PREPARATION OF DRIED HEMOGLOBIN WITHOUT LOSS OF ACTIVITY*‡§

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(Received for publication, September 3, 1947)

As an extension of the study of the properties and characteristics of hemoglobin solutions prepared as detailed in an accompanying paper (2), further experiments were carried out to determine whether such solutions could be frozen and dried without change in hemoglobin activity, and to determine the stability of such dried preparations under selected conditions as compared with the stability of the original solutions.

Morrison and Hisey (3) have shown that if a hemoglobin solution is completely deoxygenated it can be dried without formation of methemoglobin, and Drabkin (4) has shown that hemoglobin can be dried by the "lyophile" procedure without denaturing the protein. In the present work we have utilized this principle, applying the technique of first freezing and then drying *in vacuo* as utilized with plasma in order to obtain the dried material in a honeycomb structure capable of being quickly redissolved. The procedure has not been applied to large scale production, but the conditions have been defined that are necessary for preparation by its means of dried hemoglobin of minimal methemoglobin content.

Material and Methods

The *vessel* in which the hemoglobin solution was deoxygenated was of the type shown in the upper part of Fig. 1. It had at one end a two-way cock, at the other end a three-way cock with an 8 cm. length of heavy wall capillary tubing drawn at the tip to permit its insertion into a rubber tip which fits into the cup of the Van Slyke-Neill manometric gas apparatus (5). A

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

‡ The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

§ Reported in abstract at meetings of the Federated Societies for Experimental Biology and Medicine, Atlantic City, New Jersey, March 14, 1946 (1).

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three-way stopcock permits connection of the capillary delivery tube with either the contents of the vessel or a side arm connecting tube. For analysis of hemoglobin solution in the vessel, the cleaned and dried tip is placed in the cup of the Van Slyke-Neill gas apparatus under mercury, and mercury from the chamber is run up through the capillary delivery tube and into the side arm tube, thereby effectively displacing all air from the delivery tube system. The stopcock is then turned to admit the sample to a calibration mark previously made on the chamber so that the volume analyzed is 2 ml.

Deoxygenation of Oxyhemoglobin Solutions.—By repeated shaking under diminished pressure, oxygenated hemoglobin solutions were deoxygenated. Removal of all but the slightest traces of oxygen was accomplished by alternately de-gassing the solutions and saturating them with oxygen-free nitrogen. The latter gas was prepared oxygen-free by shaking with and storage over an alkaline pyrogallol solution.[1] Usually 40 ml. of the solution to be deoxygenated were transferred to an 800 ml. vessel of the type shown in the upper part of Fig. 1. By means of a Hy-Vac pump, pressure within the vessel was reduced gradually while the vessel was vigorously shaken. When frothing became severe the pressure was kept constant until with very vigorous shaking the solution became foam-free after which further pressure reduction was effected. The final pressure was 10 mm. of mercury. The solution in the vessel was adjudged to be gas-free when a distinct metallic ring was emitted upon shaking. At this time the vessel was refilled to slightly above atmospheric pressure with oxygen-free nitrogen and the procedure repeated. After four to six repetitions of evacuation to a gas-free state, the vessel was refilled with nitrogen and a sample of solution was removed for analysis of oxygen content. During the process of deoxygenation, approximately 20 per cent of the water of the solution was evaporated, and the final hemoglobin concentration was 7 to 9 per cent or greater. If, upon analysis, the oxygen content was 0.3 volume per cent or less the solution was considered to be sufficiently deoxygenated for freezing; an analysis was made for total and active hemoglobin and the preparation was ready for freezing. The entire process of deoxygenation required an average of 4 hours of continued careful and watchful manipulation.

Attempts to replace mechanical removal of oxygen by enzymic reduction were not successful.

Freezing and Drying of Deoxygenated Hemoglobin—The deoxygenated hemoglobin solutions were transferred without contact with air to a mercury-filled vessel connected to a reservoir of mercury. The vessel was then connected by means of heavy walled rubber tubing to a 25 ml. ampule. The ampule and connecting system were evacuated and filled with oxygen-free nitrogen three times; then 5 ml. of hemoglobin solution were transferred from the vessel to the ampule. The heavy walled tubing was securely clamped and the ampule and tube removed from the system. The ampule was then immersed in a bath of dry ice and alcohol and rapidly rotated until the hemoglobin solution had frozen. After this the ampules were connected to the drying apparatus, the clamp removed from the rubber tubing, and the frozen preparation was dried for 4 to 5 hours at less than 20 microns pressure. Upon completion of drying, the evacuated ampules were sealed with an oxygen flame and the preparation stored at 4°C. until required for other testing.

Redissolving of Deoxygenated Dried Hemoglobin.—It was found that even momentary contact of the dried reduced hemoglobin with atmospheric oxygen resulted in measurable methemoglobin formation. It was therefore necessary to exclude air completely during resolution of the hemoglobin. Once it had been dissolved, as was found by Morrison and Hisey (3)

[1] The nitrogen commercially available in tanks contains sufficient oxygen to prevent obtaining the degree of deoxygenation required. It was therefore made oxygen-free in a reservoir of pyrogallol from which it was then used.

access of air did not cause measurable methemoglobin formation. The apparatus shown in Fig. 1 was used for dissolving the dried deoxygenated hemoglobin without contact of the dry preparation with air.

When oxygen-free water was used to dissolve the dried hemoglobin, 10 ml. of distilled water were made air-free in the chamber of the Van Slyke-Neill gas apparatus, then trans-

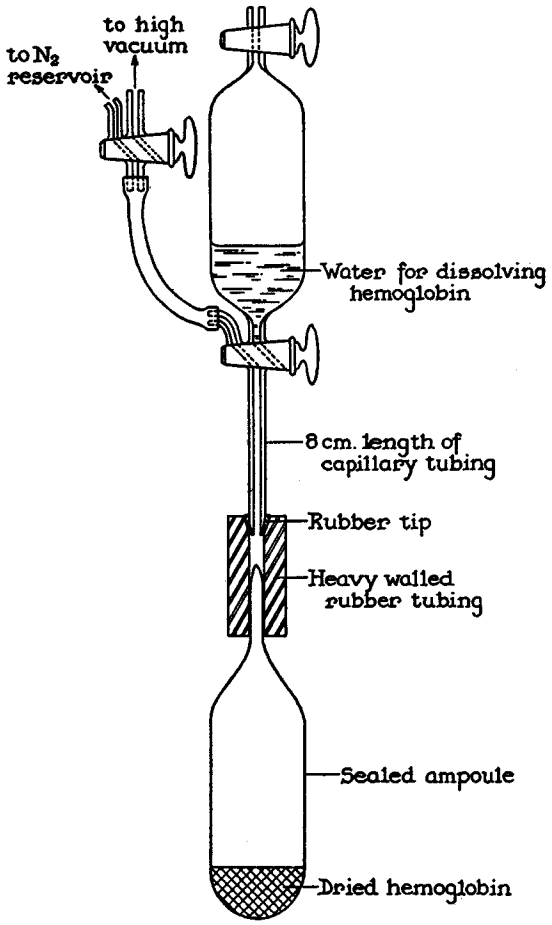

FIG. 1. Apparatus for dissolving dried hemoglobin without contact with air.

ferred to the upper vessel of Fig. 1 which had been previously evacuated to high vacuum and filled with oxygen-free nitrogen. The vessel was then connected with the sealed ampule by means of heavy walled rubber tubing as shown in Fig. 1. The dead space between ampule and vessel was alternately evacuated and filled with oxygen-free nitrogen to wash out the air. The cock of the vessel containing water was then turned to connect the vessel with the ampule, and the ampule tip was crushed by pressing the rubber tubing with heavy pliers. The water entered the evacuated ampule with considerable force, and solution was immediate. Without disconnecting, the apparatus was inverted and the solution of hemoglobin transferred from

ampule to vessel by alternate evacuation of the vessel and admission of nitrogen into the ampule. When all of the hemoglobin solution was in the vessel in an atmosphere of nitrogen, the vessel was disconnected. The tip was washed and dried through the side arm, and samples of the hemoglobin solution were measured directly into Van Slyke-Neill chambers for determination of oxygen content. The remainder of the solution was analyzed by the usual technique (6) for active and total hemoglobin, samples being measured from pipettes as usual (6).

In the experiments in which the dried hemoglobin was dissolved in air-saturated water, 10 ml. of the water were placed in the vessel (upper part of Fig. 1) without evacuating the vessel, and admitted to the ampule as described above, so that the dried hemoglobin *was covered with water before air entered the ampule.* Under these conditions, as shown by the last two columns of Fig. 4, resolution of the hemoglobin occurred without methemoglobin formation.

Freezing, Drying, and Redissolving of Oxygenated Hemoglobin.—Solutions of oxygenated hemoglobin were frozen in the usual manner in a bath of dry ice and alcohol, then dried, redissolved in water, and analyzed for total and active hemoglobin. The effects of varying quantities of added electrolytes were studied. In these experiments, the dried preparation was not stored, but was immediately redissolved and analyzed.

Analysis of Hemoglobin Solutions and Dried Preparations

Determination of Small Amounts of Oxygen in Solutions of Deoxygenated Hemoglobin.—In determining oxygen in almost completely deoxygenated hemoglobin solutions it was found necessary, needless to say, to avoid the slightest contact of air with the hemoglobin solution. For this reason samples could not be transferred to the manometric apparatus by the usual method of pipetting. The gasometric method of Van Slyke and Neill (5) for the determination of oxygen was therefore modified to permit transfer of measured samples of deoxygenated hemoglobin solutions directly from the vessel in which the deoxygenation was performed into the chamber of the manometric apparatus. The usual order of first measuring dilute ferricyanide solution into the chamber, extracting the air from it, and then measuring the blood sample, was of necessity reversed. The hemoglobin solution was first measured into the chamber, the sample being measured by a 2 ml. mark on the chamber; then 3 ml. of completely deaerated water from a mercury-sealed vessel (Fig. 2) was measured in, followed by 0.3 ml. of 32 per cent ferricyanide. In using this reversed procedure the chamber must be made completely air-free before admitting the deoxygenated sample.

Calibration of the chamber to receive a 2 ml. sample was effected by admitting into the chamber 2 ml. of water from a pipette provided with a rubber-ringed tip which was pressed through about 0.5 ml. of mercury in the cup of the chamber. The stopcock of the chamber was closed without admitting mercury from the cup so that the 2 ml. of water occupied the capillary below the cup and the chamber to a point a little above the usual 2 ml. mark. The level of the mercury meniscus was marked on the chamber.

Before analysis the chamber of the manometric apparatus was freed of air by extracting the dissolved gases from a few milliliters of water in the evacuated chamber and ejecting the evolved air and the water. Approximately 1 ml. of mercury from the chamber was then run up into the cup. The rubber-ringed tip of the vessel (upper part of Fig. 1) containing the deoxygenated hemoglobin solution was pressed into the bottom of the cup of the chamber under mercury. Mercury from the chamber was run up through the capillary delivery tube of the vessel (Fig. 1) and into its side arm, displacing air from the delivery tube. Then 2 ml. of the hemoglobin solution were admitted into the chamber to the temporary calibration mark made on the chamber, followed by 3 ml. of air-free water from the vessel shown in Fig. 2. The mercury was removed from the cup and two drops of caprylic alcohol were admitted into the capillary below the cup. Approximately 0.5 ml. of water was then placed in the cup and 0.3 ml. of the 32 per cent potassium ferricyanide was delivered into the chamber from a rubber-

tipped burette graduated in 0.01 ml., the tip of the burette being pressed under the water into the bottom of the cup above the Van Slyke-Neill chamber. The rest of the oxygen determination was carried through as described by Van Slyke and Neill (5).

Determination of Active Hemoglobin and Total Hemoglobin.—Active hemoglobin (capable of binding CO) and total hemoglobin were determined in the solution by the carbon monoxide capacity method as described by Van Slyke, Hiller, Weisiger, and Cruz (6).

Calculations.—The difference between the total hemoglobin content of the solutions and the active hemoglobin content is calculated as the inactive hemoglobin fraction hereafter designated as methemoglobin.

$$\text{Per cent active hemoglobin was calculated as } 100 \times \frac{\text{Active hemoglobin}}{\text{Total hemoglobin}}.$$

$$\text{Per cent deoxygenated hemoglobin was calculated as } 100 - \frac{O_2 \text{ content} \times 100}{\text{CO capacity}}.$$

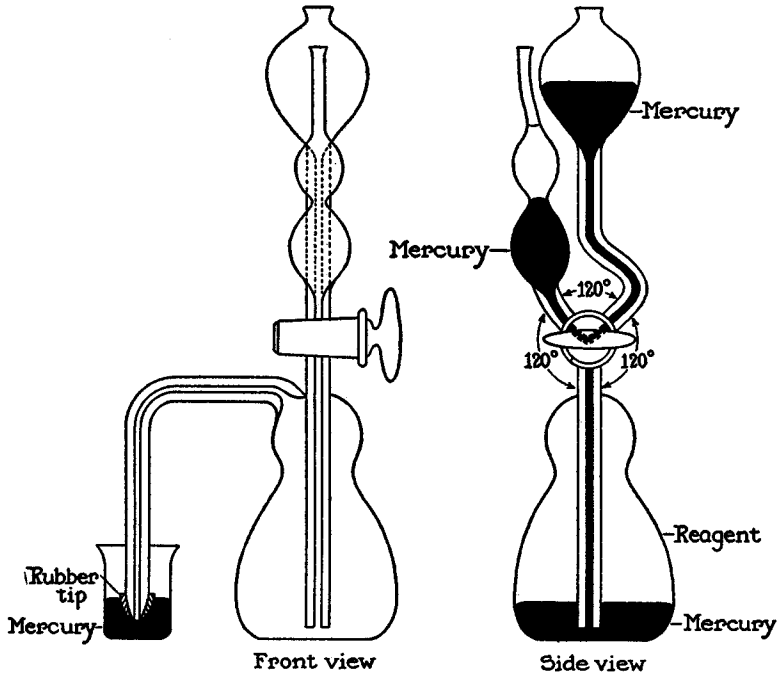

FIG. 2. Apparatus for storing air-free reagents over mercury and for delivering measured amounts into chamber of the Van Slyke-Neill gas apparatus.

Results with Oxygenated Hemoglobin Solutions

Effect of Freezing and Thawing.—Solutions of oxygenated hemoglobin which had been analyzed for active and total hemoglobin were frozen quickly by immersion in a bath of dry ice and alcohol, then permitted to thaw at room temperature and again analyzed for active hemoglobin. The results of such an experi-

ment are shown in Fig. 3. Freezing and thawing caused no change in the activity of oxyhemoglobin solutions.

Effect of Freezing and Drying Oxygenated Hemoglobin Solutions.—Solutions of oxygenated hemoglobin which had been analyzed for active and total hemoglobin were frozen, dried, redissolved in water, and again analyzed for total and active hemoglobin. The results obtained in a typical experiment of this kind are shown in Fig. 3. In all experiments of this nature, 20 to 30 per cent of the original active hemoglobin was changed to methemoglobin. Precautions taken to exclude air at time of redissolving did not change the activity. Exposure of the dried oxyhemoglobin to air for 3 minutes at room temperature and for 24 hours at 4°C. before dissolving did not alter the activity. The

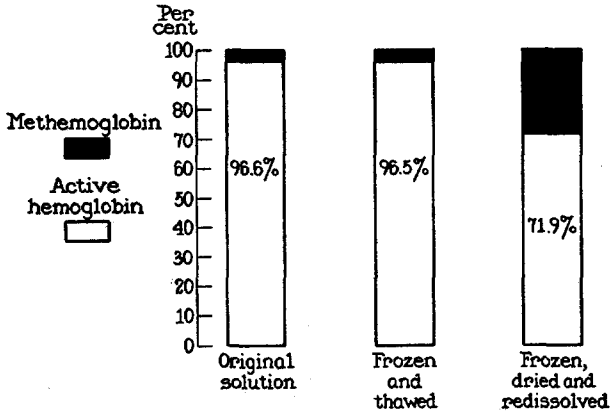

FIG. 3. Effect of freezing and drying on oxygen-binding capacity of oxyhemoglobin

addition of electrolytes to concentrations usually found in plasma slightly increased the yield of active hemoglobin. The addition of sodium oleate did not affect the formation of methemoglobin.

Results with Deoxygenated Hemoglobin Solutions

Effect of Deoxygenation on the Activity of Hemoglobin Solutions.—Deoxygenation of solutions of oxyhemoglobin caused no change in the activity measured by CO-binding capacity. The results of a typical experiment are shown in Fig. 4. In this experiment 99.7 per cent of the oxyhemoglobin was deoxygenated. The hemoglobin went through the procedure of repeated vacuum extraction of the gases without losing as much as 1 per cent of its CO-binding activity. In no such experiment was there a loss of activity greater than 1 per cent.

Effect of Freezing, Drying, and Redissolving of Deoxygenated Hemoglobin Solutions.—When dried preparations of deoxygenated hemoglobin kept *in*

vacuo were dissolved in oxygen-free water in an atmosphere of nitrogen the activity of the resulting hemoglobin solution was the same as that of the deoxygenated solution before freezing and drying, as shown in Fig. 4. Analyses of oxygen content showed that the resulting solutions were between 99 and 100 per cent deoxygenated.

When dried preparations of deoxygenated hemoglobin kept *in vacuo* were dissolved in water containing air, as described previously, in such a way that the air was removed from the space between the ampule and the vessel containing water before the ampule was opened, solution of the dried powder was immediate and resulted in a preparation of the same activity as that of the original solution of deoxygenated hemoglobin as shown in Figs. 4 and 5. The hemo-

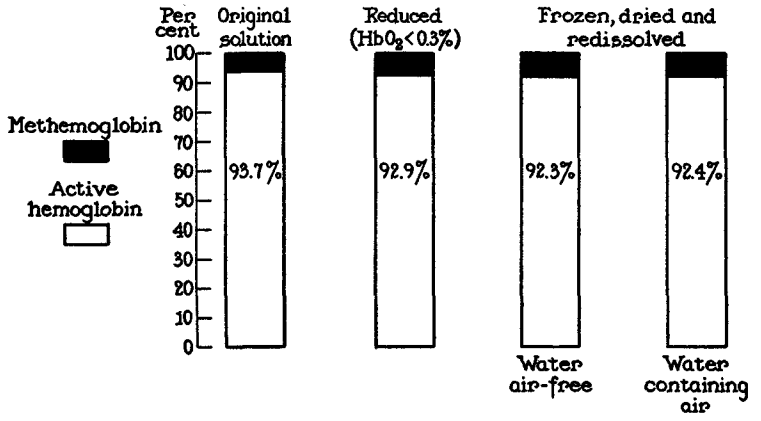

FIG. 4. Effect of freezing and drying on oxygen-binding capacity of reduced hemoglobin.

globin, after solution had been effected, could be exposed to either air or a stream of pure oxygen without any increase in the methemoglobin content of the solution. The results of three such experiments are shown in Table I.

When, however, the dried deoxygenated hemoglobin was exposed to air for 3 minutes before dissolving in water, the activity of the resulting solution was reduced by 33 per cent as shown in Fig. 5. This reduction in activity occurred whether the water was oxygen-free or saturated with air. When the dry product was exposed to air at 4°C. for 1 month before dissolving, the activity fell 60 per cent. Even momentary exposure of the dried deoxygenated hemoglobin to air at reduced pressure with subsequent rapid evacuation of admitted air, was followed by a very significant reduction in the active hemoglobin fraction amounting to approximately 10 per cent of the active hemoglobin originally present.

Once the dried deoxygenated hemoglobin had been dissolved, an increase in methemoglobin did not occur with exposure to air or oxygen (Table I) and the

concentration of active hemoglobin remained constant over a period of weeks if kept at temperatures of 4–6°C. All solutions dissolved by any of the three procedures used above were reanalyzed after a period of 3 weeks at 4–6°C.

TABLE I

Effect of Oxygenation of Redissolved Reduced, Frozen, and Dried Hemoglobin Solutions on Activity

State of solution	Oxygen content	Per cent deoxygenated	Total hemoglobin	Active hemoglobin	Activity
	vol. per cent	*per cent*	*gm. per 100 ml.*	*gm. per 100 ml.*	*per cent*
Before oxygenation	0.06	99.1	5.41	5.07	93.6
After oxygenation			5.43	5.03	92.6
Before oxygenation	0.02	99.8	6.13	5.81	94.8
After oxygenation			6.17	5.84	94.6
Before oxygenation	0.03	99.7	7.11	6.76	95.0
After oxygenation			7.16	6.82	95.4

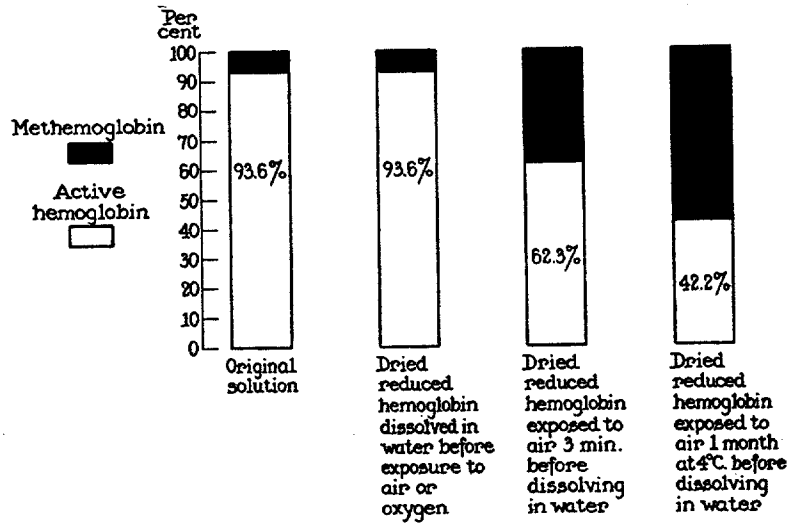

FIG. 5. Effect of air (oxygen) on oxygen-binding capacity of dry reduced hemoglobin.

and were always found to have the same quantity of active hemoglobin as when they were initially dissolved.

Stability of Dried Deoxygenated Hemoglobin Stored in Vacuo at Various Temperatures.—Ampules of dried deoxygenated hemoglobin sealed *in vacuo* were stored at 4°C. for periods of time up to 180 days, then dissolved in oxygen-free water and analyzed. In each instance there was no decrease in active hemo-

globin in any of the samples so tested over the entire 180 day period. One such experiment is shown in Fig. 6. Ampules stored at room temperature, which varied between 20° and 30°C., were removed at intervals and the dried hemoglobin was redissolved in oxygen-free water and analyzed. Again no deterioration of the product was noted with time. One such experiment in which the ampule had been stored for 180 days is shown in Fig. 6. The hemoglobin solution was found to be 99.7 per cent deoxygenated and had an activity identical with that of the original solution from which the dried product was prepared. An ampule stored at 38°C. for 1 month before dissolving was found to be 99 per cent deoxygenated, with no loss in activity. Another ampule stored at 38°C. for 92 days was found to be 87.9 per cent deoxygenated, with

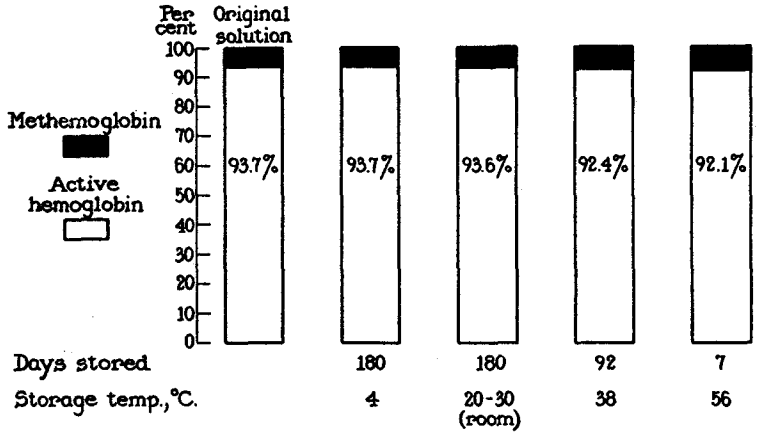

FIG. 6. Stability of dried reduced hemoglobin stored *in vacuo*.

an activity 98.8 per cent of the original solution. This is shown in Fig. 6. We believe the apparent decrease in the percentage of deoxygenated hemoglobin in this one instance was an artifact and occurred during the process of redissolving. One ampule stored at 56°C. for 7 days was found to be 99 per cent deoxygenated and had an activity of 98.3 per cent of the original solution (Fig. 6).

DISCUSSION

That crystalline oxyhemoglobin cannot be dried without loss of activity was first shown by Bohr (7) in 1892. Subsequently attempts to dry crystalline oxyhemoglobin reported by Hufner (8), Heidelberger (9), Van Slyke, Hastings, Heidelberger, and Neill (10), and Barcroft (11) were uniformly unsuccessful, as either a very large fraction or all of the hemoglobin was converted into methemoglobin by the procedure. Cannan and Redish (12) were unable to prevent from 40 to 60 per cent of the hemoglobin from being converted into methemo-

globin by drying either (1) "*in vacuo* from the frozen state," or (2) " by addition of anhydrous sodium sulfate to the crystal cake" or (3) "by air-drying a concentrated solution in a cellophane bag." Amberson, Jacobs, Hisey, and Monke (13) found on freezing and drying oxyhemoglobin solutions that methemoglobin constituted half or more of the total pigment. When the solutions were degassed prior to freezing, methemoglobin formation was definitely decreased, although far from being suppressed. When glucose was added to 5 per cent concentration to the hemoglobin solutions before freezing and drying, the amount of methemoglobin formation was reduced to about 20 per cent of the total pigment. Such solution, however, could not be completely dried. To secure a dry product only 1 or 2 per cent glucose was added and under these conditions up to 25 per cent of the hemoglobin changed to methemoglobin. Our experience with oxyhemoglobin has been similar.

In 1937 Morrison and Hisey (3) found that if hemoglobin solution was dried by distilling off the water *in vacuo* at 38°C., and was redissolved without contact with air the average loss of oxygen-binding activity was only 3 per cent. Morrison and Hisey demonstrated that oxyhemoglobin in the dry state forms methemoglobin much more rapidly than does oxyhemoglobin in solution, and that removal of oxygen, which they accomplished by distillation, is a prerequisite to drying without methemoglobin formation.

After this work was finished, Pennell, Smith, and Werkheiser (14) published a procedure for removing the oxygen from oxyhemoglobin solutions which appears better adapted to large scale preparations. These authors obtain complete deoxygenation by action of the enzymes in laked cells after addition of nicotinic acid and glucose.

SUMMARY

The technique for freezing, drying, and preserving *in vacuo* which is in common use for plasma can be successfully applied to hemoglobin solutions when the hemoglobin is first deoxygenated to the extent of 99.7 per cent or more.

In confirmation of Morrison and Hisey, the preliminary deoxygenation of the solution is found necessary to avoid formation of methemoglobin during drying. If a solution of oxyhemoglobin is frozen and dried, 20 to 30 per cent is changed to methemoglobin.

Deoxygnated hemoglobin dried and preserved *in vacuo* retained all its oxygen-binding activity for 180 days, when stored at temperatures from 4° to 30°C. Storage at 38°C. for 92 days, or at 56° for 7 days, caused no loss in activity. The dried hemoglobin had a foam structure which caused it to dissolve immediately upon contact with water.

Deoxygnated hemoglobin in the dry state was partly converted to methemoglobin by even momentary contact with oxygen. When, however, the deoxygnated hemoglobin was dissolved *before* it was exposed to air, the hemoglo-

bin in solution was relatively stable, and could be stored for months at 4° in contact with air without significant loss of activity.

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