EVALUATION OF A STROMA-FREE HEMOGLOBIN SOLUTION FOR
USE AS A PLASMA EXPANDER*

By S. FREDERICK RABINER, M.D., J. RAYMOND HELBERT;† Ph.D., HARRY
LOPAS, M.D., and LILA H. FRIEDMAN

(From the Division of Clinical Hematology, Department of Medicine, Michael
Reese Hospital and Medical Center, and the Department of Medicine,
Northwestern University Medical School, Chicago, Illinois 60616)

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Plasma, dextran, and other preparations, although effective as plasma expanders,
cannot carry oxygen and therefore are not as useful as whole blood for management of
acute hemorrhage. Blood, on the other hand, has a limited storage time and must be
typed and cross-matched prior to use. Hemoglobin in solution has several unique
properties which could be desirable for treating hemorrhagic shock. In addition to its
osmotic activity (mol wt 68,000), hemoglobin can transport and exchange oxygen
and has the advantage of not requiring typing or cross-matching (1). Despite its
potential, the use of hemoglobin solutions has not progressed past the animal experi-
mentation stage because of reports of renal damage and methemoglobin formation
following its administration (2–4).

Recent studies strongly suggest that disseminated intravascular coagulation may
play an important role in the pathogenesis of renal damage seen in shock, intravascu-
lar hemolysis, and other situations (5–8). It has also been clearly demonstrated that
hemolyzed erythrocytes can initiate blood coagulation (9, 10) and that this coagulant
activity is confined to erythrocyte stroma (11). With these observations in mind, it
may be postulated that renal damage, following administration of hemoglobin solu-
tion, could be due to coagulant activity of red cell stromal contaminants. A stromal-
free hemoglobin solution would therefore not have deleterious effects on renal function.

It is the purpose of this report to describe a method for preparation of large quan-
tities of a hemoglobin solution which is relatively free of stromal particles or lipid and
has no demonstrable coagulant activity. We also wish to report results of acute and
chronic experiments which show distribution, excretion, oxygen-carrying capacity,
and effect on renal function of this solution when it is administered to healthy mongrel
dogs.

Methods and Materials

Preparation of Hemoglobin Solution.—Erythrocytes were separated from outdated, human
whole blood and washed three times with 1.6% saline. The washed cells were lysed by adding

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Army Medical Research and Development Command.
† Dr. Helbert's present address is: Miller Brewing Company, 4000 West State Street,
Milwaukee, Wis. 53201.
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4 vol of 5 ideal milliosmolar phosphate buffer, pH 7.4 (12), to 1 vol of packed cells; gentle, but thorough mixing was effected by repeated inversion of vessel. After standing 30–60 min, the mixture was centrifuged at 16,000 g for 90 min at 5°C. Supernatant solution was removed and recentrifuged at 35,000 g in a continuous flow centrifuge at a rate of 5.0 ml/min. Supernatant was then filtered through a sterile 0.2 μm millipore filter.

To increase concentration of hemoglobin and adjust electrolytes, solution was dialyzed, under pressure, against a standard kidney dialyzing fluid, employing a Kolff twin coil artificial kidney. Careful aseptic technique was used throughout. Sodium, potassium, hemoglobin, and methemoglobin concentrations of each batch were determined and cultures for bacteria were obtained prior to storage at 5°C in sterile transfusion bottles.

Experimental Procedure.—In vivo experiments were performed on healthy mongrel dogs anesthetized with intravenous sodium pentobarbital. Urinary bladder was catheterized with an indwelling Foley-type catheter. Blood pressure and pulse were monitored from cannulated femoral artery connected to a Sanborn pressure transducer with carrier preamplifier and recorder. The femoral vein was also cannulated with a polyethylene catheter threaded into inferior vena cava. (This catheter was also employed for phlebotomy, infusion of hemoglobin solution, and to obtain samples for coagulation tests and chemical determinations.)

Arterial samples were obtained by connecting an adapter bearing a polyethylene catheter to an arm of the three-way stop cock which connected femoral artery catheter to pressure transducer. By directing channel of arterial flow through stop cock, blood samples could be collected under oil without hemolysis.

Samples of blood were collected prior to phlebotomy (control) and 5, 30, 60, 120, 180, and 240 min after hemoglobin infusion. In collecting all samples, the first 5–10 ml of blood was discarded and extreme care was taken to avoid hemolysis. Intake of intravenous fluids and urinary output were determined. Aliquots of urine were collected for hemoglobin content simultaneously with plasma samples.

Via the femoral vein catheter, 20–47 ml per kg of blood was removed in 2–13 (mean 5.4) min. Immediately following phlebotomy, the animal was infused in 4–24 (mean 13.0) min with the same volume of hemoglobin solution. After specimens of urine and blood were obtained as indicated, the experiments were terminated. The animals were sacrificed immediately and sample sections obtained from lungs, liver, spleen, and kidneys. These specimens were placed in 10% formalin and prepared for microscopic examination in usual manner. The remainder of these organs were frozen for future determination of free and red cell hemoglobin.

Prior to phlebotomy and 4 hr after hemoglobin infusion, plasma volume was determined with 131I-tagged albumin. In addition, 15 min prior to termination of experiment, 59Fe-tagged red cells, obtained from a donor dog, were administered intravenously to permit determination of tissue hemoglobin (see determination of free hemoglobin and red cell hemoglobin in tissues).

Coagulation Studies.—As previously indicated, extreme care was taken to avoid hemolysis of samples obtained for coagulation studies as well as other determinations. The first syringe with 3–5 ml of blood was discarded and the actual sample collected in a second disposable plastic syringe.

Clotting time was performed by the Lee and White method (13), except that plastic, rather than glass test tubes, were employed. To insure that clotting time would be significantly shortened by activation of the initial phase of blood coagulation, care was taken to avoid contact of plasma with any surface other than plastic (intact plasma).

1 g/100 L = NaCl—570, NaHCO3—300, KU—30, CaCl2·14, and MgCl2·6H2O—15.

2 300–500 μc of 59FeSO4 administered intravenously, 10 days later 40 ml blood was collected in ACD solution and red cell washed three times with normal saline.
Prothrombin activity was performed by the Ware and Stragnell method (14), factor V assays by the method of Biggs and Macfarlane (15), fibrinogen by a modification of the Cullen and Van Slyke method (16), and factor VIII (AHG) assay by the method of Rapaport, Schiffman, Patch, and Ware (17) with the following modifications:

1. Citric acid was not added to the anticoagulant.
2. Soy bean phosphatide (Inosithin-Associated Concentrates, Woodside, N.Y.) was employed instead of chloroform extract of brain.
3. Celite (Johns-Manville filter cell) was employed instead of Kaolin.
4. Imidazole buffer 0.04 M pH 7.35 was employed instead of veronal buffer.
5. Factor VIII-deficient plasma, obtained from a patient with classical hemophilia, was employed as substrate instead of factor XI-deficient plasma.

To study the effect of hemoglobin solution on clotting of intact plasma either 0.1 ml of hemoglobin solution or imidazole buffer (control) was added to 0.4 ml of normal intact plasma in a 12 x 75 ml plastic tube (Falcon 2003). The test tube was gently mixed, placed in a 37°C water bath, and 0.1 ml of 0.1 M calcium chloride was added. A stopwatch was started simultaneously with the addition of calcium chloride. At 1-min intervals the test tube was picked up and tilted very gently to note clot formation as in Lee White clotting time. Throughout the day repeated control clotting times were performed to insure intact state of test plasma.

Determination of Free Hemoglobin and Red Cell Hemoglobin in Tissues.—

General principles: Total tissue hemoglobin was determined colorimetrically as acid hematin in organ homogenates (18). The portion of total tissue hemoglobin derived from red cells in the vascular space was calculated on the basis of isotopic dilution of 59Fe-labeled red cells injected just before sacrifice and the gamma counting rate of tissue homogenates and whole blood. The free hemoglobin of the tissues is equal to total tissue hemoglobin minus the red cell hemoglobin.

Separation of tissues for hemoglobin analysis: 15 min after injection of labeled red cells, a sample of venous blood was obtained and the animal was sacrificed. Major vessels of organs were ligated and whole organs were removed and frozen overnight at −40°C. 5–6 g aliquots were taken from each frozen organ and homogenized with 35–45 ml of distilled water in a micro-Waring Blender, until a fine suspension was obtained.

Determination of total tissue hemoglobin: 5 ml of tissue homogenate was brought to 25 ml in a volumetric flask with acid acetone, the latter consisting of two parts 2.5% HCl and eight parts acetone (w/v). The suspension was shaken, transferred to a stoppered centrifuge tube, and centrifuged at 4°C at 11,000 g for 30 min. The transmission of supernatant was determined at 635 µ in a Beckman DB spectrophotometer. The hemoglobin content of the sample was calculated from a standard hematin curve prepared, as above, from whole blood with a known hemoglobin content. The hemoglobin content per gram of tissue is then calculated.

Determination of red cell hemoglobin content of tissue: 1 ml of the whole blood sample obtained just prior to sacrifice was hemolyzed by addition of 2 ml of distilled water, and 59Fe activity was determined in a well-type scintillation detector (Packard Auto Gamma Spectrometer). The relationship between the hemoglobin concentration of peripheral blood and the radioactivity of 59Fe was then determined. A 3 ml aliquot of each organ homogenate was counted in a similar manner. The contribution of red cell hemoglobin to total tissue hemoglobin was calculated and subtracted from total tissue hemoglobin.

Miscellaneous determinations: Plasma hemoglobin was performed by the Crosby method (19) and methemoglobin by the Evelyn and Malloy method (20). The oxygen content of blood and plasma was determined by the Peters and Van Slyke method (21) employing a Van Slyke manometric apparatus (Thomas Magnematic model). Standard methods were employed for determination of sodium, potassium, whole blood hematocrit, and hemoglobin, the latter as cyanomethemoglobin.
Renal Function Studies.—A series of experiments were performed to determine the effect of infusion of the described hemoglobin preparation on renal function. Clearances of urea, creatinine, and paraaminohippuric acid (P.A.H.) were measured by methods as described by H. Smith (23), on anesthetized mongrel dogs. Venous and arterial catheters were inserted for infusion, sampling, and to monitor vital signs.

Prior to the first clearance period each animal was infused with normal saline at a dose of 75 ml/k over a 45-min period. A series of four consecutive 30-min clearances were then performed (control periods). Between 25-28 ml/k of blood was then removed by gravity into plastic bags, the amount determined by the changes in the weight of bags.

Immediately after phlebotomy either the withdrawn blood was reinfused (whole blood animals) or an equal amount of hemoglobin solution was administered (hemoglobin animals), over a 30 min period. Each group consisted of five animals and was comparable. All infusions of saline, blood, hemoglobin, creatinine, and P.A.H. were performed via a Harvard pump at calculated rates. Following a 45 min rest period, four additional, consecutive 30-min clearances were performed (acute periods). Animals were then returned to their cages. 2-5 days later two additional periods, each consisting of four consecutive 30-min clearances separated by a 1 hr rest period (chronic periods) were performed. The animals were then sacrificed by injection of sodium pentobarbital and immediately autopsied. Following careful gross examination of kidneys, multiple specimens were fixed in formalin and histological sections were prepared in usual manner.

RESULTS

In Vitro Properties of Hemoglobin Solutions.—Since hemolysate has the ability to shorten clotting time and improve prothrombin consumption of intact plasma (22), these parameters were employed to test in vitro coagulant activity of hemoglobin preparations. It was found that significant coagulant activity persisted after centrifugation of hemolyzed erythrocytes. This coagulant activity was removed by filtration (Table I). The unfiltered preparation shortened clotting time and improved prothrombin consumption of intact plasma. This coagulant activity was not significantly reduced until the preparation was passed through 0.1-0.2 μ millipore filters.

<table>
<thead>
<tr>
<th>Filter Pore Size</th>
<th>Clotting time</th>
<th>Prothrombin consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>28.2</td>
</tr>
<tr>
<td>0.1 μ</td>
<td>25</td>
<td>30.4</td>
</tr>
<tr>
<td>0.2 μ</td>
<td>13</td>
<td>35.8</td>
</tr>
<tr>
<td>0.45 μ</td>
<td>9</td>
<td>64.5</td>
</tr>
<tr>
<td>1.2 μ</td>
<td>7</td>
<td>49.5</td>
</tr>
<tr>
<td>Unfiltered</td>
<td>7</td>
<td>112.0</td>
</tr>
</tbody>
</table>
Coagulation studies were performed employing a hemoglobin solution prepared in the manner described by Hamilton et al. (1). Although free of particulate matter, this preparation was found to be heavily contaminated with stromal lipid. When added, undiluted, to intact plasma, it acted as an anticoagulant and the resultant mixture could not be clotted. However, by diluting this preparation with saline, coagulant activity could be demonstrated and significant shortening of clotting time was found with dilutions as high as 1:250 (Table II). After this hemoglobin preparation was extracted with chloroform, it no longer had coagulant activity and furthermore, coagulant lipid could be recovered from the chloroform by evaporation. Chloroform extractions of coagulant-free hemoglobin solutions, which were prepared as described in this report, yielded little or no lipid.

**TABLE II**

*Effect of the Addition of Hemoglobin Solutions on Clotting Time of Normal Plasma in Plastic Test Tubes*

<table>
<thead>
<tr>
<th>Preparation added</th>
<th>No. of determinations</th>
<th>Clotting time deviation from control</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>7</td>
<td>-0.6</td>
<td>0.95</td>
</tr>
<tr>
<td>P. B. Hamilton, et al.</td>
<td>5</td>
<td>-11.2</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Following reverse dialysis of our hemoglobin solution, the average concentration of hemoglobin was 7.0 g/100 ml, sodium 126 meq/L, potassium 4.4 meq/L, and methemoglobin 0.4 g/100 ml.

There was no demonstrable methemoglobin formation when hemoglobin solution was stored at 5°C in sterile bottles for 8 months. In fact, methemoglobin formation did not occur when pure oxygen was bubbled through an aliquot of this solution for 12 hr.

**Oncotic Activity of Hemoglobin Preparation.**—In vivo experiments were performed on eight anesthetized dogs. Following phlebotomy, there was the expected rise in pulse and fall in blood pressure, commensurate with the amount of blood removed. Infusion of hemoglobin solution in each instance promptly restored blood pressure and pulse rate to prephlebotomy levels and this was maintained throughout the experimental period.

One can estimate the osmotic effect of infused hemoglobin solution by calculating, from intake and output measurements, the theoretical change in plasma volume that should have occurred during the experiment and comparing this value with the true change in plasma volume, as determined with 131I-labeled albumin. For these calculations we estimated the per cent of infused hemoglobin solution remaining in the plasma at the termination of the ex-
experiment (4 hr). The actual final plasma volume (\(^{131}I\)-tagged albumin) was higher than the calculated final plasma volume in six of the seven experiments. This difference varied between 16-49 ml/k and was directly proportional to the amount of hemoglobin per kilo infused.

This is illustrated in Fig. 1 which is a scatter diagram illustrating the relationship between the volume of hemoglobin solution infused per kilo and the difference between the actual and calculated plasma volume change. (The dots represent differences obtained when final volume of residual infused hemoglobin was calculated from total body distribution data while the x's are values obtained when this was calculated from change in plasma hemoglobin. The solid line is related to dots and interrupted line to the x's.) Both lines are constructed by the method of least squares, and the correlation coefficient of each is 0.8. Since this is significant between 5 and 1%, it can be assumed that the two variables are functionally related.

**Oxygen Content and Arterial Value (A-V) Difference of Infused Hemoglobin.**—The mean oxygen content of infused hemoglobin, as determined from arterial
plasma samples, was 3.60 ± 0.72 (SD) vol/100 ml. The mean predicted oxygen-carrying capacity, calculated by multiplying the plasma hemoglobin concentration by 1.34, is 3.83 ± 0.88 (SD) vol/100 ml. If we assume 97% arterial hemoglobin saturation, the mean predicted oxygen content is 3.72. There is no significant difference between actual and predicted oxygen content ($P > 0.40$). Furthermore, the individual oxygen values correlated well with their predicted values ($r = 0.9554$, $P < 0.001$) and this relationship was maintained throughout the experimental period. There was no demonstrable methemoglobin conversion of infused hemoglobin. Analysis of plasma oxygen from venous and arterial blood revealed a consistent, significant A-V difference ($P < 0.001$). The mean A-V difference was 13% of the arterial value. These observations indicate that the infused hemoglobin both transports and exchanges oxygen.

### TABLE III

**Distribution of Infused Hemoglobin after 4–5 Hr**

<table>
<thead>
<tr>
<th></th>
<th>Per cent of total hemoglobin infused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>23.4 ± 7.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>10.6 ± 10.7</td>
</tr>
<tr>
<td>Plasma</td>
<td>40.4 ± 8.5</td>
</tr>
<tr>
<td>Urine</td>
<td>9.3 ± 9.7</td>
</tr>
<tr>
<td><strong>Total recovery</strong></td>
<td><strong>88.2 ± 11.9</strong></td>
</tr>
</tbody>
</table>

**Distribution and Excretion of Infused Hemoglobin.—** The distribution of free hemoglobin was determined 4–5 hr after infusion in four animals. Indicated in Table III are the mean values, with standard deviations, of the per cent of total infused hemoglobin recovered in organs, plasma, and urine. The most significant variation occurred in the amount excreted in urine and that recovered in kidney.

The disappearance rate of hemoglobin from plasma is illustrated in Fig. 2. On the basis of data from eight experiments, the “half disappearance time” of our hemoglobin preparation in the circulating plasma is 4–5 hr. This disappearance curve appears to be biphasic with an initial rapid phase ending at 1 hr. Also shown is the disappearance of hemoglobin when infused into two animals whose ureters were tied just prior to the experiment. In these dogs the initial fall in hemoglobin concentration is parallel to and approximates the rapid phase of normal dogs. The second phase, however, is more prolonged. Although this prolongation falls within the standard deviation of the line obtained with normal dogs, the straight line relationship of the values...
speaks strongly against random distribution. Of interest is the fact that at 4 hr, the mean plasma hemoglobin of ureter-tied dogs is 9% higher than that obtained with normal dogs. This difference is in agreement with the mean value of 9.3% of the total infused hemoglobin excreted in the urine of normal dogs (Table IV).

Effect of Hemoglobin Infusion on Activation of Coagulation Mechanism.—Following intravascular coagulation, there is a rapid fall in factors V, VIII, and fibrinogen. Since these factors do not effect the prothrombin activity test (14), these factors were therefore assayed at intervals and the fluctuation of V, VIII, and fibrinogen was compared to changes in prothrombin activity following infusion of hemoglobin solution (Table IV). This comparison was felt to be important since changes in prothrombin activity should reflect endogenous or exogenous dilution. An analysis of this data is presented in Table IV. It can be seen that the difference between prothrombin activity and factors V, VIII, and fibrinogen 4 hr after hemoglobin infusion is small and cannot be considered either clinically or statistically significant.

Effect of Hemoglobin Infusion on Renal Function.—Clearance of urea, creati-
nine, and P.A.H. was measured in a series of five hemoglobin-infused dogs and
the results compared to an identical series of whole blood-infused dogs. Both
acute effects, 45 min postinfusion, and chronic effects, 2-5 days postinfusion,
were determined. The analysis of data was carried out by the following steps:

1. The mean clearance for the four 30-min values during the control period
   for each dog was computed and used as the resting value for that dog.

2. The per cent change from this resting value for each subsequent clearance
   for a given dog was then determined (four acute clearances and eight chronic
   clearances).

3. An analysis of variance of these per cent changes was used to determine
   the relative components of variation, among dogs and among 3/2-hr periods.

4. For each dogs the mean per cent change for all 3/2-hourly observations
   (four acute or eight chronic observations) was computed.

5. The mean per cent change and its standard error were then computed
   for the five dogs, in each measure of clearance, for both treatments (whole
   blood and hemoglobin), for acute and for chronic effects (Table V). None of
   these per cent changes was significantly different from 0.

6. The difference between whole blood and hemoglobin effects was then
   tested by a t test for each clearance measure and for acute or chronic effects.
   In no case was there a significant difference between these two treatments.

Employing the same analysis for urinary output per minute the mean acute
change ± SE for whole blood dogs was +46.1 ± 29.1% and for hemoglobin
dogs +227.1 ± 120.8%. The mean chronic changes for whole blood and
hemoglobin dogs were respectively +11.1 ± 23.4 and 110 ± 97.9%. T tests
of the acute and chronic means revealed no significant difference between
 treatments (P > 0.20 for acute and P > 0.40 for chronic).

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**TABLE IV**

<table>
<thead>
<tr>
<th>Coagulation factors</th>
<th>Difference between prothrombin activity (%) and activity of coagulation factors (%)</th>
<th>Mean*</th>
<th>SE of Mean</th>
<th>t†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII</td>
<td></td>
<td>-4.25</td>
<td>5.52</td>
<td>-0.95</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>4.625</td>
<td>6.85</td>
<td>0.68</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
<td>6.25</td>
<td>3.85</td>
<td>1.62</td>
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</table>

* N = 8.
† Critical value @ 0.05 = 2.306.
Examination of histological sections was performed on eight dogs sacrificed 4 hr; one dog, 2 days; two dogs, 3 days; three dogs, 4 days; three dogs, 5 days; and one dog, 6 days after hemoglobin infusion. Special attention was given to tubular changes that might have been related to hemoglobin infusion such as cellular necrosis and degeneration as well as pigmented casts (24, 25). All segments of the nephron were carefully examined and no significant changes were seen. In two animals, occasional pigmented casts were seen in the distal portion of the nephron. The extremely small number of these casts morphologically excludes the possibility of obstruction and significant interference with renal function. In addition the lack of cellular degeneration in the same tubules would indicate no noxious effect of infused hemoglobin under conditions of experiment.

### Table V

<table>
<thead>
<tr>
<th>Comp. of means</th>
<th>P value</th>
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**DISCUSSION**

The concept of employing hemoglobin as a plasma expander is not new. Extensive in vitro and in vivo experiments with a variety of preparations have been made (1-3, 26, 27). Many of these preparations, like ours, have been found to be stable and to combine with oxygen normally. These studies were abandoned mainly because of the reduction of urea clearance in recipient animals (2).

The mechanism of kidney damage following hemoglobin infusion has also been studied. Some have postulated that tubular blockage by precipitated hemoglobin is the primary cause of renal failure (24, 28-31), while other observers found either no tubular obstruction (4, 30) or regard reduction in glomerular filtration plus renal ischemia as primary causes (25, 33, 34).

Previous work in this laboratory has shown that infusion of autologous hemolytate into dogs results in production of a hypercoagulable state which can go on to actual intravascular coagulation. In addition we have been able to corroborate the observation that coagulant activity of hemolyzed blood resides in stromal rather than hemoglobin fraction and to demonstrate that stromal particles are cleared by the reticulo-endothelial system (10).
This work, plus the observations by others, of the important role of intra-
vascular coagulation in pathogenesis of renal damage due to numerous causes,
suggest the possibility that the "ischemia" implicated in the pathogenesis of
renal damage following hemoglobin infusion may be due to arterial thrombotic
lesions resulting from the initiation of coagulation mechanism by stromal
particles. This concept is supported by the fact that following infusion of our
stroma-free hemoglobin solution there was no demonstrable functional or
structural effect on the kidney. The doses employed and the experimental
conditions of previous investigators varied and for this reason it is difficult
to make direct comparisons. However, the dose employed and experimental
animal in the study by Hamilton et al. (2) is comparable to our study. They
found a fall in urea clearance which was demonstrable several hours after
hemoglobin infusion and which lasted as long as 2 wk. Urea clearance was not
significantly altered in our experiments during these same periods.

Brandt et al. found a prompt fall in P.A.H. clearance and urinary output
during infusion of hemoglobin solution to well hydrated humans (33). Although
his dose was comparable to ours, he gives no data on duration of renal effect
following cessation of hemoglobin injection. We found no changes in P.A.H.
clearance or urinary output. Goldberg also found a significant fall in urine
flow, creatinine clearance, and P.A.H. clearance following administration of
homologous hemolyzed red blood cells to dogs (31). He found no change in
renal blood flow by a gas diffusion technique using krypton. However, he in-
fused his preparation directly into the aorta above renal arteries, and used
a much smaller amount of hemoglobin (~1/10 of our dose). Jaenike found
decrease in inulin clearance and development of a renal lesion following infusion
of hemoglobin into dehydrated rats (25, 34). However, the dose of hemoglobin
employed in his experiments was approximately 25% of ours and our animals
were all hydrated prior to the first clearance period. Because of the differences
in experimental design we cannot definitely attribute the results to differences
inherent in our hemoglobin preparation. We are presently studying the effect
of various other hemoglobin preparations in a comparable series of experi-
ments.

The method described in this paper is designed to prepare large quantities
of stromal-free hemoglobin solution from outdated bank blood. To accomplish
this, we were concerned not only with removing particulate matter, but
avoiding contamination of hemoglobin solution with stromal lipid.

By utilizing coagulant activity as a test for presence of stroma, we found that
to remove all detectable stromal particles, it was necessary to first centrifuge
at high speeds and then pass the solution through an 0.2 μ millipore filter. In
order to avoid contamination of hemoglobin with nonparticulate stromal
lipid, a modification of a method designed for the preparation of hemoglobin-
free ghosts was chosen (12). This method uses, as a hemolyzing agent, a hypo-
tonic phosphate buffer of controlled osmolarity and pH. When this buffer is used essentially all stromal lipid can be recovered in the ghosts. Other workers have found that when water was employed for hemolyzing red cells of fresh ACD blood, up to 10% of phospholipid is lost from stroma and that this was increased to as much as 30% when bank blood was employed or if oxalate was used as an anticoagulant (35). In reviewing the literature, one finds that hemoglobin solutions have been used by many investigators for a variety of experiments, in addition to those designed to study hemoglobin as a plasma expander (1, 2, 26, 27). Some have investigated the effect of hemoglobin on the kidney (2, 4, 24, 25, 28–34), the antigenicity of hemoglobin (36–43), hemoglobin and iron metabolism (44–48), effect of hemolysate on blood coagulation (22), and role of intravascular hemolysis on production of a hypercoagulable state (10). Water was used to hemolyze erythrocytes in many of these studies (1, 2, 25, 29, 31, 32, 34, 41, 44–48) while other methods included the use of ultrasonic vibrations (4, 10), mechanical trauma (33), and freezing then thawing (22). Following hemolysis, efforts to further purify solution also varied. While some investigators were content to use the hemolysate without further purification (44) most felt it necessary to at least centrifuge and decant the supernatant. Others purified their preparation by chromatography (40, 42, 43, 48). While it is probable that these hemoglobin solutions were perfectly adequate for many of the studies in question, it is nevertheless important to realize that many of these solutions, by nature of their mode of preparation, were very likely contaminated with stromal particles, lipid, or both. This fact should be taken into consideration when evaluating the results of experiments where effects attributed to hemoglobin may actually have been caused by stroma.

We have studied the preparation of Hamilton et al. (1) which was used as a plasma expander and also employed to test effect of hemoglobin on the kidney (2, 4). This solution, although free of particulate matter, was heavily contaminated with stromal lipid and therefore had considerable coagulant activity. This is in contrast to the preparation described in this paper which had very little demonstrable lipid and no coagulant activity.

The amount of hemoglobin infused in our experiments was 1.4–3.3 g/k., which is far in excess of the amount employed by other investigators in experiments designed to study distribution, excretion, and metabolism of hemoglobin (45–48). It is accepted by most workers in the field that, in the circulation, extracorpuscular hemoglobin combines with haptoglobin to form a molecule which is not filterable by the renal glomerulus and is cleared by the reticuloendothelial system. When the level of hemoglobin exceeds the haptoglobin-binding capacity, the unbound hemoglobin can be excreted by the kidneys. The level of haptoglobin and its rate of productila varies among species and can be altered by disease states or chronic hemolysis (49–51). Anderson et al. administered graded doses of hemoglobin, labeled with 51Cr, to dogs and found that the plasma hemoglobin must exceed 70 mg% before
significant amounts appear in urine (48). In our experiments, initial plasma
hemoglobin levels, obtained 5 min after completion of infusion, varied from
1.6–4.3 g%. Assuming haptoglobin binding of 70 mg%, the unbound (free)
hemoglobin in these experiments varied from 57–83% of the total infused.
The per cent excreted in urine varied widely, could not be correlated with
either amount of hemoglobin infused or initial plasma hemoglobin, and was
always much less than the theoretical per cent of free hemoglobin. Also, the
per cent of total dose found in liver and spleen was also always greater than
theoretical per cent of haptoglobin-bound hemoglobin. These observations
are in agreement with those of Andersen et al. who also found that the total
amount of hemoglobin excreted in urine accounted for only a small percentage
of free hemoglobin. This suggested to them that the major portion of free
hemoglobin was cleared from plasma by body tissues (48). Our results are also
in agreement with the observation made by others that the liver is the principal
organ involved in hemoglobin clearance (45, 46).

Administration of the described hemoglobin preparation to phlebotomized
dogs resulted in a prompt restoration of vital signs which were maintained
throughout the experimental period (approximately 4 hr). This, along with
autopsy findings, supports the concept that this preparation is nontoxic. We
cannot at this time claim a beneficial effect of hemoglobin infusion on prognosis
of hemorrhagic shock. It is our hope that future experiments, employing a well-
controlled shock model, will demonstrate the effectiveness of this preparation
against shock.

SUMMARY

The preparation of large quantities of a stable, stroma-free hemoglobin
solution without coagulant activity is described. Following infusion of this
solution into phlebotomized dogs, there is no methemoglobin formation, no
adverse effects on vital signs, and no demonstrable activation of blood coagula-
tion. The hemoglobin maintains its oxygen-carrying capacity and liberates
oxygen into tissues. Acute and chronic effects on renal function following infu-
sion of this preparation were also studied and no effect on clearance of urea,
creatinine, or P.A.H. could be demonstrated. There was no change in urinary
output and histological sections revealed no lesions attributable to hemo-
globin toxicity. It is concluded that a stroma-free hemoglobin solution may
have use as a plasma expander.

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