THE RENAL EXCRETION OF HEMOGLOBIN: REGULATORY MECHANISMS AND THE DIFFERENTIAL EXCRETION OF FREE AND PROTEIN-BOUND HEMOGLOBIN * †

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Previous studies of the transport and excretion of extracorpuscular hemoglobin by the kidney have been based on the assumption that hemoglobin circulates in plasma in the free, unbound state, and within the limitations imposed by the glomerular membrane, freely diffuses into glomerular filtrate. The demonstration (2, 3) that plasma proteins possess the property of binding hemoglobin and that hemoglobin may circulate in both the free and unbound states (4–6) has invalidated the conceptual basis of these studies. Consequently many of the previous concepts of renal hemoglobin transport are no longer tenable and must be revised.

Observations of hemoglobin binding *in vivo* and *in vitro* have established that the capacity of plasma proteins to bind hemoglobin is limited (3–6). Since this capacity is near the plasma level at which hemoglobinuria characteristically occurs, it has been suggested (4, 5) that the limitation imposed on hemoglobin excretion at low plasma levels is related to protein-binding and that only when the binding capacity is exceeded and hemoglobin circulates in the free, unbound state does hemoglobinuria occur. Implicit in this suggestion is the assumption that free and protein-bound hemoglobin are transported differently by the kidney. This assumption has not, however, been subjected to experimental study and the respective roles these transport phenomena play in regulating hemoglobin excretion is unknown. The present investigation was therefore undertaken for the purpose of studying these aspects of renal hemoglobin transport and of re-evaluating the regulatory mechanisms of hemoglobin excretion in man.

METHODS

Renal hemoglobin excretion and transport were measured under conditions of a progressivly increasing plasma hemoglobin concentration in 10 adult human subjects (aged 18 to 48 years) who were free of apparent renal, cardiovascular, hematologic or other disease. All subjects were fasting at the time of the study and each had ingested 500 ml. of water approximately one hour prior to the test. A catheter was placed in the urinary bladder and an indwelling needle was inserted in the brachial artery. Arterial blood was withdrawn and hemolyzed according to methods previously described (6), and priming and sustaining infusions of hemoglobin were prepared. The priming infusions contained approximately 2.0 Gm. of hemoglobin in 50 ml. of solution. The sustaining infusion contained 2.0 Gm. of hemoglobin and 50 ml. of inulin in 1,000 ml. of 5 per cent dextrose in water. Immediately after preparation of these solutions the first of from five to seven priming injections of hemoglobin was given intravenously, along with 25 ml. of inulin, and the sustaining infusion was started. The sustaining infusion was administered intravenously at a rate of 4.5 ml. per minute (9.0 mg. of hemoglobin per minute) throughout the study. Additional priming injections of hemoglobin were given at 45 to 60 minute intervals throughout the study. These injections were given as rapidly as possible, usually over a period of from five to 10 minutes, without causing undue discomfort over the site of the vein used for injection. The total hemoglobin administered was approximately 14 to 18 Gm., given over a period of four hours.

After each priming injection of hemoglobin a period of from 15 to 30 minutes was allowed for adjustments of plasma and urinary levels, and the urine was discarded. Urine was then collected for analysis during two consecutive 15 to 20 minute periods. At approximately the midpoint of each period a sample of arterial blood was withdrawn. Standard renal clearance techniques were used for collection of the urine. The arterial blood samples were collected with care to avoid hemolysis and were heparinized and centrifuged immediately. Analysis of plasma and urine was usually made on the day of the study. When analysis was delayed plasma and urine were frozen and stored at $-10°$ C.

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Plasma and urine were analyzed for inulin by the method of Harrison (7) and the clearance of inulin during each collection period was calculated. The total hemoglobin content of plasma and urine was determined by the method of Evelyn and Malloy (8). The concentrations of free hemoglobin, protein-bound hemoglobin and methemalbumin in plasma and urine were determined by a paper electrophoretic method previously described (6). This method consisted of the separation of these moieties on paper after overnight electrophoresis utilizing a phosphate buffer, pH 7.0, ionic strength 0.05. Following electrophoresis the individual fractions were identified by staining with 1 or 2 per cent benzidine in 20 per cent acetic acid and 3 per cent hydrogen peroxide. The appearance of oxidized benzidine in the areas of the paper containing hemoglobin (or methemalbumin) permitted visualization of the separated fractions which were identified by their characteristic positional relationships.

Quantification of the individual fractions was made by photometric analysis (Spinco Analytrol®) of the stained filter paper strips, where measurements of light absorption were used to calculate the relative concentrations of each fraction. The details of these techniques have been described previously (6).

The data obtained from these analyses were used to evaluate the relationships between plasma and urinary hemoglobin and to measure two parameters of renal hemoglobin transport: glomerular clearance and tubular reabsorption. The calculations employed in these measurements are presented under Results.

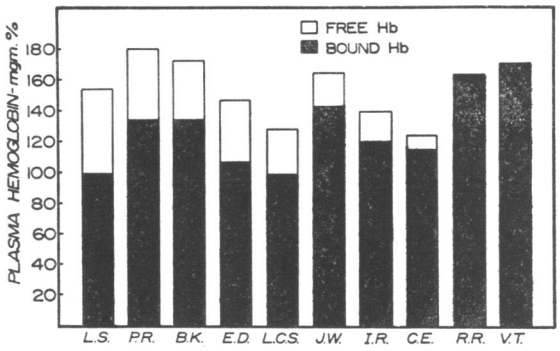

FIG. 1. THE RELATIONSHIP BETWEEN THE CONCENTRATION OF HEMOGLOBIN IN PLASMA AND THE APPEARANCE OF HEMOGLOBIN IN THE URINE

Each bar represents an individual experiment with the subject's initials at the bottom of the figure. The top of the bar represents the estimated total concentration of hemoglobin in plasma which was attained prior to the appearance of hemoglobin in the urine. The components of total hemoglobin in plasma are represented by the clear and solid portions of the bars, where the clear portion represents the concentration of free hemoglobin and the solid portion the concentration of protein-bound hemoglobin (maximal binding capacity). In each instance hemoglobinuria did not occur until the maximal binding capacity was exceeded and free, unbound hemoglobin appeared in plasma. In Subject V.T., hemoglobinuria would theoretically occur at any concentration of free hemoglobin in plasma.

RESULTS

Plasma hemoglobin

Following each priming infusion the concentration of hemoglobin in plasma increased. Between priming injections the concentration remained relatively constant. The hemoglobin administered during the early phases of the study was quantitatively bound to plasma protein and circulated entirely in this state. As the plasma level increased, the concentration of protein-bound hemoglobin rose progressively until a level was attained at which additional binding did not occur and hemoglobin appeared in plasma in the free, unbound state. This level, which may be considered as the maximal binding capacity of plasma protein for hemoglobin, varied in individual studies from 98 to 171 mg. per cent and averaged 128 ± 25 mg. per cent. As the plasma level of hemoglobin increased above the maximal binding capacity the concentration of free hemoglobin progressively rose. In all but two subjects (V. T. and E. D.) small quantities of methemalbumin (5 to 11 mg. per cent) appeared in plasma coincident with the appearance of free hemoglobin. The level of methemalbumin remained relatively constant throughout the study. At the conclusion of the test the total hemoglobin concentration in plasma varied in individual studies from 205 to 355 mg. per cent and the concentration of free hemoglobin from 101 to 263 mg. per cent.

Urinary hemoglobin

As the plasma hemoglobin concentration increased the urine remained free of hemoglobin until the maximal binding capacity of plasma proteins was exceeded and hemoglobin circulated in the free, unbound state (Figure 1). With the appearance of free hemoglobin in plasma, hemoglobin appeared in the urine. Electrophoretic studies of urinary hemoglobin at all plasma levels and excretory rates obtained revealed that in each subject the electrophoretic characteristics of urinary hemoglobin were in all respects similar to those of free hemoglobin in plasma (Figure 2). At pH 7.0 both urinary hemoglobin and plasma free hemoglobin remained at the site of application

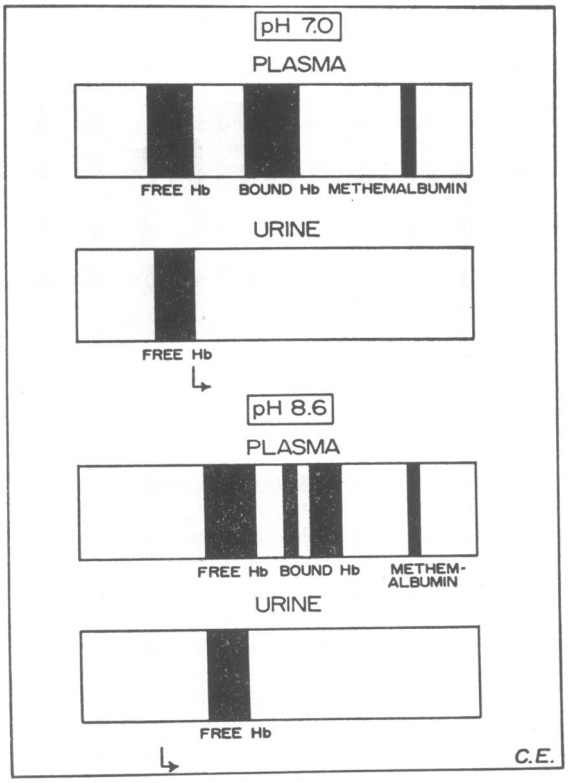

FIG. 2. THE PAPER ELECTROPHORETIC CHARACTERISTICS OF PLASMA AND URINARY HEMOGLOBIN

The positions of the various heme-fractions are shown schematically at pH 7.0 in the upper part of the figure and at pH 8.6 in the lower part of the figure. The arrows refer to the application site and direction of movement towards the anode. At both pH 7.0 and 8.6 the electrophoretic characteristics of urinary hemoglobin were the same as those of plasma free, unbound hemoglobin. The position that free hemoglobin assumes to the cathode side of the application site at pH 7.0 is due to endosmotic flow. Protein-bound hemoglobin and methemalbumin were absent from the urine. In plasma, protein-bound hemoglobin appears as two separate fractions at pH 8.6 and as one fraction at pH 7.0.

of the sample to the paper; at pH 8.6 urinary hemoglobin migrated at the same speed as plasma free hemoglobin. Thus, the circulation of free hemoglobin in plasma was associated with the appearance in the urine of hemoglobin which was, in terms of its electrophoretic characteristics, similarly free and unbound. Only free hemoglobin was excreted; in no instance was PBH or methemalbumin excreted in detectable amounts.

From a study of the relationship between changing excretory rates and plasma levels of free hemoglobin (Figure 3) estimates were made of the theoretical plasma level of free hemoglobin at which hemoglobinuria occurred. These estimates were made by an extrapolation of the curves (UV/P) shown in Figure 3, where the extrapolated intercept of the horizontal (P) axis was taken as the plasma concentration of free hemoglobin above which hemoglobin appeared in the urine. In all experiments the lowest determined value of UV used in this extrapolation was less than 1.0 mg. per minute. Thus the horizontal axis was intercepted by a short extension from determined values. Although this lessened the possibility of erroneous estimation of P, the values obtained are considered as theoretical estimates only since the relationship between UV and P at low values of each was undetermined. Within

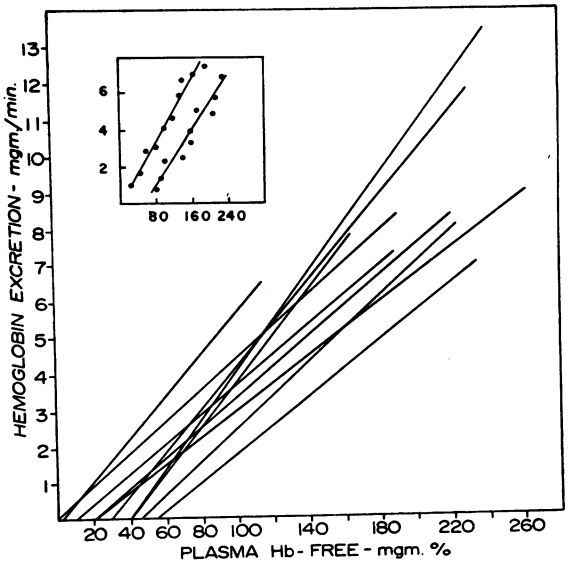

FIG. 3. THE RELATIONSHIP BETWEEN THE EXCRETORY RATE AND PLASMA CONCENTRATION OF FREE HEMOGLOBIN

Each curve represents an individual experiment. The lower portion of the curves which intercept the horizonal axis was obtained by extrapolation from determined values. In each instance such extrapolation was made from a determined excretory rate of less than 1.0 mg. per minute. The intercept of the horizontal axis represents the theoretical plasma level of free hemoglobin above which hemoglobinuria occurred. The slope of the curves (Δ UV/Δ P) represents the glomerular clearance of free hemoglobin. The determined values of UV and P from which curves were constructed in two of the experiments are shown in the insert. These two experiments represent those with the lowest and highest extrapolated P value. In all experiments the curves were drawn by visual approximation from determined values of UV and P.

these limitations and that imposed by the accuracy of estimating the relationship of UV:P, the values obtained for P, shown in Figures 1 and 3, varied in individual studies from 0 to 54 mg. per cent and averaged 27 ± 19 mg. per cent. When these levels of free hemoglobin were added to those of the respective maximal concentrations of protein-bound hemoglobin, the estimated maximal plasma level of total hemoglobin which was attained before hemoglobin appeared in the urine varied from 124 to 180 mg. per cent (Figure 1). These levels are in agreement with previous estimates of the relationship between plasma hemoglobin (total) concentration and the development of hemoglobinuria (9).

Glomerular clearance of free hemoglobin

The urinary excretion of free hemoglobin increased progressively and proportionately as the plasma level of free hemoglobin increased (Figure 3). Using this relationship, the rate at which free hemoglobin was cleared from plasma at the glomerulus was calculated from a plot of UV against P, where the slope of the curves obtained represented the clearance rate ($C^{G}_{\text{Free Hb}}$) in ml. per minute:

$$\text{Glomerular clearance (ml. per min.)} = \frac{\Delta U_{\text{Free Hb}} V}{\Delta P_{\text{Free Hb}}}. \quad 1)$$

When corrected for body surface area the glomerular clearance of free hemoglobin varied in the group of 10 subjects from 3.0 to 7.0 and averaged 5.0 ± 1.0 ml. per minute per 1.73 square meters body surface area (Table I).

The curves used to calculate these values were those constructed from determined and not from extrapolated values of UV and P. The plasma level of free hemoglobin used in these calculations was obtained by extrapolation for intrarenal delay to a point 2.5 minutes before the midpoint of each collection period. In practice such an extrapolation was seldom necessary since the plasma level changed only slightly during the 30 to 60 minute period between priming injections.

The clearance of inulin in these studies varied from 77 to 122 ml. per minute per 1.73 square meters. These values are lower than those reported in normal, basal subjects (10). This may be attributed to renal vasoconstriction with reduction in renal blood flow and glomerular filtration rate which accompanies the administration of hemoglobin intravenously (11). Progressive changes in glomerular filtration rate with each priming injection, as have been observed by others (11), did not occur and the inulin clearance remained constant throughout each study.

TABLE I

The glomerular clearance of free hemoglobin and the renal clearance of inulin *

Subject	C^{G}_{Hb}	C_{In}	C^{G}_{Hb}/C_{In}
	ml./min.	*ml./min.*	
B. K.	7	101	0.066
R. R.	6	96	0.063
I. C. S.	6	91	0.063
E. D.	5	91	0.056
V. T.	5	122	0.039
C. E.	5	87	0.053
I. R.	4	84	0.052
P. R.	4	104	0.038
L. S.	4	97	0.040
J. W.	3	77	0.043
Mean ± S. D.	5 ± 1		0.051 ± 0.01

* All values are corrected to 1.73 sq. meters body surface area. Abbreviations are as follows: C^{G}_{Hb} the glomerular clearance of free hemoglobin; C_{In}, the renal clearance of inulin.

The ratio $C^{G}_{\text{Free Hb}}/C_{In}$, tabulated in Table I, averaged 0.05 ± 0.01. This ratio expresses the glomerular permeability to hemoglobin relative to inulin.

Tubular reabsorption of free hemoglobin

The rate of tubular reabsorption of free hemoglobin was calculated as the difference between the filtered load and excretory rate at any given plasma level of free hemoglobin:

$$\text{Filtered load (mg. per minute)} = C^{G}_{\text{Free Hb}} \times P_{\text{Free Hb}}. \quad 2)$$

$$\text{Reabsorptive rate (mg. per minute)} = C^{G}_{\text{Free Hb}} \times P_{\text{Free Hb}} - U_{\text{Free Hb}} V. \quad 3)$$

At loads sufficient to induce hemoglobinuria, the relationship between the excretory rate and plasma level was linear (Figure 3) and the calculated reabsorptive rate did not, therefore, change with increasing loads. Hence, the calculated reabsorptive rate at these loads may be considered as the estimated maximal reabsorptive capacity. This capacity, obtained at load/T ratios in excess of 3.0 in all subjects, varied in individual subjects from 0.0 to 2.6, and averaged 1.3 ± 0.9 mg. per minute

TABLE II

The estimated maximal rate of tubular reabsorption of free hemoglobin *

Subject	T_{Hb}	T_{Hb}/100 ml. filtrate	C^{G}_{Hb}/T_{Hb}
	mg./min.	*mg./100 ml.*	*ml./mg.*
B. K.	2.6	2.6	2.6
E. D.	2.1	2.3	2.4
L. S.	2.1	2.2	1.9
P. R.	1.9	1.8	2.1
L. C. S.	1.6	1.8	3.6
I. R.	0.8	1.0	5.5
J. W.	0.8	1.0	4.1
C. E.	0.4	0.5	11.5
R. R.	0.3	0.3	20.0
V. T.	0.0	0.0	
Mean ± S. D.	1.3 ± 0.9	1.4 ± 1.0	6.0 ± 6.0

* All values are corrected to 1.73 sq. meters body surface area. Abbreviations are as follows: T_{Hb}, calculated maximal tubular reabsorptive rate of free hemoglobin (mg. per minute); T_{Hb}/100 ml. filtrate, mg. of free hemoglobin reabsorbed per 100 ml. of glomerular filtrate; C^{G}_{Hb}, the glomerular clearance of free hemoglobin (ml. per minute).

per 1.73 square meters body surface area (Table II). In five subjects (I.R., J.W., C.E., R.R. and V.T.) the estimated reabsorptive capacity was less than 1.0 mg. per minute and in five subjects (B.K., E.D., L.S., P.R. and L.C.S.) greater than 1.0 mg. per minute. The intercept of the curves obtained from a plot of UV against P was at or near zero in those subjects with a low estimated reabsorptive capacity and was displaced from zero in those subjects with a reabsorptive capacity in excess of 1.0 mg. per minute (Figure 3). There was no correlation between estimated reabsorptive capacity and glomerular clearance (Table II).

When reabsorption was expressed in terms of glomerular filtration rate, an average of 1.4 ± 1.0 mg. of hemoglobin was reabsorbed per 100 ml. of glomerular filtrate (Table II).

DISCUSSION

This study has demonstrated, as predicted (4, 5), that hemoglobin is not excreted in the urine until the plasma hemoglobin concentration reaches a level which exceeds the capacity of plasma proteins to bind hemoglobin. When this capacity, which averages 128 mg. per cent, is exceeded, free, unbound hemoglobin appears in plasma and is excreted in the urine. Under these circumstances, and as the plasma level changes, the urine contains free hemoglobin, but does not contain protein-bound hemoglobin, which is not excreted.

The rate at which free hemoglobin is excreted is dependent upon three variables: *1*) the plasma level of free hemoglobin, *2*) the rate of clearance from plasma by the glomerulus, and *3*) the rate of tubular reabsorption. Under conditions of a progressively increasing plasma concentration of free hemoglobin the excretory rate increased in the present study in proportion to the change in plasma level. At any given plasma level the rate of excretion appeared to depend in large measure upon the rate at which free hemoglobin was filtered by the glomerulus and only to a negligible, if any, extent on tubular reabsorptive activity. Estimates of glomerular clearance made from the relationship between the changing excretory rate and plasma concentration ($\Delta UV/\Delta P$) revealed that on the average 5 ml. of plasma was cleared of free hemoglobin per minute by the glomerulus. Since the clearance of inulin averaged approximately 100 ml. per minute, the glomerulus was 5 per cent as permeable to free hemoglobin as to water (inulin). Or, expressed in another way, the concentration of free hemoglobin in glomerular filtrate averaged 5 per cent of that in plasma. The permeability of the glomerulus to hemoglobin is therefore much less than previous estimates which were made without consideration of the physicochemical state of plasma hemoglobin (9).

Although the glomerular clearance was small, considerable amounts of hemoglobin were filtered. Calculated filtered loads reached 8 to 10 mg. per minute in most studies. It appeared that most, if not all, of this filtered free hemoglobin was excreted in the urine. The highest estimated maximal tubular reabsorptive rate observed was 2.6 mg. per minute, and in one-half of the subjects the rate was less than 1.0 mg. per minute. Thus the composition of glomerular filtrate with respect to free hemoglobin appeared to change but little during the passage of urine down the renal tubules. Indeed, the values obtained for maximal reabsorptive capacity are so small that it is uncertain whether the composition changed at all. The physiologic validity of the calculations of reabsorptive rate is dependent upon an accurate determination of two variables: load and excretory rate. Since these variables were large in relation to the differences between them (reabsorptive rate), inaccuracies in the determination of either would affect to a con-

siderable extent estimates of tubular reabsorptive activity, the physiologic significance of which would depend upon the direction and magnitude of the errors involved. Without knowing the extent and direction of such errors, the values obtained for reabsorptive capacity appear to be of insufficient magnitude in relation to possible errors in their estimation to justify definite conclusions regarding the quantitative aspects of tubular reabsorption. The results are consistent with a low rate of tubular reabsorption of free hemoglobin but do not distinguish between small or absent reabsorptive activity.

If a precise quantitative estimate of tubular reabsorption cannot be made, these observations indicate that tubular reabsorption of free hemoglobin is at least small and does not play a determinant role in regulating hemoglobin excretion. It would therefore be anticipated that hemoglobin would appear in the urine at low plasma levels of free hemoglobin. This was, in fact, observed in the present study where estimates of the plasma level above which hemoglobinuria occurred averaged 27 mg. per cent. Since, however, calculations of this value also depended upon an accurate estimation of the slope $\Delta UP : \Delta P$, and upon an extrapolation as well, this figure must be interpreted cautiously. Nonetheless, in several subjects this estimated plasma level exceeded 20 mg. per cent and in one subject approached 60 mg. per cent (Figure 3), values which would be difficult, because of their magnitude, to attribute to errors in estimating the slope alone. These figures are therefore more suggestive of tubular reabsorptive activity than are the calculated reabsorptive rates, on the assumption that it was necessary to elevate the plasma concentration of free hemoglobin to these levels before tubular reabsorptive activity was saturated and hemoglobinuria occurred.

These considerations are not conclusive, however, and the evidence for tubular reabsorption in man remains doubtful. Although experiments in animals suggest that hemoglobin finds its way into renal tubular cells (12–14) the evidence in man is less convincing and appears to consist principally of the demonstration of hemosiderin in renal tubular cells (15), in the urine and in urinary casts (16) in patients with hemolytic disorders. There is little histologic evidence of tubular reabsorption in man (17) and the present study does not provide unequivocal physiologic evidence. More definitive studies are therefore necessary before it can be concluded that tubular reabsorption of free hemoglobin occurs in man.

The failure to detect protein-bound hemoglobin or methemalbumin in the urine in the present study when these constituents were present in plasma may be attributed to lack of filtration or to complete tubular reabsorption of these hemeproteins. In view of what is known concerning glomerular permeability and tubular reabsorption of large molecular substances, these heme-protein complexes would be expected to traverse the glomerular membrane with difficulty and would be unlikely to appear in glomerular filtrate in significant concentration under normal circumstances (18, 19). The molecular weight and size of methemalbumin is certainly equal to that of albumin (20), to which the normal glomerulus is believed to be in large measure impermeable (18, 19). Although the physical characteristics of protein-bound hemoglobin have not been definitely established, the molecular weight of this hemoglobin-α_2 globulin (?) complex appears to be in excess of 300,000 (4), suggesting that it also is unlikely to traverse the glomerular membrane readily because of its size. These considerations therefore suggest that the limitation imposed upon the excretion of these substances is attributable, at least in large part, to the permeability characteristics of the glomerular membrane rather than to avid tubular reabsorption activity.

The probem raised by Smith (10) as to why the glomerulus is as permeable as it appears to be to (free) hemoglobin, which in molecular weight, at least, is similar to albumin (20), is not resolved. The lower estimates of glomerular permeability made in this study are more consistent than previous estimates (9) with the molecular weight and physical characteristics of free hemoglobin (20). However, the question is unanswered as to whether the glomerulus is permeable to the free hemoglobin molecule as such or whether hemoglobin enters glomerular filtrate only after dissociating into smaller parts.

SUMMARY

A study of urinary hemoglobin excretion and renal hemoglobin transport was made in 10 normal

human subjects. Under conditions of a progressively increasing plasma hemoglobin concentration, hemoglobinuria did not occur until the plasma level exceeded the capacity of plasma proteins to bind hemoglobin. When this capacity, which averaged 128 mg. per cent, was exceeded, free, unbound hemoglobin appeared in plasma and was excreted in the urine.

A linear relationship was demonstrated between the excretory rate and plasma level of free hemoglobin as the plasma concentration increased. From this relationship calculations were made of the glomerular clearance and tubular reabsorption of free hemoglobin. The glomerular clearance averaged 5 ml. per minute per 1.73 square meters of body surface area and averaged 5 per cent of the inulin clearance. The estimated maximal reabsorptive rate averaged 1.0 ml. per minute per 1.73 square meters of body surface area. This value was too small to be considered as conclusive evidence of tubular reabsorptive activity.

Neither protein-bound hemoglobin nor methemalbumin was excreted in the urine when these constituents were present in plasma. This was attributed to a failure of these large heme-proteins to traverse the glomerular membrane.

ACKNOWLEDGMENTS

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ADDENDUM

Since this work was completed Vanderveiken, Gueritte, de Myttenaere and Lambert have reported (Effets de la liason hemoglobine-haptoglobine sur l'excretion de l'hemoglobine. J. d'Urol. 1958, **64**, 137) on a failure to find physiologic evidence of tubular reabsorption of free hemoglobin in the dog.

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