THE DISTRIBUTION OF EXTRACORPUSCULAR HEMOGLOBIN IN CIRCULATING PLASMA * †

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(Submitted for publication October 1, 1958; accepted November 6, 1958)

The addition of hemoglobin to plasma is accompanied by a number of physiochemical and biologic reactions. Among the important reactions which may occur is the interaction of hemoglobin with one or more of the plasma proteins in which hemoglobin forms a complex with, and becomes bound to, plasma protein. This phenomenon was first described by Polonovski and Jayle (2–4), and has been studied extensively (5, 6). To the protein (or proteins) having the property of binding hemoglobin Polonovski and Jayle gave the name haptoglobin (7). The identification of this protein(s) has not been definitely established, but tentative evidence suggests that it may be an α_2 globulin (5, 6). Utilizing *in vitro* and titra-metric techniques, Jayle and Conas (5) and others (6) have observed that plasma protein is capable of binding hemoglobin in concentrations up to approximately 150 mg. per cent; at concentrations in excess of this, additional binding does not occur. This limitation of binding capacity was later observed by Laurell and Nyman (8) and Allison and ap Rees (9) who used paper electrophoretic techniques and who also demonstrated that hemoglobin appeared in plasma in the free, unbound state when added *in vitro* in amounts which exceeded the maximal binding capacity.

The quantitative aspects of hemoglobin binding *in vitro* and of the relationships between binding capacity and the appearance of free hemoglobin have been established (8, 9). However, the methods available have not been suitable for *in vivo* studies and, consequently, quantitative information concerning these phenomena and the distribution of hemoglobin in *circulating* plasma is lacking. It was the purpose of the present study to provide such information. To this end an electrophoretic method was developed for the quantitative analysis of free and protein-bound hemoglobin concentrations in plasma. Using this method, a study was made of the electrophoretic characteristics of plasma hemoglobin and of the distribution and transport of intravenously administered hemoglobin.

METHODS

The total hemoglobin concentration in plasma was determined by the method of Evelyn and Malloy (10). The relative concentrations of free hemoglobin, protein-bound hemoglobin (PBH), and methemalbumin were determined by photometric analysis of benzidine-stained filter paper strips following separation of the individual hemeprotein fractions by paper electrophoresis.

Paper electrophoresis. Standard techniques were employed in which 10 μl. of plasma containing hemoglobin was subjected to overnight electrophoresis (5 ma., 110 volts) in a Durrum type cell employing hanging filter paper strips (Whatman No. 3). Two different buffer solutions were used: *1*) veronal buffer, pH 8.6, ionic strength, 0.075, and *2*) sodium phosphate buffer, pH 7.0, ionic strength, 0.05. For quantitative studies the phosphate buffer was routinely used.

Staining. The property of hemoglobin to catalyze the oxidation of benzidine into its colored, quinoid derivative in the presence of hydrogen peroxide was utilized for staining the separated hemoglobin fractions. On completion of electrophoresis the paper strips were placed in an oven at 120° C. for 30 minutes in order to fix the hemoglobin to the paper. After removal from the oven, the filter paper strips were individually immersed in a solution of 1 or 2 per cent benzidine in 20 per cent acetic acid which was prepared fresh each day. The strength of the benzidine employed varied with the hemoglobin concentration. At hemoglobin concentrations less than 200 mg. per cent, 2 per cent benzidine was used; at higher concentrations 1 per cent benzidine was employed. When thoroughly saturated with benzidine (immersion for 15 to 30 seconds) the paper strips were removed and were blotted thoroughly to remove excess benzidine. They were then immediately immersed, again individually, in a freshly prepared solution of 3 per cent hydrogen peroxide in water. The paper strips were thoroughly saturated

* Supported by grants from the National Heart Institute, United States Public Health Service; and the American Heart Association.

† Presented at the Eastern Section Meeting, American Federation for Clinical Research, New Haven, Conn., December 7, 1957 (1).

with peroxide but were not immersed for longer than 30 seconds since progressive elution of oxidized benzidine from the paper occurred during immersion for longer periods. On removal from hydrogen peroxide the strips were rinsed quickly with distilled water to remove excess peroxide and were partially dried by blotting. The paper strips used for quantitative studies were set aside to allow the oxidation of benzidine to reach a maximum before photometric analysis was made. This required approximately five to seven minutes.

Quantitative analysis. When the oxidation of benzidine was complete and color intensity reached a maximum, measurements of the light absorption of the stained fractions were made. The damp filter paper strips were placed in a clear, celluloid envelope and were inserted in an automatic recording densitometer (Spinco Analytrol) for scanning. The celluloid envelope was used to prevent sticking or tearing of the damp filter paper during scanning. Using a 500 mμ interference filter, the light absorption of the stained paper strips was recorded automatically and measured. Using these measurements, the relative concentrations of free hemoglobin, PBH and methemalbumin were computed. The absolute concentrations of these fractions were calculated from the relative concentrations when these were related to the determined total hemoglobin concentration.

EXPERIMENTAL PROCEDURE

Hemoglobin was administered intravenously in either single or multiple injections to 13 healthy, fasting adult subjects who showed no evidence of cardiovascular, infectious, hematologic or renal disease. Hemoglobin was prepared from autogenous blood which was withdrawn in heparinized syringes and hemolyzed by the addition of 2.5 volumes of distilled water to each volume of whole blood. Using a blood transfusion set to prevent the introduction of clots, a total of approximately 14 to 16 Gm. of hemoglobin was administered as a 5 per cent solution. In 10 subjects this amount of hemoglobin was given over a two or three hour period in seven to nine evenly spaced injections, each injection containing approximately 2 Gm. of hemoglobin. In three subjects hemoglobin (14 to 16 Gm.) was administered in a single injection over a period of 30 minutes. Arterial blood samples were taken every 20 to 30 minutes following the initial injection and all subsequent injections, if given, over a period of from two to six hours. Blood was collected carefully in dry syringes utilizing the pressure of the arterial flow, care being taken to avoid hemolysis, and was placed in heparinized tubes, mixed gently and centrifuged. Following centrifugation the plasma was separated and was frozen and stored at −10° C. until analysis for hemoglobin content could be made.

OBSERVATIONS AND RESULTS

Electrophoretic characteristics

Plasma hemoglobin was separated electrophoretically into two components: free hemoglobin and protein-bound hemoglobin (PBH). The electrophoretic characteristics of these heme-proteins and their positional relationships to plasma proteins at pH 8.6 and at pH 7.0 are illustrated in Figures 1 and 2, respectively. At pH 8.6 free hemoglobin migrated between α_2 and β globulin and PBH migrated with, or slightly slower than, α_2 globulin. At pH 7.0 free hemoglobin did not migrate but remained at the site of application of the sample to the paper, assuming a position to the cathode side of the application site as a result of endosmotic flow. At this pH, PBH migrated with α_2 globulin. These electrophoretic characteristics are similar to those previously described (8, 9, 11, 12).

In addition to free and protein-bound hemoglobin, a third benzidine reacting material was separated electrophoretically. This fraction, tentatively identified as methemalbumin, migrated with albumin at pH 8.6 and 7.0 (Figures 1 and 2).

The identification of these fractions as free hemoglobin, PBH, and methemalbumin, respectively, was based upon the following considerations:

Free hemoglobin. *a*) The electrophoretic characteristics of this moiety were similar to those of preparations of hemoglobin in aqueous solution which were studied under similar conditions and are those to be expected of unbound hemoglobin with an isoelectric point of 6.9 (8); *b*) The glomerulus is permeable to this fraction which is excreted in the urine (13), indicating that it is diffusible and suggesting that it circulates in the free, unbound state.

Protein-bound hemoglobin. *a*) The electrophoretic characteristics of this moiety were similar to those of protein-bound hemoglobin as described by Smithies and Walker (11, 12), Laurell and Nyman (8) and Allison and ap Rees (9), and are those to be expected of hemoglobin which is believed to be bound by α_2 globulin (5); *b*) The demonstration, as described below, of a limiting, maximal concentration of this fraction, with the appearance of another moiety (free hemoglobin) at higher hemoglobin concentrations, is consistent with a protein-binding reaction as has been described for other constituents of plasma (14); *c*) This fraction is not excreted in the urine (13), an observation consistent with hemoglobin binding by a large-sized plasma protein to which the glomerulus is impermeable; *d*) The absorption spec-

trum of this fraction (Figure 3) was similar to that of oxyhemoglobin (15), suggesting that the benzidine reacting material which is bound to plasma protein is hemoglobin and not a derivative thereof.

Methemalbumin. The identification of this moiety, which migrated with albumin at pH 8.6 and 7.0, as methemalbumin was based upon its electrophoretic characteristics. These characteristics were similar to those of preparations of methemalbumin (acid hematin plus albumin) which were studied under similar electrophoretic conditions. Attempts at a more definitive identification by demonstration of the characteristic absorption spectrum of methemalbumin were unsuccessful, presumably because the concentrations involved were too small to detect spectroscopically.

A distinct electrophoretic separation was consistently obtained between free hemoglobin, PBH and methemalbumin at both pH 7.0 and 8.6. However, a greater separation was obtained between free and protein-bound hemoglobin at pH 7.0 owing to the stationary position of free hemoglobin at this pH. For this reason pH 7.0 was selected for routine quantitative analysis. Another factor in the selection of this pH for quantitative studies was that identification of the separated fractions was further simplified owing to the appearance of protein-bound hemoglobin as a single, easily recognizable broad band at pH 7.0, whereas at pH 8.6 PBH often appeared in double bands (Figure 1), the slowest moving of which was in close proximity to free hemoglobin. The appearance of PBH in multiple bands [up to four have been described using *in vitro* techniques and starch electrophoresis (9, 11, 12)] has been attributed to genetically determined differences of the protein(s) having the property of binding hemoglobin (11, 12). The merging of separate bands of PBH into a single homogenous fraction

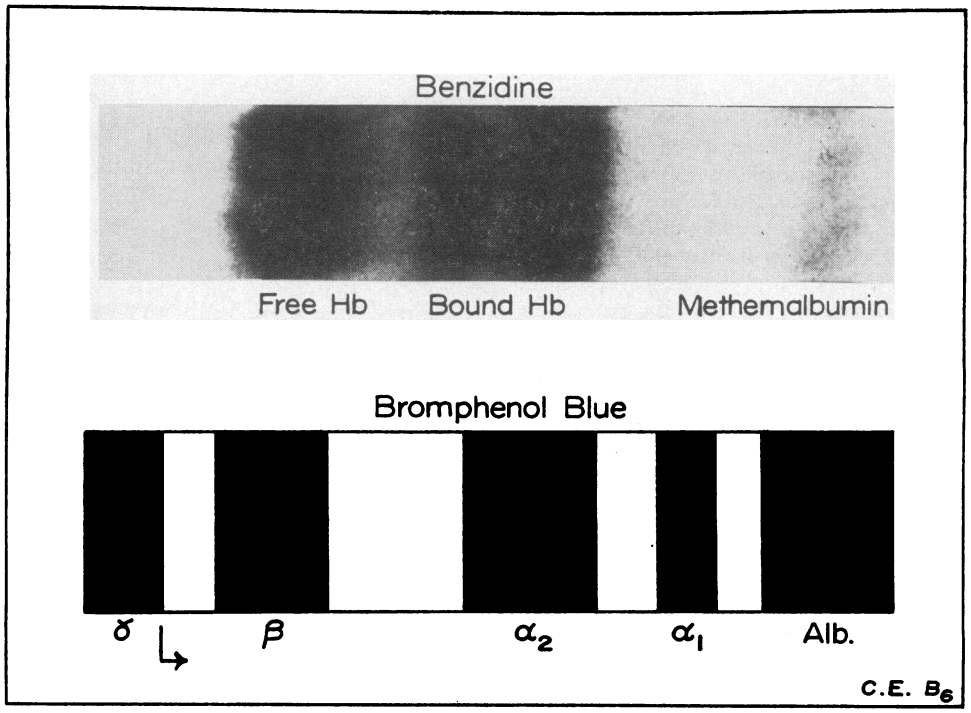

FIG. 1. THE ELECTROPHORETIC CHARACTERISTICS OF PLASMA HEMOGLOBIN AND PLASMA PROTEINS AT PH 8.6

The upper strip, stained with benzidine, shows the position of free hemoglobin, protein-bound hemoglobin (PBH), and methemalbumin. At this pH, PBH appeared as a double band. The lower strip shows schematically the positions of plasma proteins as determined by staining with bromphenol blue. The arrow refers to the application site and direction of movement towards the anode.

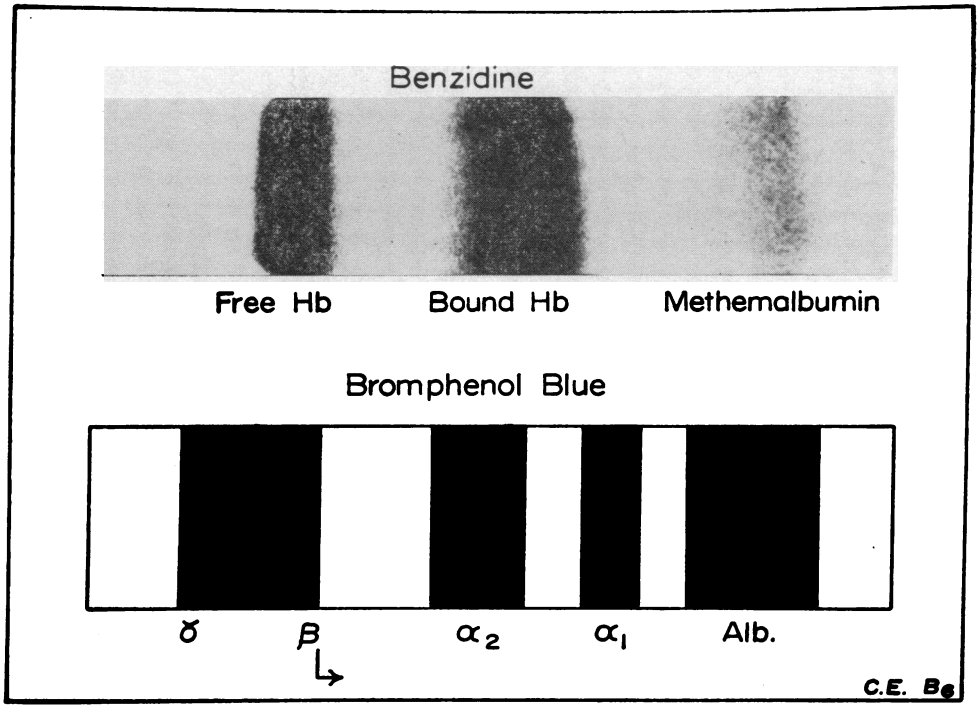

FIG. 2. THE ELECTROPHORETIC CHARACTERISTICS OF PLASMA HEMOGLOBIN AND PLASMA PROTEINS AT pH 7.0

At this pH, free hemoglobin assumed a position to the cathode side of the application site owing to endosmotic flow. PBH appeared as a single homogenous band.

at pH 7.0 may be attributed to a general reduction in electrophoretic resolution at this pH.

Staining characteristics

The blue color imparted to the filter paper strips appeared in the areas of the paper containing hemoglobin (or methemalbumin) within a few seconds after immersing the benzidine-saturated paper in hydrogen peroxide. The intensity of the color increased progressively with time after removal of the strips from peroxide, reaching a maximum in all fractions in from five to seven minutes, as determined photometrically. After this time interval the color remained stable for 10 to 15 minutes, during which time measurements of light absorption were made. After 15 to 30 minutes the background of the paper became discolored as a result of the decomposition of benzidine and photometric measurements were no longer possible.

The relative intensities of the three fractions did not change with variations in the exposure time to benzidine or hydrogen peroxide or with variable concentrations of these reagents as employed. The color intensity of each fraction was greater when 2 per cent benzidine was used as compared with 1 per cent, but the relative intensities of the three fractions did not change. Similarly, variability was encountered from day to day in the intensity of the color reaction obtained, but each fraction varied in a similar manner and the relative intensities remained unchanged. The factors accounting for this day to day variability were not established, but in view of the sensitive nature of the catalytic reaction involved in the oxidative transformation of benzidine, numerous factors may have contributed, such as minor variations in temperature, exposure time, saturation of the paper and concentration of the reagents employed.

During the development of color a slow diffusion of oxidized benzidine occurred through the wet filter paper. Such diffusion did not obscure the background between the separated fractions and had ceased at the time of photometric measurements when partial drying had taken place.

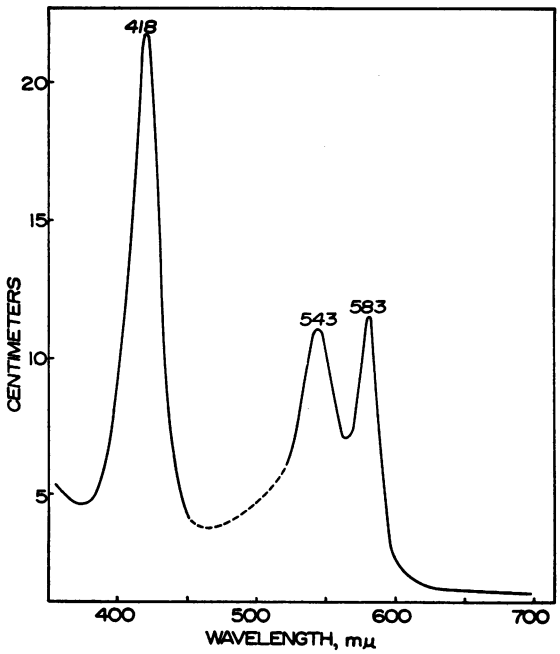

FIG. 3. ABSORPTION SPECTRUM OF PROTEIN-BOUND HEMOGLOBIN

The peaks at 543 and 583 mμ are characteristic of oxyhemoglobin. These peaks were determined at a plasma dilution of 1:1; the Soret band (418 mμ) was determined at a dilution of 1:7. The ordinate refers to the distance (cm.) of the excursion of the recording pen above the baseline, which was a function of extinction of light.

Quantitative analysis

The characteristic curves obtained when the light absorption of the benzidine stained paper strips was measured are illustrated in Figure 4. In order to establish the validity of the use of these curves for the determination of the relative concentrations of free hemoglobin, PBH and methemalbumin, studies were made of the relationship between hemoglobin concentration and the color intensity of the benzidine stained fractions. The purpose of these studies was twofold: *a*) to determine if the relationship between hemoglobin content and color intensity, as determined by light absorption, was linear, and *b*) to determine if the relationship between hemoglobin content and light absorption was the same for free hemoglobin, PBH and methemalbumin.

In order to study the first of these relationships, known concentrations of hemoglobin in aqueous solution were applied to filter paper, color was developed with benzidine and hydrogen peroxide in the usual manner, and photometric measurements were made. At concentrations of hemoglobin of 12.5 to 200 mg. per cent, the relationship between

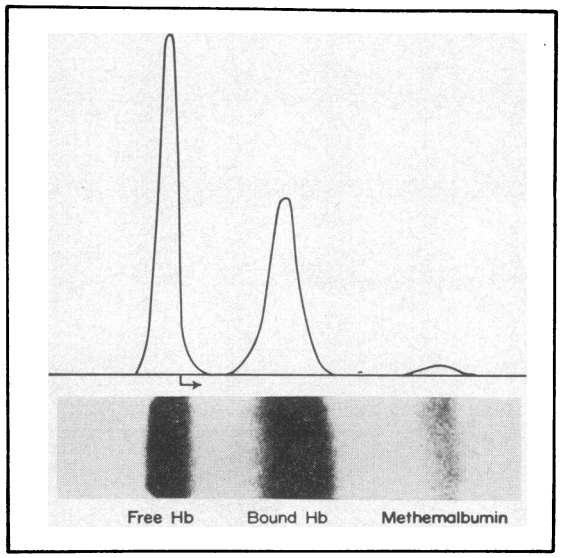

FIG. 4. CURVES OF THE LIGHT ABSORPTION OF BENZIDINE-STAINED HEMOGLOBIN FRACTIONS

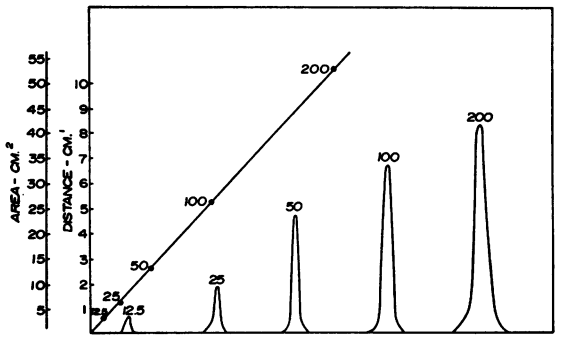

FIG. 5. RELATIONSHIP BETWEEN THE CONCENTRATION OF FREE HEMOGLOBIN AND LIGHT ABSORPTION FOLLOWING STAINING WITH BENZIDINE-HYDROGEN PEROXIDE.

Hemoglobin was applied to a filter paper strip in concentrations of 12.5 to 200 mg. per cent. The numbers within the enclosed box represent hemoglobin concentrations. The curves represent recorded measurements of the light absorption of the respective hemoglobin concentrations following staining with benzidine-hydrogen peroxide. The height of these curves is expressed as the distance (cm.) of the excursion of the recording pen above the baseline. The area under each curve is expressed as "area—cm.²" The diagonal line is a plot of the concentration of hemoglobin against the area under the respective curve of light absorption. The relationship shown between concentration and light absorption is linear.

concentration and light absorption was linear (Figure 5), indicating that the color intensity of the benzidine oxidatively transformed was directly proportional to the content of hemoglobin on paper.

In order to compare the relationship between hemoglobin concentration and light absorption for free and protein-bound hemoglobin, the following study was made. Hemoglobin was added to plasma *in vitro* in increments of from 2 to 5 mg. per cent over a concentration range of 80 to 240 mg. per cent. Following incubation of plasma at 37° C. in order to promote protein-binding, electrophoresis, staining and photometric analysis were carried out in the usual manner. Protein-binding was maximal in this experiment at a hemoglobin concentration of 112 mg. per cent. At concentrations greater than 112 mg. per cent, free hemoglobin appeared in increasing concentrations. Assuming that the concentration of PBH remained at 112 mg. per cent at all concentrations in excess of this maximal binding capacity, the concentration of free hemoglobin at these higher (112 to 240 mg. per cent) concentrations was calculated as the difference between the known *total* hemoglobin concentration and the concentration of PBH (112 mg. per cent). Knowing, therefore, the actual concentrations of free and protein-bound hemoglobin, the light absorption of each fraction was measured and compared at each concentration studied. Figure 6 illustrates that at all concentrations studied the relationship between hemoglobin concentration and light absorption of the oxidized benzidine bands was linear and was the same for free and protein-bound hemoglobin. Calculations of the concentrations of free and protein-bound hemoglobin as determined by the photometric technique were, therefore, in close agreement with the actual concentrations of these components (Table I).

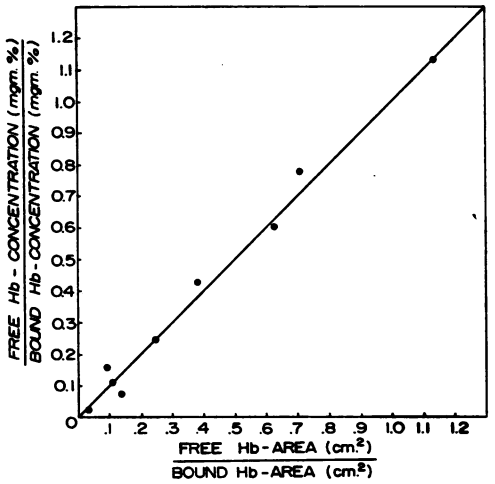

FIG. 6. RELATIONSHIP BETWEEN CONCENTRATION AND LIGHT ABSORPTION (BENZIDINE STAIN) OF FREE HEMOGLOBIN RELATIVE TO PROTEIN-BOUND HEMOGLOBIN

The concentration of free hemoglobin relative to the concentration of protein-bound hemoglobin is plotted against the respective ratios of the light absorption of these constituents (when stained with benzidine) at each ratio of concentrations studied. The straight line relationship shown indicates that the relationship between concentration and light absorption is the same for free and protein-bound hemoglobin.

A similar study of methemalbumin revealed that the relationship between concentration and

TABLE I

In vitro study of the recovery of free and protein-bound hemoglobin (Hb) as determined by photometric analysis *

Total Hb	Free Hb		Free (calculated) / Free (actual)	Bound Hb		Bound (calculated) / Bound (actual)
	Actual	Calculated		Actual	Calculated	
110	0	0	1.00	110	110	1.00
112	0	0	1.00	112	112	1.00
115	3	3	1.00	112	112	1.00
120	8	14	1.75	112	106	0.95
125	13	12	0.93	112	113	1.01
130	18	12	0.67	112	118	1.05
140	28	30	1.07	112	110	0.98
160	48	44	0.92	112	116	1.04
180	68	69	1.01	112	111	0.99
200	88	90	1.02	112	110	0.98
240	128	128	1.00	112	112	1.00

* Hemoglobin values are expressed as concentration in mg. per cent.

TABLE II

In vitro study of the recovery of free hemoglobin (Hb) and methemalbumin as determined by photometric analysis *

Free Hb		Methemalbumin	
Actual	Calculated	Actual	Calculated
5	7	5	3
10	11	10	9
20	20	20	20
30	28	30	32
40	41	40	39

* All values are expressed in mg. per cent.

light absorption was the same for this heme-protein as for free hemoglobin (and, by inference, therefore, the same as PBH). In this experiment methemalbumin, prepared by adding acid hematin to albumin, was mixed with equal concentrations of free hemoglobin in aqueous solution over a range of concentration of each of from 5 to 40 mg. per cent. The similar relationships between light absorption and concentration obtained for free hemoglobin and methemalbumin resulted in a close agreement found between the actual and calculated concentrations of these constituents when light absorption measurements were used to estimate concentrations (Table II).

These experiments therefore satisfactorily established the validity of the quantitative photometric method as employed. The necessary criteria for validation were fulfilled: The relationship between concentration and light absorption was linear and was the same for free hemoglobin, PBH and methemalbumin.

The sensitivity of this quantitative method was such that hemoglobin concentration was accurately measured within ± 5 mg. per cent (Table I). At concentrations in excess of 20 mg. per cent the experimental error of this method, as determined by multiple analysis of single samples of plasma, was ± 7.0 per cent. These figures apply to calculations of free hemoglobin, PBH and methemalbumin.

Application of quantitative method—The distribution of intravenously administered hemoglobin

The distribution of hemoglobin in plasma following intravenous administration is illustrated by representative experiments in Figures 7 and 8. These figures illustrate, respectively, the distribution of hemoglobin when the plasma level was

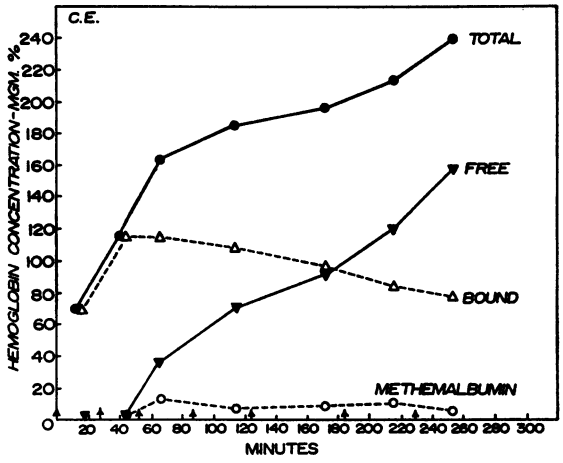

FIG. 7. THE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED HEMOGLOBIN

At each point in time indicated by an arrow approximately 2 Gm. of hemoglobin was given intravenously. With time the total hemoglobin concentration progressively increased. Initially all hemoglobin circulating in plasma was bound to plasma protein. When the maximal binding capacity was exceeded (114 mg. per cent) free hemoglobin appeared in plasma, the concentration of which progressively rose. Accompanying the appearance of free hemoglobin, a small quantity of methemalbumin was formed. With time, the concentration of PBH fell progressively.

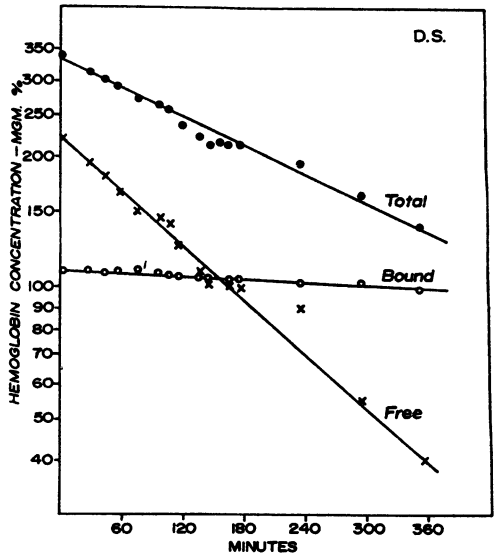

FIG. 8. THE DISTRIBUTION OF HEMOGLOBIN IN PLASMA FOLLOWING A SINGLE LARGE INTRAVENOUS INJECTION

Both free and protein-bound hemoglobin were present initially. The concentration of each decreased with time. The disappearance of both free and protein-bound hemoglobin was exponential, but free hemoglobin disappeared at a faster rate.

gradually increased, and the disappearance of hemoglobin from plasma following rapid elevation of the plasma level.

When hemoglobin was introduced into circulating blood it was immediately and, insofar as could be determined, instantaneously bound to plasma protein (Figure 7). When small quantities were administered, binding was quantitative and hemoglobin circulated entirely in the protein-bound state. As hemoglobin was given in increasing amounts, the quantity bound increased until a concentration was attained at which additional hemoglobin given was not bound but instead circulated in the free, unbound state (Figure 7). This limiting concentration, which may be considered as the maximal binding capacity of the hemoglobin-binding protein, varied in individual studies from 98 to 171 mg. per cent (Table III) and averaged 128 ± 25 mg. per cent. These capacities are similar to those observed *in vitro* by Laurell and Nyman (8) and Allison and ap Rees (9).

TABLE III

In vivo distribution of hemoglobin (Hb) in plasma *

Subject		Total Hb mg. %	PBH mg. %	Free Hb mg. %	Methemalbumin mg. %
V. T.	1)	171	171	0	0
	2)	355	145	210	0
R. R.	1)	162	162	0	0
	2)	258	142	101	5
J. W.	1)	143	143	0	0
	2)	325	53	263	9
B. K.	1)	133	133	0	0
	2)	230	68	157	5
P. R.	1)	133	133	0	0
	2)	265	74	182	9
C. E.	1)	114	114	0	0
	2)	239	77	157	5
I. R.	1)	111	111	0	0
	2)	269	60	200	9
E. D.	1)	107	107	0	0
	2)	320	79	241	0
L. S.	1)	99	99	0	0
	2)	205	43	151	11
L. C. S.	1)	98	98	0	0
	2)	280	43	228	9

* As determined under conditions of a progressively increasing hemoglobin concentration. 1) Refers to the hemoglobin concentrations at the maximal binding capacity of plasma protein for hemoglobin. 2) Refers to the final concentrations of hemoglobin at the completion of the experiment.

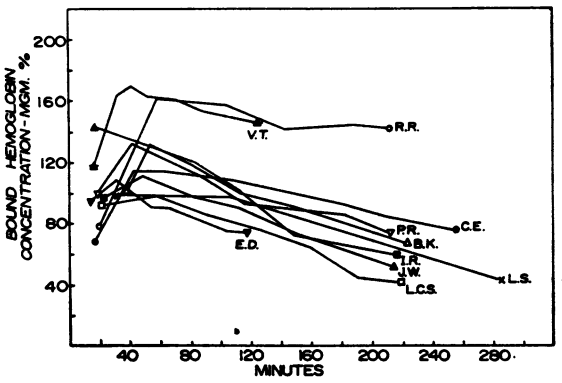

FIG. 9. DISAPPEARANCE CURVES OF PROTEIN-BOUND HEMOGLOBIN FROM PLASMA

These curves, obtained from 10 separate experiments under conditions of a progressively increasing *total* plasma hemoglobin concentration, illustrate the initial rise in the concentration of PBH to a maximal level with subsequent progressive fall in concentration with time.

The limitation imposed by this maximal binding capacity resulted in hemoglobin appearing in plasma in the free, unbound state when the amount of hemoglobin administered was in excess of that which could be bound. Under these circumstances, a progressive increase occurred in the concentration of free hemoglobin circulating in plasma as additional hemoglobin was administered intravenously. No limitation was imposed upon the transport of hemoglobin in the unbound state. Concentrations up to 263 mg. per cent were obtained (Table III).

Accompanying this rise in total and free hemoglobin levels in plasma, the concentration of PBH diminished with time (Figure 9). The rate at which this decrease in concentration of PBH occurred varied in individual studies from 6 to 25 mg. per cent per hour and averaged 15 mg. per cent per hour. In five subjects (E.D., P.R., B.K., I.R. and V.T.) PBH disappeared from plasma at a single exponential rate ($T_{1/2}$, 250 minutes). In the remaining five subjects the disappearance was linear and not exponential. The disappearance rate, however, was approximately the same in these subjects as in those in whom an exponential disappearance occurred. The disappearance of PBH from plasma has been studied by Laurell and Nyman (8) and was observed by them to be accompanied by a parallel decrement in the concentration

of α_2 globulin, suggesting that hemoglobin is removed from the circulation in combination with the protein to which it is bound. Since PBH is not excreted in the urine (13) this removal must occur at extravascular sites within the body, presumably within the reticuloendothelial system (16). The capacity of this or other systems to remove PBH has not been established, but Laurell and Nyman have suggested that this capacity may be of a low order of magnitude in view of a constant rather than an exponential disappearance rate as observed by them in two subjects (8). The present observations do not clarify this supposition since the characteristics of the disappearance curves were not consistent. The explanation of this inconsistency is not clear. Conceivably, differences in disappearance rates might occur as a result of differences among individual subjects in the characteristics of the hemoglobin-binding protein which have been described (11, 12). However, the differences in disappearance curves observed may be artificial owing to an inadequate time of observation. In view of the slow disappearance rates, a longer period of observation is necessary to establish more clearly the precise characteristics of the removal of PBH from plasma.

Accompanying these changes in plasma hemoglobin, methemalbuminemia was observed in 8 of 10 studies (Table III). The appearance of methemalbuminemia was dependent upon the presence of free hemoglobin in plasma. Methemalbumin was not formed until the binding capacity was exceeded and hemoglobin appeared in the free, unbound state (Figure 7). This observation, also made *in vitro* by Allison and ap Rees (9), is consonant with the view (9, 17) that methemalbumin is formed *in vivo* from heme molecules which are derived from free but not from protein-bound hemoglobin.[1] There was, however, no correlation observed in the present experiments between the plasma level of methemalbumin and the concentration of free hemoglobin. When methemalbuminemia developed, it did so early, when free hemoglobin first appeared, or not at all (Figure 7). The quantity of methemalbumin formed was small. The plasma levels varied in individual studies from 5 to 11 mg. per cent (Table III). These levels remained relatively constant during the period of observation and neither decreased nor increased significantly as the level of free hemoglobin changed. This suggests that methemalbumin was probably formed and removed from the circulation at a relatively constant rate.

In Figure 8 a representative experiment is illustrated of the disappearance from plasma of free and protein-bound hemoglobin following rapid elevation of the plasma levels of these constituents by a single, rapid, intravenous injection of hemoglobin. In this and two other similar experiments the initial total hemoglobin concentration was greater than 200 mg. per cent and both free and protein-bound hemoglobin were present in the initial samples. Methemalbuminemia did not occur in these three studies. With time the concentrations of free and protein-bound hemoglobin decreased. At the completion of the period of observation (five to six hours) the concentration of PBH had fallen by 27, 18 and 32 per cent, respectively, in the three studies and free hemoglobin by 62, 82 and 59 per cent. Free hemoglobin disappeared from plasma at an exponential rate (average $T_{1/2}$, 207 minutes). The characteristics of the disappearance curves of PBH were variable. In Subject D.S. (Figure 8), PBH disappeared at a slow exponential rate. In the other two subjects, the disappearance was constant and was not exponential. These differences, as noted above, remain unexplained. The faster disappearance of free hemoglobin (Figure 8) than of PBH may be attributed, in part, to its excretion in the urine (13) and to its smaller molecular size with consequent larger volume of distribution in body fluids. Whether free and protein-bound hemoglobin are removed differentially by the reticuloendothelial or other systems remains to be determined.

SUMMARY

Utilizing paper electrophoretic techniques, a method was developed for the partition and quantification of plasma hemoglobin. Electrophoretically, plasma hemoglobin was separated into a free and a protein-bound component. These fractions were identified, when benzidine and hydrogen

[1] Small quantities of methemalbumin were detected in plasma containing only PBH after storage in the frozen state for longer than two to three months. Thus, heme molecules appear to be released from PBH *in vitro*, but not *in vivo*.

peroxide were used for staining purposes, by their characteristic positional relationships on paper and were quantified by photometric analysis of the stained filter paper strips.

Using these techniques a quantitative study was made in human subjects of the distribution and transport of intravenously administered hemoglobin. The observations made showed that hemoglobin was transported in plasma in a protein-bound form (PBH) and in a free, unbound state; that the binding of hemoglobin by plasma proteins was limited to a maximum hemoglobin concentration which averaged 128 ± 25 mg. per cent; that free hemoglobin did not appear in plasma until this binding capacity was exceeded; and that methemalbuminemia often developed, but only in association with the appearance of free hemoglobinemia.

From an analysis of rising and falling plasma hemoglobin levels, PBH was found to disappear slowly from circulating plasma and was distributed in extravascular sites, but was not excreted. Free hemoglobin disappeared from plasma at a faster rate than PBH and was excreted in the urine.

These observations emphasize the differential transport and distribution of free and protein-bound hemoglobin in circulating plasma. The methods employed provided quantitative information concerning these phenomena and may be used for further study of hemoglobin metabolism and physiology.

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