney, and will state in passing that Schmidt (9) and Sellards and Minot (10) have definitely established that the excretion of hemoglobin does not in itself cause any damage to the organ.

Technique.

Histological Methods.—There is comparative histological evidence, such as the reaction with red blood cells, which indicates the selective affinity of hematoxylin under certain conditions for hemoglobin, but an ideal microchemical method for the recognition of the substance would be one of which the chemical basis is more definitely understood. Such a method has been worked out by Brown (11), who has shown that oxidation by hydrogen peroxide of many of the complex iron compounds found in tissues allows their reaction with potassium ferrocyanide. A typical Berlin blue reaction is thus obtained with iron which is ordinarily masked.

Like all microchemical reactions, the method is capricious, and negative results must be controlled by repeated experiments. In our experiments various methods of fixation of the tissue were tried, and boiling in either water or Müller's solution for 2 minutes was found to be most satisfactory. This was followed by the usual dehydration in alcohols and embedding in paraffin.

Before staining sections which contain iron in the form of hemoglobin added by the process of the experiment, it is first necessary to determine the normal iron content of the cells. As Brown has shown, practically all tissues contain iron compounds reacting to some degree by this method. Longer periods of oxidation are necessary, however, to prepare such compounds for the reaction with the

ferrocyanide than are required to show it in elements which, like red blood cells, contain hemoglobin. This latter substance may therefore be stained differentially by determining the time necessary for sufficient oxidation to cause the iron to react, yet insufficient for the preparation of the masked iron of the protoplasm of tissues in general. This time will vary, depending on the strength of the peroxide solution and on the method of fixation, so that in practise certain results are obtained only if one stains at the same time and with the same reagents a known negative control.

In detail our method was as follows: Sections were freed of paraffin, run down through the alcohols to distilled water, and placed for 18 hours in a 3 per cent solution of peroxide. After brief rinsing in distilled water, they were placed in a mixture of equal parts of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid for 1 hour. The sections were finally counterstained with carmine. The control section, known to be free of hemoglobin except in its red blood cells, was either mounted on the same slide, or run through on a separate slide with the experimental sections.

Besides the method described above, Miller's hematoxylin stain was also used for the purpose of comparison. No modification in the details of the method was made.

Determination of Hemoglobin in the Urine.—The amount of hemoglobin in the urine was determined by Newcomer's method, with a colored glass standard and the Duboscq colorimeter. To 5 cc. of 1 per cent HCl a sufficient amount of filtered urine containing hemoglobin was added, the mixture allowed to stand for 15 minutes, and the comparison made. The hemoglobin was expressed in percentage concentration or in milligrams by the usual formula of the method. No trouble was caused by the urinary pigments as they are greatly diluted in the determination. In these experiments the animals were, moreover, excreting a dilute urine which, except for the hemoglobin, is quite pale.

This method was checked by diluting a known solution of hemoglobin with various amounts of urine and a satisfactory agreement found.

EXPERIMENTAL.

Variations in the Amount of Hemoglobin Excreted in the Urine.

The following was the general type of experiment followed.

A male rabbit was given a moderate intravenous injection of normal saline or 100 cc. of water by stomach tube. ½ hour later the animal was catheterized and

an intravenous injection of hemoglobin solution in normal saline given. At 15 minute intervals the catheterization was repeated, each time the bladder being washed out with a known amount of water. In these specimens the amount of hemoglobin was determined.

The amount of hemoglobin injected was in each case 0.06 mg. per kilo, twice the amount which Pearce, Austin, and Eisenbrey (12) have shown to be the threshold for excretion of this substance through the kidneys. The solution was prepared from the animals' own blood by laking with distilled water the red blood cells which had been collected in isotonic oxalate. This solution was made isotonic with NaCl and the stromata were removed with the centrifuge. In the first experiments toxic effects and even death followed the injections. It was found that these results could be avoided by manipulating the blood as little as possible and injecting the final solution of hemoglobin promptly after its preparation. We made no further study of the cause of these ill effects as soon as we had found a way of obviating them.

In all, ten experiments were done. The details of a typical experiment are given below and the results of the entire series are summarized in Text-fig. 1 and in Table I.

Rabbit 5.—Male; weight 2,625 gm. Bled and a 2.8 per cent hemoglobin solution in normal salt solution prepared. 15 cc. of normal salt solution were injected intravenously and 30 minutes later 11.1 cc. of the hemoglobin solution injected into the ear vein. This amount of solution contains 0.315 gm., twice the threshold dose of 0.06 gm. per kilo. The animal was then catheterized every 15 minutes for eight periods, and then after 30 and 75 minutes. The amounts of hemoglobin and water excreted are found in Table I.

A comparison of the series of curves derived from the experiments shows several points of interest. In the first place, the amount of urine in the different periods is quite variable and the time of occurrence of the maximum output varies widely. It must be remembered in this regard that the amount of water given in these experiments was comparatively small. A marked diuresis was not desired, and the fluid administered was only for the purpose of providing a sufficient amount of urine for the determinations. Doubtless administration of larger amounts would have produced a more regular output of water, and certainly longer collection periods would have had a smoothing effect on the curves.

TABLE I.

Rabbit No.	Duration of period, min.	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
1	Water, cc.	2.5	2.0	6.0	1.5		25.1						16.0			17.0
	Hemoglobin, mg..	12.0	13.0	10.0	69.0		17.0						9.0			19.0
2	Water, cc.	3.0	1.0	0.5	0.5	0			5.0				0			
	Hemoglobin, mg..	8.0	2.0	7.0	49.0	0			11.0				Tr.			
3	Water, cc.	4.5	7.8	10.5	17.0	1.0	0.3	4.5		Injected 20 cc. of NaCl.				0.5	5.3	2.2
	Hemoglobin, mg..	11.1	16.7	9.8	7.4	5.5	0.9	6.8						0.2	1.7	1.3
4	Water, cc.	2.6	2.4	4.0	2.8	5.0	2.0	3.8	23.5	15.8			2.7	2.1		
	Hemoglobin, mg..	6.0	10.1	10.9	8.1	7.8	4.2	4.0	15.8	8.0			1.2	2.0		
5	Water, cc.	6.0	5.3	9.5	15.5	11.9	4.7	3.8	7.7	15.8		32.6				
	Hemoglobin, mg..	7.3	22.7	12.6	11.2	11.5	8.0	4.0	1.0	8.0		3.0				
6	Water, cc.	4.7	3.7	8.8	8.0	9.4	9.6	5.0	2.5	5.5		4.9				
	Hemoglobin, mg..	7.7	11.8	12.8	7.0	7.3	6.7	4.0	2.6	5.2		2.6				
7	Water, cc.	21.0	15.2	11.9	8.3	5.2	7.2	6.4	12.7	14.0	11.1		3.5	2.0		
	Hemoglobin, mg..	13.0	13.4	10.0	8.5	5.7	5.9	3.9	3.7	3.6	2.1		1.5	1.7		
8	Water, cc.	2.5	3.5	1.4	1.0	3.0	1.3	0.5	0.7	1.8						
	Hemoglobin, mg..	11.6	15.9	5.8	5.5	8.2	5.7	2.3	1.7	4.6						
9	Water, cc.	6.8	1.4	2.0	6.0	6.0	3.0	Injected 38 cc. of NaCl.	22.0	9.5	3.5		3.0	1.0		
	Hemoglobin, mg..	13.8	17.3	24.8	11.9	5.2	3.9		15.4	2.8	1.1		1.0	Tr.		
10	Water, cc.	1.6	2.6	1.8	3.7	Injected 35 cc. of NaCl.	4.4	5.8	4.2	8.0	4.5					
	Hemoglobin, mg..	7.5	5.3	4.7	3.6		4.5	3.2	3.0	4.8	3.0					

At the beginning of each experiment twice the ordinary threshold dose of hemoglobin was injected intravenously.

The amount and rate of hemoglobin excretion are much more stable. There is a steady rise in both, reaching the maximum in seven out of the ten experiments in the second or third period and then falling gradually with occasional levels of constant excretion or even slight rises (Rabbit 9). For the purpose of description, we shall refer to the peak formed by the excretion of the maximum amount of hemoglobin as the major curve, and the lesser variations as the minor curves.

From a consideration of the characteristics of these two curves, the one regular in its course and the other variable, it is obvious that there can be little conformity between the two. In all the experiments save two, the major curve of hemoglobin excretion bears no constant relation to the amount of water excreted. In the two exceptional experiments (Rabbits 7 and 8) the unusual regularity of the water excretion causes it to coincide more or less with the output of hemoglobin.

A certain relation, however, can be found between the curves of excretion of the two substances. This consists in a coincidence in the majority of the experiments of the minor curves of hemoglobin excretion with increases in the amount of water. This is most strikingly demonstrated in Rabbit 9 in which a late diuresis, following an injection of NaCl solution, produced a sharp and definite increase in the excretion of hemoglobin. In other cases (Rabbits 8 and 10) this increase in the minor curve is only slight, while in others (Rabbits 3 to 6) the increase in water is accompanied by only a level of higher hemoglobin excretion than would be expected from the preceding slope of the curve. In one experiment (Rabbit 7) a definite increase in the amount of water coincided with a continually decreasing excretion of hemoglobin.

The significance of these relations between the output of water and hemoglobin will be discussed later when the histological distribution of the excreted hemoglobin has been described.

Demonstration of Hemoglobin in the Kidney.

As stated above, the microchemical demonstration of the iron of hemoglobin sometimes fails. Fortunately two controls are always at hand, first the red blood cells in the vessels of the kidney under

test, which when stained indicate whether or not the hemoglobin in the section has reacted positively, and secondly, the tissue of a normal or control kidney which possesses the masked iron common to all cells, that may be liberated by too long oxidation.

As an example of the distribution of hemoglobin in a kidney which is excreting hemoglobin, the detailed findings in a typical experiment are given.

Rabbit 11.—10 cc. of 0.9 per cent NaCl solution given intravenously. ½ hour later 20 cc. of approximately 3 per cent hemoglobin solution, representing twice the threshold value, were injected into the ear vein and 1½ hours later the animal was killed. The bladder urine was stained a dark red by hemoglobin. Small pieces of kidney were fixed at once for 2 minutes, in boiling Müller's fluid, embedded in paraffin, and sections stained, along with the normal control, for hemoglobin by the method previously described.

Microscopic Findings (Fig. 1).—The red blood cells throughout both the experimented and control kidneys were stained a light blue. Otherwise there was no blue staining in the control kidney. The following description applies only to the one which was excreting hemoglobin.

Cortex.—In the capsular space of the glomeruli there is a fine granular precipitate which stains a faint but definite blue. The lining epithelium of Bowman's space and the endothelium are negative, while in the capillaries of the vascular tuft are many blue-tinged erythrocytes. The proximal convoluted tubules as a rule show an obliterated or narrow lumen. The protoplasm of their thick epithelium is definitely blue, and in some the striations due to the batonnets can be made out. The nuclei of these cells stain only with the carmine. The distal convoluted tubule with its narrower epithelial lining shows a more definite lumen which contains bluish granular material. The protoplasm of the cells is also stained a definite blue.

Medulla.—The descending limb of Henle's loop contains bluish granular material, but its lining epithelium is stained red by the carmine counterstain. The broad ascending limb, however, resembles in its reaction the distal convoluted tubule, the protoplasm of its epithelium being blue with the usual red nucleus. In the lumina of the collecting tubules and in the ducts of Bellini are large masses of deep blue homogeneous or granular material. The most intense blue of the entire section is found in these cast-like appearances. The protoplasm of the epithelial cells is either red or faintly tinged along the free border of the cells with a deposit of blue color.

Animals killed at intervals of from 15 minutes to 2 hours showed the same picture; and the renal findings by Miller's method of staining were similar to those by Brown's. The convoluted tubules and ascending limbs of Henle's loops were heavily stained as well as

the masses of hemoglobin in the ducts of Bellini. The reaction in these structures was very pronounced, so that black hemoglobin contrasted well with practically unstained background (Fig. 2). In Bowman's capsule, where the hemoglobin was dilute, the reaction was not pronounced enough to allow one definitely to recognize the granular material as hemoglobin.

From the above description it will be seen that hemoglobin occurs in the lumen of the renal units in increasing concentration from the capsular space of the glomerulus to the duct of Bellini. In the protoplasm of the renal cells it is found in considerable concentration in the proximal and distal convoluted tubules and in the ascending limb of Henle's loop. Small amounts are also seen on the internal surface of the cells of the collecting tubules, but never here in a concentration approximating that found in the cells of the tubules mentioned above. Such a distribution is found 15 minutes after the beginning of the excretion of hemoglobin and continues to be the typical condition for 2 hours.

DISCUSSION.

It is evident that a consideration of either the functional or the anatomical findings leaves considerable uncertainty as to the route taken by the hemoglobin in its passage from the blood stream to the urine. If either set of observations is considered in the light of the other, however, certain of these complexities may be cleared.

From the microscopic findings it is certain that hemoglobin passes through the glomerular membrane, though apparently only in low concentration, and that it occurs in considerable amount in the cells of the convoluted tubules and in the ascending limb of Henle's loop. By the same means we are certain that a great increase in the concentration of hemoglobin in the urine occurs by the time it has reached the ducts of Bellini, at which point we may assume it has reached its final concentration.

The explanation of this increase in the hemoglobin concentration may be made in two ways: either (a) there is an absorption of water by the tubules, or (b) there is an added excretion of hemoglobin by the tubules into the urine as it passes down the tube. Let us examine the likelihood of both processes.

(a) From the histological examination of kidneys of animals which are excreting hemoglobin, we know that there is a considerable amount of this substance in the cells of certain of the tubules. Postulating that there is no excretion of hemoglobin by them, we must assume that this hemoglobin has been absorbed from the lumen along with the water. Although an absorption of the two components of the urine, water and hemoglobin, would be a very inefficient method of raising the concentration of the latter, such a method is possible.

But a comparison of the curves of water and hemoglobin excretion presents further complications. In Rabbit 5 (Text-fig. 1), for example, if the large amount and high concentration of hemoglobin in the second 15 minute period is the result of the production of a large amount of glomerular filtrate and a subsequent absorption of the water, then we must assume a converse small degree of hemoglobin absorption to produce such a high hemoglobin concentration. Yet sections of the kidney at such a time regularly show a marked accumulation of hemoglobin in the cells of the tubules. Moreover, at the third period, a reversal must have taken place in the relative degree of absorption of the two substances, as the amount of hemoglobin is now decreased, yet the amount of water is increased. Inspection of the curves of other experiments will show that this same sudden shifting of the relative degree of absorption of one or the other of the two substances is required again and again, if the variations in relative amounts of the two substances, as they appear in the urine, are to be explained by any such process.

(b) If the second explanation is accepted we assume two concentrating processes, occurring more or less independently, an absorption of water by certain tubules and an excretion of hemoglobin by others. The comparative constancy of the hemoglobin curve is explained by a corresponding constancy of its excretion by the cells of the tubules, the amount decreasing gradually as the available supply of free hemoglobin in the plasma becomes smaller. This accounts for the relatively large amounts of hemoglobin seen in the cells if they are examined in the early stages of the experiment. The filtration and absorption of water occurring independently of this excretion by the tubules will obviously form a curve which has little relation to the absolute amount of hemoglobin excreted, and the
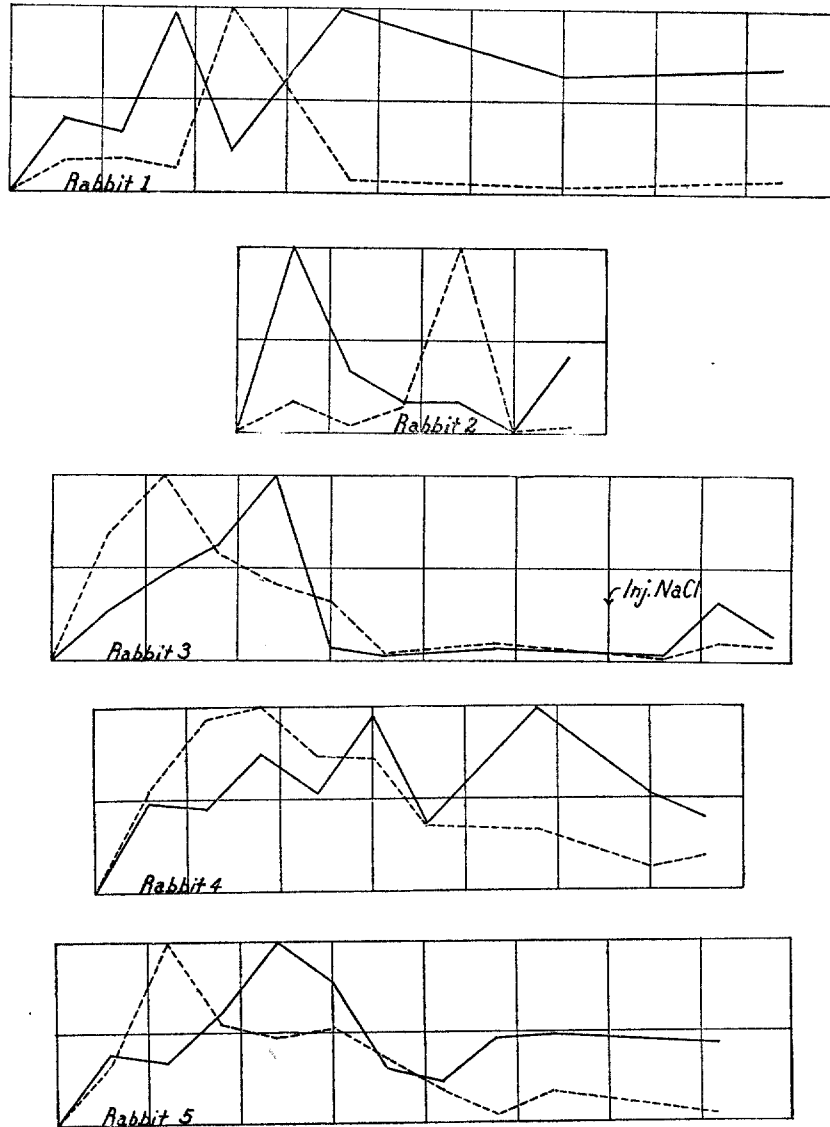
———— Excretion of water.

- - - - - - Excretion of hemoglobin.

TEXT-FIG. 1. Excretion of water and hemoglobin. The abscissæ represent periods of time following the intravenous injection of hemoglobin, in most instances 15 minutes. The ordinates show the amount of water or hemoglobin. The
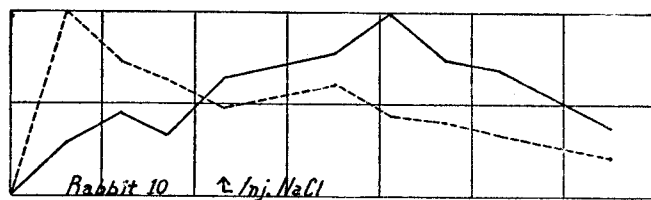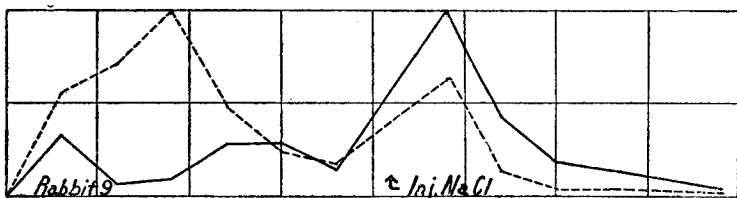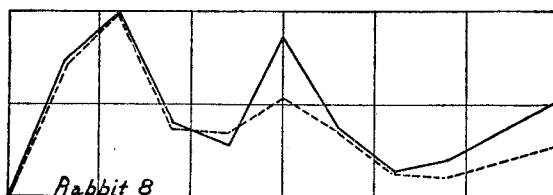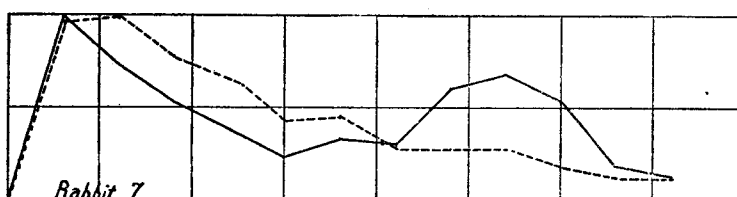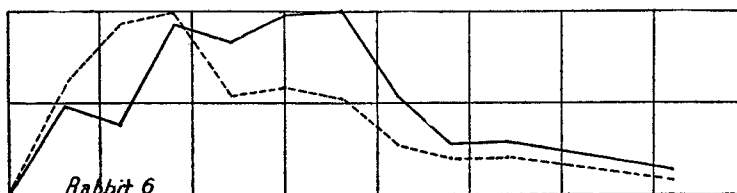
greatest output of the substance in any one 15 minute period is taken as 100, and the amounts for the other periods expressed as percentages of this maximum sample. The absolute amounts of the two substances for each period are shown in Table I.

small amount of hemoglobin which does filter through the glomerular membrane will account for the slight secondary increases in the amount of hemoglobin which are seen following sudden increases in the amount of water put out (Rabbit 9), and the levels of constant hemoglobin excretion seen after less exaggerated increases in the water excretion (Rabbits 3 to 6, 8 and 10). This effect of diuresis on the excretion of hemoglobin has been pointed out by Haessler (13) who demonstrated that increases in water excretion cause the appearance of hemoglobin in the urine even in animals whose plasma contains so small an amount of this substance that under ordinary conditions of urine formation it cannot pass the renal barrier. It is also worthy of note that the effects of diuresis in our experiments were marked only in the latter parts of the experiments, a time when the hemoglobin content of the plasma was low, the condition which obtained in Haessler's experiments.

Of the two possible methods of hemoglobin concentration described above, the second seems preferable to us. By such a method the renal findings, both anatomical and functional, are more directly explained than by the assumption that the tubule cells absorb substances but do not excrete them. As has already been shown by one of us (14) a similar mechanism of glomerular filtration and tubule excretion operates in the excretion of urea.

CONCLUSIONS.

1. The anatomical and functional findings in hemoglobin excretion are best explained by the assumption of a filtration of this substance through the glomerulus and an additional excretion of it by the tubule cells.

2. Absorption of water aids in the concentrating process, and is most marked in the collecting tubules.

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EXPLANATION OF PLATE 8.

Fig. 1. Rabbit 11. Kidney removed 1½ hours after the intravenous injection of hemoglobin. Stained by Brown's method, the hemoglobin appears as a light blue. The lower portion of the cortex and outer zone of the medulla with a medullary ray are shown. Hemoglobin is seen in the vessels of the glomeruli, in the cells of the convoluted tubules, the terminal portion of the proximal convoluted tubules, and the ascending limbs of Henle's loop and in the lumen of the ducts of Bellini. The process of reproduction from the original colored drawing has somewhat exaggerated the contrast between those cells which contain hemoglobin and those which do not. Camera lucida drawing.

Fig. 2. Same section as Fig. 1, stained by Miller's method. The outer zone of the medulla is shown, in which the terminal portions of the proximal convoluted tubules are seen stained a heavy black by the hematoxylin.
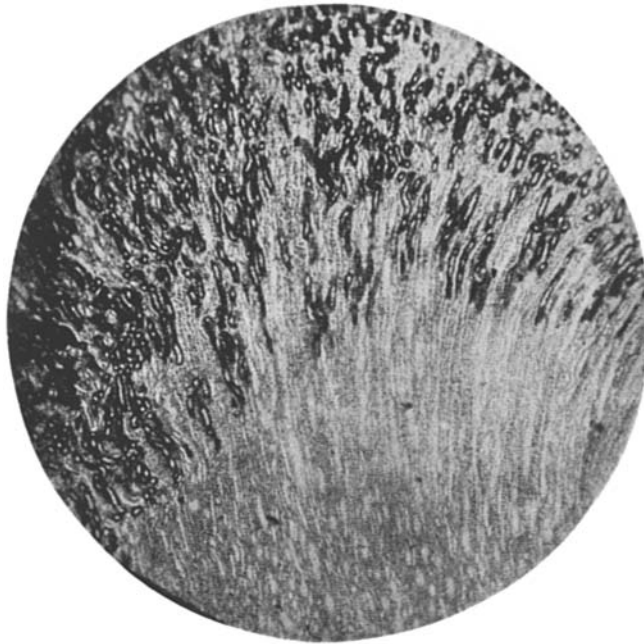
FIG. 1.

FIG. 2.

(Fukuda and Oliver: Excretion of hemoglobin by kidney.)