

## PREPARATION OF HEMOGLOBIN SOLUTIONS FOR INTRA- VENOUS INFUSION\*†§

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The infusion of hemoglobin solutions to replace lost blood has been tested in experiments on both animals (2-15) and man (4, 16-19). Beneficial results have been obtained in restoring the volume of circulating blood, in carrying oxygen to the tissues (3), and in supplying iron for regeneration of erythrocytes and nitrogen for regeneration of blood and tissue proteins (12). For infusions hemoglobin solutions have advantages over whole blood in that the former do not need to be typed and cross-matched, and that longer preservation is possible. However, in both patients and animals, some of the infusions have been followed by unfavorable results, particularly on the kidneys. It has appeared uncertain whether these effects have been due to injury by the hemoglobin itself, caused during its rather rapid excretion through the kidneys, to admixture of methemoglobin with the active hemoglobin (hemoglobin capable of reversible combination with oxygen), to the presence of cell stroma material in the hemoglobin solutions used, or to bacterial contamination or products.

The present work was undertaken to provide a method whereby large volumes of hemoglobin solution could be prepared in a sterile state, free from stroma material, of electrolyte content approximating that of plasma, and capable of preservation without change of the active hemoglobin to methemoglobin.

### *Analytical Methods and Tests*

The *carbon monoxide-binding capacity* of the hemoglobin solutions was determined by a modification of the method of Van Slyke and Hiller (20) described by Van Slyke, Hiller,

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Weisiger, and Cruz (21). The hemoglobin fraction capable of binding carbon monoxide is hereafter designated as "active hemoglobin."

*Total hemoglobin content* of the solutions was determined by the above carbon monoxide capacity method after reduction of the solution with sodium hyposulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) according to the modification of the method of Van Slyke and Hiller (22) described by Van Slyke, Hiller, Weisiger, and Cruz (21).

The difference between the total hemoglobin content of the solutions and the active hemoglobin content gives the inactive hemoglobin fraction hereafter designated as methemoglobin.

*Total lipid content* of the solutions was determined by extraction of the lipids by the method of Folch and Van Slyke (23) and subsequent estimation of the extracted lipids by determining their carbon by the method of Van Slyke and Folch (24).

The *pH* of the preparations was determined by the glass electrode.

*Sodium and potassium* concentrations of solutions were determined with a flame photometer (25).<sup>1</sup>

Tests for pyrogenic substances, for which we are indebted to Dr. R. B. Pennell of Sharp and Dohme Company, were carried out in accordance with the directions of the U. S. Pharmacopoeia XII (26).

Sterility tests were carried out by culturing 5 ml. aliquots aerobically and anaerobically. We are indebted to Dr. Frank Horsfall, Jr., of the Rockefeller Institute Hospital for most of these tests and to Dr. Pennell of Sharp and Dohme for the remainder.

Toxicity tests were carried out on all earlier preparations. One half ml. of the solution to be tested was injected intraperitoneally into each of ten 15 gm. Swiss mice. The criterion was survival. For later preparations, made after experience gained with the solutions, this test was omitted; but in other experiments the effects of administration to dogs in large quantities intravenously provided evidence for the absence of toxicity factors.

#### *Preparation of Hemoglobin Solutions*

The procedure consisted of the following steps: (1) laking the cells with water, (2) precipitation of the stroma material by acidifying to pH 5.7-5.8, (3) ion exchange of most of the potassium for sodium by treatment with sodium zeolite, (4) removal of stroma and zeolite by centrifugation, (5) addition of NaCl and  $\text{NaHCO}_3$  to obtain physiological salt concentration and pH, (6) sterilization by filtration through a Seitz filter.

*General Measures to Insure Sterility.*—Throughout the procedures required in the preparation of the solutions a strict aseptic technique was followed. All containers were sterilized by autoclaving prior to use. When a vessel was opened to receive a solution, the mouth was flamed, and the flaming was repeated before reinsertion of the sterile plug used as a stopper. All water used in preparations was freshly double distilled under conditions making it pyrogen-free. Salts were added to solutions immediately before passage through a Seitz sterilizing filter. All procedures were carried out at 4°C., and the solutions were stored at 4°C., unless otherwise stated. Cultures for aerobic and anaerobic organisms were taken prior to passage through the Seitz sterilizing filter. Bacterial contamination of the solution at this point was

<sup>1</sup> We are greatly indebted to Dr. R. Bowling Barnes and Dr. J. W. Berry of the American Cyanamid Company, Stamford, Connecticut, for the extended use of one of their own research models of the flame photometer, considerably in advance of detailed publication or commercial manufacture of their instruments.

never encountered. The sterility tests were repeated on solutions just prior to use in physiological experiments. Specific procedures are detailed below.

*Source of Red Blood Cells.*—Red blood cell residues discarded from Red Cross donor centers<sup>2</sup> after withdrawal of plasma were used for making human hemoglobin solutions. Solutions of dog hemoglobin were made by bleeding dogs, heparinizing the blood, sterilely aspirating the plasma after centrifugation, and utilizing the residues in the same manner as with human blood.

*Laking of Red Blood Cells and Precipitation of Stroma Material.*—One volume of fresh red blood cell residues was suspended in an equal volume of sterile isotonic saline and repacked by centrifugation for 30 minutes at 3000 R.P.M. The supernatant fluid and buffy layer were aspirated off with aseptic technique and discarded. One volume of washed cell residue was then laked by mixing and standing for 1 hour with two volumes of distilled water. Tenth normal HCl was then added in small portions, usually ten, until the pH of the mixture fell to 5.7–5.8. Approximately 0.6 volume of acid was needed, and it was convenient to add 0.5 volume of acid before determining the pH. During the addition of the acid, the mixture was stirred by swirling the contents of the flask but violent mechanical agitation was avoided; this procedure produced a bulky precipitate which filtered off readily. After addition of the acid, the solution was made up to a volume equal to four times the original volume of red cells used. The 0.1 N HCl used was made up by diluting reagent grade concentrated HCl with pyrogen-free, sterile, distilled water.

*Removal of Excess Potassium.*—Without removing the suspended precipitate, the mixture obtained by the above acidification was treated with sodium zeolite (decalso), to reduce, by ion exchange for Na, the potassium concentration to a physiological plasma level. The decalso, 50 to 80 mesh, was first washed with 5 per cent NaCl solution, dried, and heat-sterilized.<sup>3</sup> Thirty grams of this decalso were added per liter of cell solution, and the mixture was gently stirred several times to insure adequate mixing. Exchange of potassium of the solution with sodium of the decalso approached equilibrium in a few minutes,<sup>4</sup> and, with the amount of decalso used, the concentration of potassium in the treated solution was lowered to approximately 4 milliequivalents per liter.

After the addition of decalso, the mixture was let stand for 1 hour to allow maximum aggregation of the bulky, almost granular precipitate and then the decalso and previously precipitated stroma were removed together by filtration through a coarse filter paper. The filtrate volume approximated 70 per cent of the volume of mixture filtered; the yield by centrifugation was not found sufficiently greater to compensate for using it in place of filtration.

*Adjustment to Final pH and Electrolyte Content.*—The clear deep red filtrate of pH 5.7 to 5.8 contained approximately 4 milliequivalents of potassium and 30 milliequivalents of sodium per liter, chiefly as the chlorides. Solid sodium bicarbonate was added to the solution in an amount sufficiently in excess of the previously added HCl to provide a final concentration of

<sup>2</sup> The authors are greatly indebted to Dr. Canby Robinson of the American Red Cross for permission to use discarded red blood cell residues, and to Dr. Boynton of the American Red Cross for constant effort to insure that an adequate supply of fresh cell residues was maintained to this laboratory.

<sup>3</sup> Decalso, trade name for a synthetic sodium-aluminum-silicate zeolite, manufactured and obtained from the Permutit Company, 330 West 42nd Street, New York 18, New York.

Drying and heat sterilizing decalso for 1 hour at 160° to 170°C. apparently causes some diminution in its effectiveness in exchanging sodium for potassium; the 30 gm. per liter of solution recommended allows for this slight loss of activity.

<sup>4</sup> That potassium is exchanged for sodium, atom for atom, was established by analysis of the solution for sodium and potassium before and after treatment with decalso.

20 millimolar bicarbonate. In addition, enough solid sodium chloride was added to bring the final concentration of sodium to approximately 145 milliequivalents per liter, and sufficient calcium and magnesium as chlorides to give concentrations of 5 and 2 milliequivalents per liter respectively. The pH of the final solution usually lay between 7.1 and 7.6, depending on the residual amount of carbon dioxide liberated by reaction with the HCl. A sample of the filtrate was taken for sterility tests.

*Sterilization by Seitz Filtration.*—The solution was filtered through a Seitz filter using an S-1 filter pad under 15 to 20 pounds pressure per square inch. Filtration was rapid; 2 liters filter through a pad 120 mm. in diameter in about 15 to 20 minutes. Passage through the Seitz filter also insured removal of any precipitated stroma and zeolite that might not have been removed in the first filtration. The solution was conveniently filtered into 500 ml. or liter bottles, which were then closed with sterile rubber stoppers and kept at 4°C., until used. Portions of the filtrate were taken for sterility and pyrogen tests and additional tests for sterility were done on a number of individual bottles after varying periods of storage.

#### *Properties of the Hemoglobin Solutions*

*Composition and Physical Properties.*—Solutions of hemoglobin prepared according to the method just described were clear, of deep red color, and contained approximately 7 gm. of hemoglobin per 100 ml. If the entire procedure was carried out at 4°C., 95 to 98 per cent of the hemoglobin was active. If the procedure was carried out at room temperature with solutions previously chilled to 4°C., and removed from the refrigerator as used, the percentage of active hemoglobin was slightly lower, 92 to 95 per cent. Analysis for lipid carbon showed that 90 to 95 per cent of all lipids had been removed. Numerous tests of human hemoglobin solutions mixed with human blood cells of all four types have never shown any red cell agglutinating properties. No particulate material could be detected on dark field illumination. In the absence of evaporation when kept at 4°C., the solutions remained free of precipitate for 6 months or longer. Some solutions ultimately developed small amounts of a fine reddish precipitate which was observed only after standing 6 months or longer; the formation of precipitate is discussed below.

#### *Stability of Solutions Stored at Various Temperatures.*—

Ten ml. portions of a hemoglobin solution were transferred with a sterile pipette into 20 ml. sterile pyrex glass ampules, and the ampules sealed in the oxygen flame. Sealed ampules were stored at 4°C., at 20–25°C. (room temperature), and at 38°C. respectively. From time to time up to 47 days, ampules stored at these temperatures were opened and analyzed for total and active hemoglobin. The results are shown in Fig. 1. It will be seen from these data that the solutions were completely stable at 4°C. but that at room temperature methemoglobin (total—active hemoglobin) formation was appreciable, and that it was still faster at 38°C. It was observed that the formation of precipitate in the solution also depended upon the temperature of storage. Considerable precipitate slowly formed in the solutions stored at 38°C., less in those stored at room temperature, and none was formed in the solutions stored at 4°C. over the 47 day period. Solutions stored under CO<sub>2</sub> at room temperature showed more precipitate than those stored under air at the same temperature.

Two ampules after 6 months' storage at 4°C. showed 3 and 15 per cent formation of methemoglobin respectively. One ampule of hemoglobin solution in which 99.7 per cent of the

active hemoglobin had been deoxygenated by removal of  $O_2$  before the ampule was sealed showed no change in methemoglobin or active hemoglobin after 6 months' storage at  $4^\circ C$ . None of these ampules had developed any sediment in the 6 months' storage period.

Other stability experiments were carried out on 200 cc. lots of hemoglobin solution stored at  $4^\circ C$ . in sterile bottles closed with rubber stoppers. Samples of solution were removed with aseptic precautions at weekly intervals and analyzed for total and active hemoglobin. No methemoglobin formation was observed over a period of 10 weeks; and no sediment was observed at the end of 6 months.<sup>5</sup>

From these experiments it appears that no methemoglobin is formed over a period of  $2\frac{1}{2}$  months; over a 6 month period a small and variable amount of methemoglobin may be formed. Dr. R. B. Pennell of Sharp and Dohme Company<sup>6</sup> has informed the authors that he has succeeded in storing solutions of hemoglobin, prepared in the manner described in this paper, for over 12

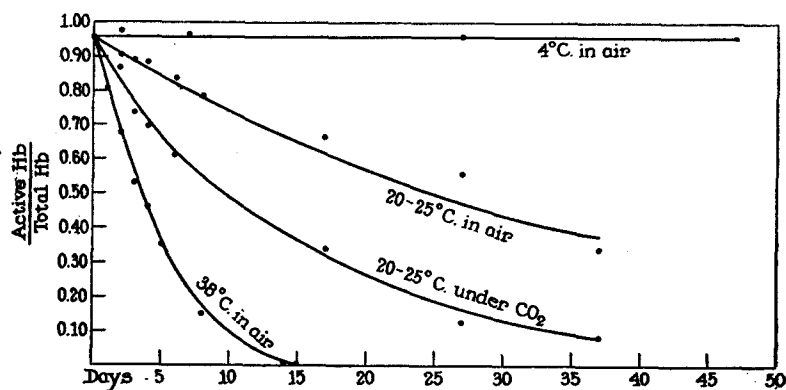


FIG. 1. Decrease in activity of hemoglobin in solutions standing at different temperatures.

months without detectable methemoglobin formation and without any precipitate formation providing the solutions were kept frozen solid at temperatures of  $-5^\circ$  to  $0^\circ C$ . It therefore appears probable that the methemoglobin and sediment formation observed in our experiments was due to storage at too

<sup>5</sup> Anomalous behavior was occasionally noted in a bottle of the thirty or forty that were usually filled from one preparation. Some of these bottles exhibiting anomalous behavior showed on storage fairly rapid conversion of hemoglobin to methemoglobin which on further storage showed slow spontaneous reconversion to active deoxygenated hemoglobin. Other bottles exhibited consumption of oxygen with the formation of deoxygenated hemoglobin without previous conversion to methemoglobin. These changes were always visibly evident, the solution becoming brown with the formation of methemoglobin, and a deep purple hue with the formation of the deoxygenated hemoglobin. Examination of these bottles that behaved anomalously never revealed bacterial contamination.

<sup>6</sup> The authors are indebted to Dr. R. B. Pennell of Sharp and Dohme Company, Glenolden, Pennsylvania, for much helpful information which he has freely placed at our disposal throughout the course of this work.

high a temperature; the minimum temperature of our storage facilities was 4°C., in refrigerators that were opened frequently and which did in fact suffer considerable elevations of temperature above this minimum as a result of temporary interruption of refrigeration service.

*Effect of Storage under a CO<sub>2</sub> Atmosphere.*—Ten ml. portions of hemoglobin solutions were transferred, with precautions for sterility, to a few ampules which were then evacuated with a water pump and refilled with CO<sub>2</sub> four times before sealing of the ampule. These were stored at room temperature only. In these CO<sub>2</sub>-filled ampules, formation of methemoglobin was more rapid than in the air-filled ampules (Fig. 1). This observation is in keeping with the findings of Brooks (27) who showed that at 25°C. decrease of pH accelerates the conversion of hemoglobin to methemoglobin. On filling ampules with CO<sub>2</sub> the pH was lowered from 7.6 to 6.8 or 7.0; solutions in ampules sealed with air and stored had pH values of 7.3 to 7.6 when opened subsequently. It is pertinent that Brooks (27) showed that the conversion of hemoglobin to methemoglobin by oxygen under these conditions was the only detectable reaction; *i.e.*, he could not detect conversion of hemoglobin or methemoglobin to other denatured pigments.

#### DISCUSSION

The two major problems which had to be overcome in the preparation of these solutions were the complete removal of stroma and reduction of the potassium content to a concentration approximately that of normal plasma. No adequate tests exist for the detection of stroma material, since the substance has not been sufficiently characterized chemically. We found that simple addition of salt to the laked cell suspension (8, 13, 15, 17, 18) did not remove all material precipitable by toluene or by acid at a pH of 5.9. Cooling the laked cell suspension and bubbling carbon dioxide through it precipitated the stroma material, but the precipitate was so fine that frequently it was impossible to separate it by filtration from the solution, and it blocked a Seitz filter pad. The CO<sub>2</sub> treatment also increased the proportion of methemoglobin. Removal of stroma with aluminum hydroxide (28) was not found satisfactory. Although toluene as used by Heidelberger (29) and a number of subsequent workers (2-4, 9, 16, 30), appears to remove stroma material quantitatively, the resulting solutions could be rendered free of toluene only with difficulty and uncertainly. Crystallization of hemoglobin and preparation of solutions from such crystallized material was avoided because of the extra steps involved, and because hemoglobin crystallized by Heidelberger's method (29) has been found to contain 7 to 15 per cent of methemoglobin (31). The acidification technique originally described by Jorpes (32) for the isolation of stroma material was found to be efficient and easily adaptable to the present investigation. However, it was found that if the HCl is added slowly as from a burette with vig-

orous mechanical stirring a very fine precipitate forms, presumably stroma, which is very difficult to centrifuge down and impossible to filter off, even though the pH has been brought to 5.7 to 5.8. If to this unfilterable suspension of stroma material 1 to 5 per cent of acid-denatured hemoglobin is added rapid and complete flocculation of the stroma occurs in a form that can be readily filtered off. Since the technique of acidification used in our preparations produced an easily filterable precipitate it is inferred that there must have been small amounts of acid-denatured hemoglobin formed on the addition of the HCl. These observations indicate that adjustment to the isoelectric point of stroma is not the only factor involved in the precipitation of stroma on the addition of acid.

Although removal of excess potassium in solutions made from dog hemoglobin is not necessary because of the low potassium content of red blood cells of the dog, it is required with solutions made from human red blood cells, or the resulting solution will have a potassium content close to toxic levels (33) for plasma. Ion exchange with sodium zeolite proved to be a rapid and economical means of reducing the  $K^+$  concentration to normal physiological levels by replacement with  $Na^+$ .

The importance of keeping bacterial contamination and growth low hardly needs emphasis, for toxic substances are rapidly produced when bacteria multiply in solutions of hemoglobin. Most investigators previously have preferred to prepare solutions for immediate use, sacrificing rigid aseptic technique for speed of preparation and relying on low temperatures to retard bacterial growth till the moment of injection. None have mentioned carrying out tests for pyrogenic substances, and in the absence of such tests it is difficult to assay reactions, such as chills and fever, which have been reported to follow intravenous administration of hemoglobin solutions into human subjects (13, 15-18).

Deoxygenated hemoglobin provides a more stable form than oxyhemoglobin for storage in solution. However, complete deoxygenation is a long and tedious process, and since the oxyhemoglobin solutions have been found to be stable at 4°C. over a period of at least 6 months, we do not believe that the advantages to be gained by deoxygenation are equivalent to the increased labor of preparation. Hemoglobin solutions prepared for freezing and drying must be completely deoxygenated, as noted in the accompanying paper (34) to prevent methemoglobin formation during the process of dehydrating the frozen mixture.

#### SUMMARY

A procedure has been detailed for the preparation of sterile non-pyrogenic solutions of oxyhemoglobin which have the approximate protein content and electrolyte composition of plasma.

Large volumes of solution can be rapidly prepared, with 95 to 98 per cent of the hemoglobin in the active form capable of combining with oxygen. The

solutions contain no particulate matter; 95 per cent of total blood lipids are removed.

Solutions stored at 4°C. showed no conversion of hemoglobin to methemoglobin over a period of 2½ months; over a 6 month period a small and variable amount of methemoglobin may be formed.

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