PREPARATION OF STABILIZED SOLUTIONS OF HEMOGLOBIN

By Robert B. Pennell, Ph.D., and William Elliott Smith, B.S.

During the recent war the need for whole blood for its oxygen-carrying capacity and the supply of human red cells derived from the plasma program revived interest in the long controversy as to the usefulness of hemoglobin solutions in therapy. That there should have been such a controversy was due in large part to the lack of availability for study of a standard hemoglobin solution of high stability prepared with adequate bacteriologic control, and to the consequent difficulty of interpreting much of the published work. This has been emphasized by Hamilton, et al. We had the privilege of working with two of the groups which undertook the reinvestigation of this problem, that of Dr. William R. Amberson of the University of Maryland Medical School and that of Dr. Donald D. Van Slyke of the Hospital of the Rockefeller Institute, N. Y. From the work of each of these groups a standard hemoglobin solution suitable for clinical study was developed. Stability of these solutions could be maintained, however, only by special treatment, exhaustion of oxygen by high vacuum in the one instance and refrigeration in the other.

Hemoglobin in solution exists in three forms which are in equilibrium, two of which, oxyhemoglobin and reduced hemoglobin, are physiologically active (i.e., they can act as oxygen carriers) and one of which, methemoglobin, is physiologically inactive. One of the physiologically active forms, reduced hemoglobin, is stable and the other is not. The instability of hemoglobin in solution is first manifested by its conversion to the physiologically inactive methemoglobin. The three forms of hemoglobin also occur within the red cell, which is so constructed, however, that the hemoglobin within it is maintained in an active oxygen-carrying form, the inactive methemoglobin being kept at an extremely low level. In the course of our studies we found methods of preparing solutions of hemoglobin by which the mechanism for maintenance of its oxygen carrying capacity was preserved. This resulted in a hemoglobin solution which would convert itself to the stable reduced hemoglobin form, thus obviating the special treatments usually necessary for storage of the solutions. The present study is concerned with delineation and demonstration of the factors of importance to the preparation of this type of solution.

METHODS, TESTS AND EQUIPMENT

Total hemoglobin and methemoglobin contents of hemoglobin solutions were determined with the Evelyn photoelectric colorimeter, employing the method of Evelyn and Malloy. The values so obtained were found to check well with those obtained in other laboratories by other methods. The pH of solutions was determined by glass electrode without dilution of the hemoglobin solutions. Sodium and potassium concentrations of solutions were determined by the flame photometer.

Tests for pyrogenic substances were carried out in accordance with the Minimum Requirements for Pyrogen Tests on Biologic Products from Blood Serum, Nov. 19, 1945, National Institute of Health.

From the Department of Immunochemistry, Sharp and Dohme, Inc., Glenolden, Pennsylvania.
Sterility tests and animal safety tests were carried out in accordance with the Federal Register of September 16, 1947, as amended in January 1948. Since hemoglobin solutions form precipitates when added to the culture medium used in the sterility test, subcultures were made at the end of one week and the final test was read two weeks after the date of testing.

Pyrogen free water was prepared by double distillation, followed by immediate use, or by immediate storage at 2°C for not more than sixteen hours.

Total reducing substances and nonfermentable reducing substances were determined by the method of Benedict. Inorganic phosphorus was determined by the method of Embden and Fetter. Lipid phosphorus was determined by the method outlined in "Quantitative Clinical Chemistry," by Peters and Van Slyke, vol. II, page 884, 1st Edition, 1931.

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Source of Blood Cells. Sterile human red blood cell residues were obtained from commercial bleedings from the plasma processing unit of Sharp and Dohme. The bleedings were drawn in sodium citrate solution and approximately seventy-two hours elapsed between the time of bleeding and the time that the red cell residues were available for hemoglobin preparation.

General Measures Observed. Rapidity of operation and maintenance of optimal working conditions were employed rather than aseptic handling during preparation of the solutions. Starting with sterile red cells, the entire operation was invariably completed and the final solutions sterilized within the course of eight hours. All work was performed at 2°C in a laboratory equipped with Sterilamps. All equipment coming in contact with the solutions was carefully cleaned and rinsed with pyrogen free water immediately before use. Pyrogen free water was used throughout for all dilutions and all solutions added to, or coming in contact with, the cells and hemoglobin. All hemoglobin solutions were submitted for sterility, and for pyrogen and safety testing immediately after preparation.

Washing and Laking of Red Blood Cells. From 2 to 5 liters of packed human red cells were washed by suspension in 2 to 5 volumes of 6 per cent dextrose solution containing 0.15 per cent nicotinic acid amide and 0.006 per cent ammonia, followed by centrifugation in a laboratory model Sharples Super Centrifuge using the Sharples blood separator bowl. With this bowl the wash solution is delivered from one outlet and the washed cells from another. The cells were ruptured during centrifugation and were caught in a container holding a small amount of nicotinic acid amide solution (containing sufficient nicotinic acid amide to provide 0.13 per cent in the estimated final volume of solution). The washed, ruptured cells were diluted with 2 volumes of dextrose solution (sufficient dextrose is used to provide 6 per cent in the estimated final volume of hemoglobin solution).

Precipitation of Stroma. The mixture was adjusted to pH 5.7-5.8 with 0.1 N hydrochloric acid at which pH the stroma was readily removed by centrifugation in a large Sharples Super Centrifuge, using a clarifying bowl. The addition of the acid was accompanied by brisk mechanical stirring. The acid was allowed to run in a thin stream from a capillary pipette near the vortex of the stirring solution. Approximately 250 cc. of 0.1 N hydrochloric acid per liter of solution may be added before determining the pH.

Removal of Excess Potassium. The centrifuged mixture was treated with sodium zeolite (declalso) to reduce the potassium content. Approximately 30 Gm. of sodium zeolite per liter of solution was added and the mixture was stirred gently for 4 hours, after which the sodium zeolite was allowed to settle for ten to fifteen minutes. The solution was then decanted. Adjustments to the Final pH and Composition. Sufficient solid sodium bicarbonate was added to the solution to neutralize the hydrochloric acid added and to provide a slight excess. It was found that 7.9 Gm. of sodium bicarbonate per liter of solution provided a pH of 7.2 to 7.3 at this point. The final concentration of hemoglobin was adjusted to approximately 7 per cent, that of dextrose to 6 per cent and that of nicotinic acid amide to 0.15 per cent. To the solution was added, per liter, 5 cc. of ammonium hydroxide solution (5 parts of Baker's A.C.S. ammonium hydroxide to 100 parts of water) to provide a concentration of 0.006 per cent NH₃, 4 cc. of 1 per cent merthiolate (to provide a concentration of 1:15,000), 14 mg.

* Manufactured by the Permutit Co., New York, N. Y. Since earlier work had indicated the occasional presence of pyrogenic material in declalso it was always washed as described by Smith and Pennell in J. Bact. 54: 715, 1947.
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MgSO$_4$·7H$_2$O (1.1 cc. of 2 per cent solution), 23 mg. CoCl$_2$·6H$_2$O (1.15 cc. of 2 per cent solution), and 19.8 mg. MnCl$_2$·4H$_2$O (0.99 cc. of 1 per cent solution), providing 0.1 millimolar concentration of each of the metals, and 20 mg. of nile blue (1 cc. of 1 per cent solution). In a few of the later solutions 1 Gm. per liter of the calcium salt of hexose diphosphate* was added before stirring with decalso. The Ca$^{2+}$ ions were removed from solution by the ion exchange agent.

Sterilization by Sitz Filtration. The solution was filtered through K6 clarifying pads and sterilized by filtration through SSf pads using a Republic filter press. The clear red filtrate was caught in a sterile bottle containing a sterile syphon which could be used for filling the solution into a series of small containers. Samples taken at the time of filling these small containers were tested for pyrogenicity, sterility and safety. The solutions were held at 25 C. until the completion of these tests.

PROPERTIES OF HEMOGLOBIN SOLUTIONS

Solutions prepared according to the procedure just described were crystal clear and of deep red color. The hemoglobin of the solutions was more than 98 per cent active. Thorough examination indicated complete inability of the solutions to agglutinate A, B or O cells. Examination of the serum of patients before and two weeks after the injection of these solutions has shown no increase in the titer of anti-A and anti-B isohemagglutinins. Lipid phosphorus determinations showed that upon removal of the stroma less than 10 per cent of the lipid phosphorus remained in the solution, a figure corresponding closely to the 5-10% of lipid carbon reported by Hamilton, et al. In a typical preparation the solution contained 5.56 milliequivalents of potassium, as compared to 19 milliequivalents before decalso treatment. These solutions, when in sealed containers with little air space were completely converted to reduced hemoglobin in one to two days at 37 C., in two to three days at 25.27 C., or in seven to eight weeks at 2 C. During this conversion the presence of methemoglobin in quantities greater than 1-3 per cent of the total pigment was not detectable by daily examination. The reduced hemoglobin so obtained has been observed for twenty-four months at 25-27 C. without a change in the percentage of active hemoglobin. There may be, however, gradual deposition of a sediment during this time, the amount of sediment being a function of the speed of disappearance of oxygen, the amount of oxygen to be consumed, i.e., the amount of air space in the bottle, and the temperature of storage. No noticeable sediment has been encountered in solutions stored at 2 C. (Cursory examination of the sediment has shown it to be in part carbohydrate in nature, giving no reduction before hydrolysis and indicating a mixture of aldo- and keto-sugars after hydrolysis.) Shaking of the solutions, with consequent foam formation, may result in the appearance of films due to surface denatured protein. None of these phenomena has been found to have influence, detectable by the methods employed on the activity of the hemoglobin itself. These solutions lend themselves readily to lyophilization as will be described in a subsequent publication.

EFFECTS OF VARIOUS STEPS ON THE CAPACITY OF THE SOLUTIONS TO FORM REDUCED HEMOGLOBIN

Neill$^t$ showed that since oxyhemoglobin and reduced hemoglobin have different colors, hemoglobin solutions in sealed containers could act as indicators of the

* Schwartz Laboratory, Inc., New York, N. Y.
† Republic Filter Corp., Paterson, N. J.
loss of oxygen from solution due to the action of enzyme systems which he added. Warburg had similarly made use of this phenomenon in following respiration of intact avian red cells. Neill showed that when removal of oxygen from solution was rapid, the accumulation of methemoglobin did not occur. It has since been shown by many workers that methemoglobin itself may be reconverted to active hemoglobin by the action of enzyme systems. Evelyn and Malloy developed methods based on the light absorption of the cyan derivatives of these pigments which allow this interconversion to be followed quantitatively. The aging data reported below were obtained by measurements of the accumulation and disappearance of methemoglobin by the method of Evelyn and Malloy in sterile hemoglobin solutions stored in sealed vials with a small amount of air space. A separate vial was opened for each determination and was then discarded. The aging test

Table 1.—Changes in Reducing Substances and Inorganic Phosphorus During Preparation of Hemoglobin Solutions

<table>
<thead>
<tr>
<th></th>
<th>Total reducing substances</th>
<th>Nonfermentable reducing substances</th>
<th>Inorganic P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>13.4</td>
<td>11.5</td>
<td>0.98</td>
</tr>
<tr>
<td>II</td>
<td>2.6</td>
<td>0.90</td>
<td>2.4</td>
</tr>
<tr>
<td>III</td>
<td>1.7</td>
<td>1.28</td>
<td>2.55</td>
</tr>
<tr>
<td>IV</td>
<td>1.7</td>
<td>0.81</td>
<td>3.05</td>
</tr>
<tr>
<td>V</td>
<td>1.5</td>
<td>0.48</td>
<td>2.61</td>
</tr>
<tr>
<td>VI</td>
<td>1.42</td>
<td>0.42</td>
<td>2.11</td>
</tr>
</tbody>
</table>

for a particular solution was considered to be completed when the accumulated methemoglobin had been reconverted to hemoglobin, or, when methemoglobin accumulation was not detected, upon the appearance of the typical grape-juice color of reduced hemoglobin.

The data to be presented are strictly comparable only when obtained from a single pool of cells. In table 1 it is evident that the amount of fermentable reducing substances in the cells obtained from the plasma laboratory was much less than in cells from a fresh bleeding, and at the same time, the inorganic phosphorus was elevated. It is well known that after exhaustion of the available substrate, red cells cannot be restored to their original state of metabolism. The status of the red cells to be used for preparation of the particular type of hemoglobin solution under discussion will, then, be very important in its effect on the properties of the solution. Uncertainty as to the exact state at which a given batch of cells might be, necessitated the finer comparisons being made only from a single lot of cells.

In the solutions to be described below, with the exception of that of figure 1, the procedure of preparation was as described above, with only the variations noted in each case. None of the solutions with the exception of that of figure 8 contained hexose diphosphate.
Figure 1 gives the aging at room temperature of a hemoglobin solution made by the method of Hamilton et al. Methemoglobin accumulated steadily. Such solutions examined after one year at \(-10\) °C. showed no accumulation of methemoglobin over that of the original solution. After two years of storage at \(-10\) °C., however, such solutions have from 30 to 50 per cent of their total pigment in the form of methemoglobin.

Figure 2 gives aging data of a typical solution prepared by the methods just described. In the lower curve the hemoglobin was completely reduced on the seventh day. At the time this particular study was made, the methemoglobin content was being determined at weekly intervals. Later studies with daily determinations have revealed no deviation from this curve. The upper curve demonstrates that during storage there was no loss in total pigment content. Examination of these solutions for the presence of reducing substances following conversion of the pigment to reduced hemoglobin has revealed a drop in reducing substances from the original 6 per cent to 0-3 per cent.

Figure 3 illustrates that when dextrose was used, both in washing the cells and in the final solution, but neither nicotinic acid amide nor ammonia were used, methemoglobin gradually accumulated and then disappeared with reconversion to reduced hemoglobin. If no dextrose was used, but both nicotinic acid amide and ammonia were used there was no reconversion of methemoglobin. If the cells were
Fig. 1—Aging at Room Temperature of a Hemoglobin Solution Prepared by the Methods Described Above.

- - Per cent of hemoglobin appearing as methemoglobin
- - - Grams per cent of total hemoglobin in the solution

Fig. 3.—Effect of Dextrose on the Aging of Hemoglobin Solutions at Room Temperature.

- - - Aging data for a hemoglobin solution in the preparation of which dextrose alone was used and from which the stroma was removed without adjustment of pH.

- - - Cells washed in 1 per cent sodium chloride solution but the solution made up as usual.

- - - Cells washed as usual but no dextrose added to the solution after washing.

- - - - No dextrose added at any stage of preparation.
washed in salt solution but the final solution contained dextrose reconversion of methemoglobin was again seen. If the cells were washed with 6 per cent dextrose solution but the solution was made up in physiological salt solution reconversion was slow and partial. These data indicated to us that the presence of dextrose in the final solution was essential, and its presence in the wash water desirable. It is interesting to note that maintenance of the cells and the hemoglobin con-

![Graph showing the effect of nicotinic acid amide on aging of hemoglobin solutions at room temperature.](image)

**Fig. 4.** Effect of Nicotinic Acid Amide on Aging of Hemoglobin Solutions at Room Temperature

- - - - Aging data for a solution made without addition of nicotinic acid amide at any stage.
- - - - 0.075 per cent nicotinic acid amide in final solution
- - - - 0.15 per cent nicotinic acid amide in final solution
- - - - 0.3 per cent nicotinic acid amide in final solution

stantly in the presence of dextrose alone will bring about reconversion of the methemoglobin. Three lots of cells were used in these preparations.

Figure 4 shows the effect of nicotinic acid amide on reconversion of methemoglobin to reduced hemoglobin. The upper curve shows aging data from a solution made without nicotinic acid amide at any stage. Reconversion of the methemoglobin formed was slow and incomplete. The three curves at the bottom of this graph represent a single hemoglobin solution, divided into 3 portions to which 0.075 per cent, 0.15 per cent and 0.3 per cent nicotinic acid amide were added respectively. Although as seen from figure 3 dextrose is essential, the efficacy of nicotinic acid amide is self evident.
Figure 5. The solid curve represents aging data obtained with a hemoglobin solution in which the neutralization of hydrochloric acid and adjustment to pH 7.3 was achieved with ammonium hydroxide. Neutralization with sodium hydroxide and the addition of ammonia was also effective. The two higher curves represent neutralization with sodium hydroxide and potassium carbonate with no ammonia addition. While not essential, ammonia is obviously advantageous to reconversion of methemoglobin to hemoglobin. Curves in which an attempt was made to evaluate the optimum amount of ammonia all coincided with the base line, and are not shown.

Figure 6. The use of an ion exchange agent made it seem likely that traces of metals essential to some of the enzyme systems might be removed. Mg²⁺ and Mn²⁺ ions are well known to be important to the action of some enzymes and it has been suggested that Mg²⁺, Mn²⁺ and Co²⁺ may have protective action against certain types of inhibition of enzyme systems. The central curve represents aging data obtained with a solution to which no metal ions were added. The dotted line represents another portion of the same solution to which Mg²⁺, Mn²⁺ and Co²⁺ ions...
were added. Reconversion was much quicker in this solution. To separate vials of the control solution, sterile solutions of manganese chloride alone, cobalt chloride alone and magnesium sulfate alone were injected. The aging data were similar for each set and are represented by the solid line curve. The long lag period preceding the appearance of methemoglobin was unexpected and its significance is not apparent to us.

Figure 7. A hemoglobin solution was made containing no nile blue. To one portion of this solution nile blue was added. The results are evident from this graph.
Figure 8. As noted above hexose diphosphate has been incorporated in some of the more recent hemoglobin solutions. This figure shows aging data obtained with a solution to one portion of which hexose diphosphate was added. This portion...
demonstrated a somewhat more active reconversion of methemoglobin than did the portion not containing hexose diphosphate.

Figure 9 demonstrates the importance of the type of filter pad used for sterilization of hemoglobin solutions. A hemoglobin solution was divided before sterilizing filtration, one portion being filtered through a Republic S6 pad and one portion being filtered through the finer meshed Republic S3 pad. The ratio of the volume of hemoglobin solution filtered to the pad surface was identical for each portion. It can readily be seen that the tighter pad removed something from the solution that was important for the reconversion of methemoglobin to hemoglobin.

**DISCUSSION**

It has been reported by all previous workers that the ability of the erythrocyte to utilize dextrose as a substrate is lost at the time of, or soon after, the disruption of the cell. The present data suggest that these previous findings must be qualified, for if the cells are maintained in the presence of dextrose during hemolysis, the ability to utilize dextrose continues. It is true that when the only precaution taken is maintenance of the cells in the presence of dextrose the utilization of dextrose after removal of the stroma is extremely slow. When, however, ammonia, a known stimulant of respiration$^{49}$ and nicotinic acid amide, a known protector of enzymic action,$^{71}$ are also present, utilization of dextrose and consumption of oxygen are appreciably accelerated. In the presence of dextrose, nicotinic acid amide and ammonia the additional contributions of added metals, nile blue and hexose diphosphate to the speed of consumption of oxygen from the solutions is relatively slight. The data suggest that the success of the preparation of the type of hemoglobin solution under discussion is dependent on the maintenance of a high state of metabolic activity in the red cell during the preparation. This approach may well lead to the development of solutions of still higher activity. One definite limitation to the activity of such solutions is suggested by the past emphasis on the importance of the cell structure for the activity of the cellular enzymes.$^{51}$ $^{64}$$^{66}$ $^{69}$ The data in figure 9 give indication that some of the structurally important elements are necessary to the highest activity and can be removed by further treatment.

The respiratory activity of the adult mammalian red cell is known to be small. It was first clearly demonstrated by the use of dyes of proper oxidation-reduction potential.$^{65}$ $^{72}$ Methylene blue, the dye most studied in this connection, not only catalyzes the action of the cellular enzyme systems but also catalyzes the formation of methemoglobin from hemoglobin. Kiese$^{48}$ pointed out that nile blue acted as a catalyst for the enzyme systems but did not catalyze the other reaction. It was used in these studies for that reason.

Since the entire cell residues from plasma have been used in these studies, it cannot be stated with accuracy that the white cells do not contribute to the self reduction noted. The work of Bird$^{74}$ suggests that their contribution would not be a major one.

Although the solutions under discussion readily utilize dextrose as a substrate, the fact that added hexose diphosphate further increases the rate of disappearance...
of oxygen is of importance since aging studies have indicated that the appearance of the reduced solutions improves in inverse proportion to the time of reduction. The presence of specific enzymes capable of bringing about the actions observed has not been proven in this study. The factors affecting the data reported are highly suggestive that this action is enzymic, however. It is hoped to pursue this study further at a later date. It is also hoped to find something of the nature of the by-products formed.

Summary

It is possible to prepare self-stabilizing solutions of hemoglobin from human erythrocytes by the use of dextrose, nicotinic acid amide and ammonia during the preparation and in the final solutions themselves. Co++, Mn++ and Mg++ ions, nile blue and hexose diphosphate contribute to the speed of stabilization of these solutions. Stabilization is obtained by the faculty of the solutions, presumably by enzymic action, to convert the hemoglobin to the reduced form and to maintain it in this form. The hemoglobin solutions described are suitable for intravenous administration.

Acknowledgment

We wish to acknowledge our deep indebtedness to Miss Lois Priester and to Mr. Edward Smith for their technical assistance in this work. We wish also to express our gratitude to the Biological Production Division and to the Biological Control Division of Sharp and Dohme for assistance in many phases of the work.

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