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PRESERVATION OF RED CELLS IN THE FROZEN STATE WITH THE AID OF SUGARS AND MACROMOLECULAR ADDITIVES -- EFFECT OF LACTOSE, DEXTRAN AND ALBUMIN ON RECOVERY AND SURVIVAL OF FROZEN RED CELLS.

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ABSTRACT

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2. Title of Report: Preservation of Red Cells in the Frozen State with the Aid of Sugars and Macromolecular Additives - Effect of Lactose, Dextran and Albumin on Recovery and Survival of Frozen Red Cells

3. Principal Investigator: Max M. Strumia, M.D.

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Modification of red cells with lactose solution, removal of the modifying agent, and freezing in Teflon containers with the addition of low molecular weight dextran results in the recovery of 98.5% of the red cells; when albumin is used the recovery rate of red cells is 98.57%. A mean survival of 69.9% of red cells has been obtained 24 hours posttransfusion, when dextran was used as the additive after modification with lactose.

Dimensional changes, stability and O_2 dissociation of frozen red cells are described; the relationship of the mean corpuscular volume to survival is discussed.

There is evidence that the chromium tag of red cells, frozen with the addition of lactose followed by dextran, is labile; this suggests the necessity to re-evaluate the posttransfusion survival of red cells, frozen with the present technique, with the nonagglutinable cell method.

INTRODUCTION

Addition of dextrose-lactose mixtures to whole blood has been shown to exert a protective effect on erythrocytes during rapid freezing and storing at temperatures of about -93°C., rapid thawing and transfusion without further modification (1, 2). Solutions of human serum albumin and of dextran of different molecular weights, and of other macromolecular substances, have also been found to be highly effective in protecting human erythrocytes against the damage of freezing and thawing (3).

In the present work we have studied the effect of albumin and dextran, with and without previous modification with lactose, on the recovery and posttransfusion survival of red cells subjected to rapid freezing and thawing. Also studied was the effect of "Teflon" containers, and of the M.C.V. of the erythrocytes.

Potassium loss, stability, dimensional changes and O_2 dissociation of frozen red cells were determined; the complex interaction of lactose, dextran and albumin and the effect of cooling and warming velocities are discussed.

METHODS

Collection of Blood

Blood collected in the low dextrose, isotonic ACD solution (4)* was used within one to four days of collection. The ACD blood was centrifuged at 4°C. at 5000 r.p.m. for 5 minutes; enough plasma was removed to obtain an hematocrit of approximately 80. This material was mixed prior to freezing with modifying and additive solutions.

Modifying and Additive Solutions

Lactose as modifying agent was used in concentrations from 5 to 15% in saline solution, and was added to the packed red cells in equal volumes. In some experiment this mixture was frozen and thawed without further changes (1,2).

The packed red cells-lactose mixture was kept at room temperature for 5 minutes, centrifuged at 4°C. and at 5000 r.p.m. for 5 minutes. Enough supernatant was then removed to obtain an hematocrit of about 80.

In most instances lactose solutions were used only as a means to "modify" the red cells prior to freezing with macromolecular solutions: albumin or dextran.

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*The formula is:

	100 ml. contains	63 ml. contains
Trisodium citrate, dihydrate	2.55 gm.	1.6 gm.
Citric acid, monohydrate	0.80 gm.	0.5 gm.
Dextrose, anhydrous	1.20 gm.	0.756 gm.

pH 5.0

63 ml. are used for 500 ml. of blood

The "albumin" used was the "normal serum albumin (human) salt poor" processed by E. R. Squibb and Company, obtained through the American Red Cross as a 25% solution. Albumin was made isotonic by adding 5 ml. of 17% NaCl to 100 ml. of 25% solution. The albumin solution thus obtained (approximately 24% concentration) was added to the red cell suspension. Physiologic saline solution was added to the isotonic 24% solution when lower concentration was desired.

Dextran, molecular weight 40,000, obtained from the Pharmachem Corporation of Bethlehem, Pennsylvania, as a 30% solution in saline, was diluted with .85% sodium chloride solution to obtain the desired concentration. All solutions were sterilized by heating at 120°C. for 30 minutes.

The amount of albumin or dextran solution added to the lactose treated red cell mass is calculated to result in a final hematocrit of about 25%.

In another series of experiments packed red cells were mixed with albumin or dextran solutions without previous treatment with lactose.

Containers

The containers used for recovery studies were of a variety of sizes and material For screening in in vitro studies, cylindrical aluminum containers were used, with a thickness of approximately 0.25 mm. Layers of blood of varying thickness, from 1.3 3.2 mm., were obtained by varying the volume of blood. Flat metal containers were used, similar to those previously described (1), for larger quantities of blood. The were maintained in agitation during cooling and warming.

Containers made of "Teflon", 0.051 mm. in thickness, were also used.* The bags measured 10 x 30 cm. and were held in a metal frame to maintain a layer of blood of 0.2 to 0.4 cm. A more uniform thickness of blood was obtained by using channelled bags (Figure 1) or bags contained between two fluted copper plates, maintained at the proper distance. The blood in Teflon bags was frozen by immersion in a coolant maintained in very active motion.

Freezing and Thawing

Cooling has been obtained with a CO_2 ice-ethanol mixture at an average temperature of -73°C.; with Freon 11 cooled with liquid nitrogen to a temperature of -85 to -106°C., and with Freon 21, cooled to a temperature of -130°C. with liquid nitrogen. When liquid nitrogen was used directly as the coolant, the metal containers were coated with glycerol and methanol followed by "Santocell", (Monsanto Chemicals) (5).

Thawing was invariably carried out in the water bath at 40°C. for one or two minutes, according to volume. Motion was employed only with metal containers.

When frozen blood was not immediately thawed for use, it was stored at -90°C. ± 4.

Recovery

Measure of recovery requires the measure of the hematocrit, of the total hemoglobin and of the supernatant:

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*Obtained from the DuPont Company, Wilmington, Delaware

Measure of free hemoglobin. Initially the free hemoglobin was measured by a benzidine method (6); because of instability of the reagent, the carbonate method was adopted, using the Beckman B spectrophotometer. Spectral analysis demonstrated a hemoglobin peak at 420 mu with minimal absorption at 480 mu. A straight line was obtained when hemoglobin concentrations were plotted against O.D. in the range from 0.08 mg. to 10.0 mg. of hemoglobin per 100 ml. of 0.1% carbonate solution. Interfering substances had a constant absorption from 420 to 480 mu. They could thus be eliminated by taking the readings from diluted plasma at 420 and 480 mu.:

O.D. 420 mu. − O.D. 480 mu. X dilution factor = Hemoglobin mg.%

When mixtures of hemoglobin with lipemic (cloudy) plasma were made, recoveries of hemoglobin ranged from 100 to 106%. This means that hemoglobin concentrations as low as 2 mg.% could be measured with an error of ± 6%.

Measure of the hematocrit, hemoglobin and red cell count. The hematocrit was measured by a micro method previously described (7). The total hemoglobin was determined as oxyhemoglobin checked with the red cell iron content and O_2 capacity. The red cells were counted electronically (Coulter counter), checked daily by accurate chamber counting.

Recovery at each step is obtained as follows:

$$\frac{\frac{100 - \text{hematocrit}}{100} \times \text{supernatant Hb. gm./100 ml.} \times 100}{\text{Total Hb. gm./100 ml.}} = \text{Hb. loss \% of total}$$

In the measurement of losses of red cells through hemolysis at each step involving resuspension of red cells, carry over of free hemoglobin from one suspension to the next was taken into consideration. The amount of free hemoglobin carried over is represented by:

$$\frac{100 - \text{hematocrit X free Hb. \%}}{100}$$

This amount was subtracted from the amount of free hemoglobin measured in the successive step of the process. In this manner adding of the percent hemoglobin loss at each step gives a true value for the total hemoglobin loss in all the process, taking the initial value of the ACD blood as 100%. However, the value thus obtained would be slightly less than the amount of hemoglobin actually present in the supernatant at each step. The actual quantity of free hemoglobin present in the material used for transfusion is of considerable importance, and it has been measured.

Survival

For the measure of the survival of transfused red cells, a measure of the cell volume of the recipient is required which is determined independently of the transfusion. We have used routinely the T-1824 dye indirect method, as previously described (8).

$$\text{Expected 100\% specific Activity at 0 Time} = \frac{\text{Total counts in cells to be transfused}}{\text{Recipient's red cell volume (ml.)}}$$

$$\text{Observed specific activity at any time posttransfusion} = \frac{\text{Counts/minute/ml. whole blood}}{\text{Hematocrit}}$$

$$\text{Percentage survival at any time posttransfusion} = \frac{\text{100 X observed specific activity}}{\text{Expected 100\% specific activity at 0 time}}$$

The measure of the posttransfusion survival with radiochromium^{51} is carried out as follows:

One hundred ml. of ACD blood, not over 24 hours old, were centrifuged in a refrigerated centrifuge, plasma was removed to obtain an hematocrit of about 80, and the packed red cells were mixed with 500 microcuries of high specificity $Na_2Cr^{51}O4$. The total amount of chromium did not exceed one microgram/ml. of packed red cells.

The packed red cells were placed in a water bath at 37°C. for 20 minutes; the radioactivity uptake varied from 93 to 97%. The blood was then frozen and thawed as described. After mixing, 10 ml. of blood were transfused in a period of 8 to 12 seconds. The syringe was rinsed once by aspirating and re-injecting 2 or 3 ml. of the recipient's blood. The remainder of the tagged blood was used for radioactivity standard and other in vitro measurements.

Samples were taken at 1, 3, 5, 10 and 60 minutes, and at 24 hours posttransfusion. In many instances samples were taken on subsequent days, to determine the T 1/2. The hematocrit of the blood transfused and of each sample obtained was determined. Radioactivity was determined in 3 ml. samples, from which plasma was removed.

All experiments were autotransfusions performed in healthy young subjects; all but one were males.

O_2 Dissociation

Samples of blood were equilibrated in a rotating, constant water vapor pressure tonometer* at 37°C. with known mixture of nitrogen and oxygen. The carbon dioxide tension was kept at 40 mm. hemoglobin and the oxygen concentration was varied by means of two flowmeters.**

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* Obtained from Instrumentation Laboratory, 9 Galen Street, Boston 72, Massachusetts

**Fisher-Porter Company, Warminster, Pennsylvania.

After equilibration the blood samples were removed from the tonometer by means of a built-in syringe, which was capped immediately. An aliquot was removed anaerobically by injecting the blood into a rubber capped van-Slyke pipette containing mineral oil and used for the determination of the oxygen content and saturation. The blood remaining in the syringe was delivered into a tensiometer* for the direct determination of pH, pCO_2 and pO_2.

Oxyhemoglobin dissociation curves were constructed by plotting pO_2 venous oxygen saturation values and obtaining a best-fit line.

Measure of Relative Viscosity

The measure of the relative viscosity was carried out by a method previously described (9).

Sedimentation Rate

The sedimentation rate was measured with the Cutler method (10).

Pre and Post Freezing Agglutinability of Red Cells

Blood was drawn from four individuals, and the red cells from a portion were modified with lactose and frozen with the addition of dextran. The red cells, after thawing, were resuspended in saline, to a 5-10% concentration, after one to three washings with saline. Serial titrations were done by the same technicians, with one pipette. For the albumin titer, three drops of albumin solution were added to each tube after incubation and prior to centrifugation. The same vials of anti-A

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*Obtained from Instrumentation Laboratory, 9 Galen Street, Boston 72, Massachusetts.

serum*, saline anti-D serum**, and slide anti-D serum** were used throughout. Control included patient's cells, frozen and not frozen, versus patient's plasma in saline and albumin.

In addition to the standard hand procedure, the agglutinability of frozen and controlled cells was tested with the "Autotechnicon" apparatus, the amount of agglutination being read by a visible end point and by a point at which 50% of the cells were agglutinated.

RESULTS

General Considerations

The process of preservation of red cells at low temperatures may involve the following steps in vitro: collection of blood in an anticoagulant; centrifugation and removal of plasma; addition of a modifying agent, followed by centrifugation in most cases, removal of supernatant and mixing with an additive; freezing; storing in the frozen state; thawing; resuspension.

For the purpose of this paper, the term "modifying agent" is used to indicate a substance which improves the recovery or survival of red cells submitted to the process of freezing and thawing. It may, or may not, be removed and replaced, before freezing, by an additive which protects the red cells during the actual process of freezing and thawing.

Each of these procedures induces certain biochemical and dimensional changes in the red cells, and may result in damage and loss of cells.

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*From the Philadelphia Serum Exchange

**From the Dade Company

The losses in vitro may be purely mechanical, such as in the removal of supernatant fluid, or the result of hemolysis. The mechanical losses in the process of centrifugation and transfer can be reduced to a minimum with proper technique, and will not be reported in detail. In any case, they need not exceed 0.1%. Losses by hemolysis are by far the most important, since they generally involve damage to remaining, apparently intact cells. Additionally, large amounts of free hemoglobin may be detrimental to the recipient of the transfusion.

Recovery

Lactose followed by dextran. Packed red cells with an hematocrit of 85.5, obtained from ACD blood, were submitted to the action of 5% lactose, followed by centrifugation. The supernatant was removed, leaving packed red cells with an hematocrit of 76.2, to which 20% dextran was added, the resulting red cell suspension having an hematocrit of 24.6, hemoglobin of 9.3 gm./100 ml. Forty-five ml. of this red cells suspension were frozen at -80°C. in a Teflon bag, divided into three longitudinal pockets (see Figure 1). In all, three such bags were used, so that nine separate units from the same original material were available for study. The results are shown in Table I.

This procedure resulted in suspensions of red cells which were used for study of stability (resuspension) or for autotransfusion. The sum of the percent losses of hemoglobin at each step represents the true total loss of hemoglobin expressed in percent of the initial hemoglobin of the ACD blood. The concentration of lactose may vary from 5 to 15% without appreciable change in results. The velocity of cooling was 5.1°C./second, the coolant bath being -80°C.; the velocity of warming was 3.3°C./second, the warming bath being 40°C.

Lactose followed by albumin. The recovery of red cells was similarly determined when albumin was used as the additive. Packed red cells with an hematocrit of 87.8, obtained from ACD blood, were submitted to the action of 15% lactose, followed by centrifugation. The supernatant was removed, with a resultant hematocrit of 76.7; albumin solution in physiological saline was added, the resulting red cell suspension having an hematocrit of 23.4, hemoglobin of 9.8 gm./100 ml. The freezing was carried out exactly as described for the dextran treated cells; the results are shown in Table II.

Similar results have been obtained with lactose concentrations of 5 and 10%; the velocities of cooling and warming were the same as used in the previous experiment.

Lactose-Dextran and Lactose-Albumin Mixtures. The recovery of red cells after freezing and thawing was similarly tested when the red cells were added to a solution containing both lactose (5%) and dextran (20%) in physiological saline, having in view the possible elimination of one step.

As seen from Table III the rate of recovery is slightly but significantly higher than obtained with the red cells treated separately with lactose followed by dextran. Very similar results were obtained when packed red cells were frozen in a mixture of lactose 5%, albumin 24%.

Survival

Survival is defined as the amount of radioactivity of Cr^{51} tagged red cells remaining in circulation 24 hours after transfusion, expressed in percent of the 100% level calculated as previously described.

Forty-five autotransfusions of small quantities of blood have been given to determine the survival of frozen-thawed red cells; seventeen autotransfusions were carried out with blood frozen in metal containers, using various temperatures of cooling and various concentrations of additives. The results of these preliminary experiments can be summarized as follows: 11 units of blood were frozen with the aid of 15% lactose followed by 24% albumin, with an average recovery of 92.5% and a survival of 54.8%. Using 20% dextran in place of albumin the recovery averaged 95.5% and the survival 51.9% in six cases.

Using the experience obtained by these studies, later experiments were carried out with a more uniform technique employing Teflon bags 10 X 30 cm., without agitation of the material being frozen, but maintaining the cooling and warming fluids in motion.

Lactose followed by dextran. Plasma was removed from ACD blood to obtain an hematocrit averaging 80%; 5 to 15% lactose solution was added in equal volumes to the packed red cells, the supernatant was removed after five minutes, obtaining an hematocrit averaging 78%. Twenty percent low molecular weight dextran was added. The amount of blood frozen averaged 50 ml., the resulting thickness of the layer of blood was irregular. The temperature of freezing varied between -72°C. and -100°C. After thawing the resulting suspension of red cells was autotransfused without further modification and within one hour of thawing.

All recipients were young, healthy males, with a single female recipient; results are shown in Table IV. It will be noted that the recovery averaged 1.44% less than reported in Table I; this is due to the fact that freezing of all but one specimen in this series (#106) was carried out with Teflon bags without "channelling"

Under these conditions the thickness of the layers of blood is much more irregular, and optimal cooling and warming velocities are not obtained uniformly.

Variations in the initial concentrations of lactose (5-15%) are reflected in the final concentration of the suspension used for transfusion.

Lactose followed by albumin. Four units of blood were frozen and thawed by the same technique as the experiment with dextran but 24% albumin was used in place of the dextran. The temperature of the cooling bath varied from -70 to -95°C. Results are shown in Table V; the survival rate appears to be significantly below that obtained with the use of dextran.

Survival of frozen red cells following the first twenty-four hour loss. The survival in circulation of red cells following the initial 24 hour loss was investigated in 14 autotransfusions of frozen red cells. The results are shown in Table VI. Variations on the mean recovery and mean survival of cases shown in Table VI compared to those in Table IV are due to the fact that the procedure for freezing and thawing used in the cases reported in Table VI varied in details from that used to obtain the data of Table IV.

Freezing with addition of albumin or dextran alone. It was previously found (3) that the use of albumin alone as an additive for freezing of blood resulted in a survival definitely below that obtained when the red cells were first modified with the use of lactose followed by freezing with albumin. Three units of blood were frozen with the addition of albumin alone and two were frozen with the aid of dextran alone. The results shown in Table VII indicate that the survival of red cells frozen with the aid of albumin preceded by modification with lactose is significantly better than the survival of red cells frozen with addition of albumin alone;

dextran used alone results in a survival considerably below that obtained with dextran preceded by modification with lactose.

Lactose-dextran mixture and apparent competitive effect of lactose and albumin. Good recovery but poor survival is obtained when lactose and dextran are mixed together before the packed red cells are added. In a typical experiment a solution containing 5% lactose and 20% dextran was prepared and then to this solution packed red cells were added. Freezing was obtained in a Teflon bag at -91°C.; the recovery was 96.1 but the survival was only 23.1%.

Stability of Frozen Red Cells after Thawing and Resuspension

The stability of nine units of red cells subjected to freezing and thawing with lactose and dextran (see Table I) was studied. Immediately after thawing each unit of red cells was divided in three aliquots, and treated as follows: 1) allowed to remain undisturbed in the lactose-dextran solution; 2) resuspended in own plasma, in proportion of 1.9; 3) resuspended in saline solution in proportion 1:9. The high dilution was chosen to simulate the dilution effect of a transfusion in a 75 kg. man. Determination of the total and free hemoglobin, red cell count, hematocrit and recovery were carried out immediately after resuspension and 24 hours after storage. Results are shown in Table VIII.

The frozen thawed red cells are relatively stable when left in the original additive -- thus, with an immediate recovery of 98.5% (all losses included), the average recovery at 24 hours was 97.4% (S.D. 0.088) representing an additional loss of 1.1% (S.D. 0.2). Resuspension in autoplasma of red cells with the same post freezing recovery of 98.5% resulted in an immediate mean recovery of 87.8% (S.D. 0.82) and a 24 hour recovery of 80.6% (S.D. 2.1). With saline solution resuspension of the same red cells the immediate recovery was 77.7% (S.D. 1.3) and at 24 hours 71.7% (S.D. 2.3).

Resuspension in neutral citrate, low dextrose solution* resulted in an immediate recovery of 96.8%, the recovery immediately after freezing and thawing being 97.64%. At 24 hours the recovery of resuspended cells was 82.1%.

No relationship was found between recovery after resuspension in plasma and survival posttransfusion.

Dimensional Changes of Frozen Red Cells

Dimensional changes, expressed as M.C.V. have been reported in Tables I to V. The variations in the mean diameter of red cells, when modified with lactose and frozen with 20% dextran, is shown in Figure 2. The mean diameter of the frozen, thawed red cells (5.9 micra) is considerably below that of the fresh ACD cells (7.1 micra); the aspect of the frozen red cells, smeared and stained, is similar to that of the ACD cells, but the frozen red cells showed a wider range of diameter.

The use of the Coulter "Particle Size Distribution Plotter" was attempted, but the apparatus requires resuspension of frozen cells in saline or Eagle solution, in both of which the stability of frozen red cells is poor; the apparatus cannot distinguish well preserved cells from ghosts.

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* This solution is used essentially for preparation of red cell suspensions and washing of red cells:

Trisodium citrate, dihydrate	30.0 gm.
Citric acid, monohydrate	0.050 gm.
Dextrose, anhydrous	1.0 gm.
Water to	1000 ml.
pH after steam sterilization	7.0

Microscopically, the lactose treated cells are uniformly reduced in size, about one-third showing variably increased crenation and some being more definitely spherocytic.

Addition of dextran shows complete disappearance of crenation, the cells remaining smaller than normal but mostly with visible central depression. About ten percent appear "cup" shaped and a similar number are "drop" shaped.

Cells treated with lactose followed by albumin have a more uniform biconcave appearance than those treated with lactose followed by dextran.

Resuspension in plasma shows appearance of five to ten percent of large spheroidal forms and a few ghosts. Very few crenated forms remain. Resuspension in saline shows more uniform population, with fewer of the macrocytic forms.

When the aspect of red cells frozen and thawed is compared with red cells stored in ACD for several days, the significance of these changes is minimized, since the appearance of ACD stored red cells is very heterogeneous. There appears to be no significant relationship between survival and morphological changes revealed by microscopy of stained specimens.

Effect of the M.C.V. of Frozen Cells on Survival

The effect of the M.C.V. of red cells on their posttransfusion survival has previously been discussed. It was found that the effect was related to the nature of the mechanism producing the reduction of the M.C.V. (3).

The present studies indicate that optimal survival is obtained with a M.C.V. of less than 100; lower values of the M.C.V. are not necessarily related to improved recovery or survival. On the other hand, M.C.V. exceeding 100 is generally accompanied by lowered recovery and survival (Figure 3).

O_2 Dissociation Curve

We have observed that the oxygen equilibrium curve of one day old blood (in ACD) was nearly identical with that of fresh heparinized blood, when corrected in respect to pH. With storage there was a definite shift to the left in outdated ACD blood. ACD cells in fresh heparinized plasma and fresh heparinized cells suspended in ACD plasma, when corrected in respect to pH gave values which fell on the "normal" curve.

Figure 4 shows that red cells submitted to freezing and thawing with lactose followed by dextran behave more like 20 day old ACD blood than fresh heparinized blood. Further studies are in progress.

Cooling and Warming Velocities

Definitive conclusions concerning the optimal cooling and warming velocities have not been reached, due to the numerous changes in the technique. It appears from the work so far done that very fast cooling, as obtained with liquid N_2, with the small volumes of blood used results in recovery and survival less satisfactory than obtained with slower rates of cooling.

Fairly satisfactory results have been obtained with cooling rates between 7 and 8°C./second and the warming rates of 5 to 6°C./second. These figures cannot be considered optimal; the cooling velocity has been measured between -4 and -40°C. and the warming velocities between -40 and 0°C. Figure 5 shows a typical temperature curve obtained from the middle channel of three channels of a Teflon bag during freezing and thawing of 50 ml. of blood, resulting in optimal recovery.

Effect of Addition of Dextran or Albumin on the Viscosity of the Blood and on the Sedimentation Rate of Red Cells

Resuspension of packed ACD red cells in dextran and albumin solutions in saline greatly increases the viscosity. (Figure 6).

Thus, the relative viscosity of ACD blood with an hematocrit of 32.7 was 2.3 centistokes; when the red cells were suspended in 20% dextran with the same hematocrit, the relative viscosity was 14.4 centistokes. Likewise, the relative viscosity of ACD blood with an hematocrit of 30.5 was 2.2 centistokes; when the red cells were suspended in 24% albumin with the same hematocrit, the relative viscosity was 5.7 centistokes.

The rate of sedimentation of fresh red cells in ACD solution is 7 mm./hour. When packed red cells with an hematocrit of 80 are resuspended in solutions of albumin of 3 to 24% or dextran of 3 to 20%, the rate of sedimentation is 1.2 mm./hour. The same rate of sedimentation of red cells is obtained when packed red cells are suspended in saline solution. In each instance, the retardation of sedimentaion of red cells is due to the lack of plasma globulins, essentially of fibrinogen.

Agglutinability of Frozen Red Cells

The results of the standard agglutinability test, which included red cells A_1 Rh positive, A_2 Rh positive and two samples of O positive Rh cells indicated no difference between the frozen red cells and the control red cells. The study made with the "Autotechnicon" apparatus showed slight decrease between frozen and control red cells.

The controls used with the autotechnicon studies included pure albumin solutions versus a mixture of cells frozen and not frozen (see Table IX).

DISCUSSION

In assessing the possible benefits of any attempt to improve red cell preservation, the end use of the material must be defined. It was previously shown that the simple addition of lactose-dextran mixtures to whole blood permits freezing and storing at low temperatures for a period of at least three years, with survival 24 hours posttransfusion averaging 77% of the original amount of red cells obtained from the donor (2). When large quantities of such red cells need be transfused, consideration must be given to lactose toxicity. Transfusions of blood stored with addition of lactose have shown that 15 gm. of lactose given at one time are entirely well tolerated (11); not much is known about the toxicity of lactose in man. However, removal by centrifugation of the supernatant plasma-lactose-dextran solution would permit transfusion of several units of blood thus preserved. Except under special conditions, this simple operation would not entail specialized apparatus or a great deal of time.

There are, however, conditions in which it would be desirable to transfuse blood, preserved at low temperature, without need of any preparation other than thawing. Obviously, this would apply to military medicine and to any form of massive casualties.

The primary purpose of the present study was to find additives which have a protective effect on red cells during the process of freezing and thawing but which are non-toxic to the human, the final goal being the development of a technique which would permit the transfusion of frozen blood after thawing without additional preparation, or would yield cells which after thawing could be resuspended and stored for a period of time with conventional refrigeration. We also wished to determine the relationship between cell size expressed as M.C.V. and the rate of survival, to obtain a method for in vitro screening.

Teflon containers were finally chosen because of the simplicity of technique, the stability of the material and the uniformity of the high recovery rate.

We did not fully investigate the stability of the frozen red cells beyond 24 hours; the stability in media other than saline solution and plasma; the effect of the cooling and warming rates; the effect of shaking during freezing and thawing the stability of the red cells after prolonged storage at low temperature.

Results indicate that dextran, and to a lesser extent albumin, possess good protective properties for red cells, and that the red cells thus processed have fair posttransfusion survival rate and have an oxygen dissociated curve comparable to stored ACD blood. However, it would appear that both purified human albumin and dextran possess such properties only when added to red cells which have been previously modified with lactose. It remained to be explained why with the high rate of recovery of red cells frozen and thawed with albumin or dextran alone, the posttransfusion survival should be poor. (See Table VII) Likewise, it is difficult to explain why good recovery but poor survival was obtained when lactose and dextran were mixed together before adding the packed red cells.

Possible explanations of this phenomenon have been investigated. In one experiment red cells from ACD blood were washed three times with cold saline and then submitted to the effect of lactose, followed by freezing and thawing with dextran. The recovery was 97.0 and that of control red cells from the same unit of blood treated with lactose without previous washing was 96.6. However, the M.C.V. at the end of the process was found to be 80 for the washed red cells and 92 for the unwashed control cells. This suggested that the presence of plasma proteins lessens the effectiveness of lactose in controlling the re-entry of water in the red cells.

It was also observed that red cells swell more when resuspended in plasma than when resuspended in salt solution after thawing (see Table VIII). This phenomenon could be attributed to a change of the cells' surface induced by lactose, which resulted in a diminished permeability of the red cells to water after thawing, this effect being apparently reduced in the presence of albumin and of dextran. The increased red cell size in turn was considered as decreasing the chances for survival.

Subsequent experiments, however, led us to consider the possibility that the differences noted between recovery and survival might simply be due to a variable degree of stability of the chromium tag in the presence of various additives. In a typical experiment packed red cells from ACD blood were tagged with Cr^{51}, treated with lactose followed by dextran and then submitted to the standard procedure of freezing and thawing. The red cells were then twice washed with cold neutral citrate solution and resuspended with the same solution. The hemoglobin of the original tagged red cells, before additives, freezing and thawing, had a specific activity of 4,500,865 counts/1 gram of hemoglobin. The hemoglobin of the supernatant after resuspension in neutral citrate dextrose solution had a specific activity of 3,185,900/1 gm. of hemoglobin, indicating the probable presence of free chromium.

In a similar experiment Cr^{51} tagging was carried out on the packed red cells obtained after freezing and thawing, but before washing. After washing the loss of hemoglobin of the intact red cells averaged 12.8%; the radioactivity loss was 24.3%. An in vivo experiment was set up to simulate the results of the in vitro study. The red cells were frozen and thawed using lactose followed by dextran exactly as the cases reported on Table IV, except that tagging of red cells was done after freezing and thawing. The recovery was 96.13%; the survival was only 43, or nearly 5 S.D. less than the mean obtained when tagging was done on the packed ACD red cells before freezing and thawing.

These results suggest the necessity to re-evaluate the posttransfusion survival by a technique other than the Cr^{51} tagging. Preliminary studies indicate that the nonagglutinable red cell method using an automatic apparatus is well applicable to transfusions from 100 ml. to 500 ml. These studies are under way.

SUMMARY

Dextran and albumin exert a protective action for red cells during freezing and thawing, with a recovery rate of 98.5 and 98.57 respectively. A mean survival of 69.14 of red cells was obtained 24 hours posttransfusion when the red cells were previously treated with lactose.

There is evidence that under the experimental conditions, the Cr^{51} tag is labile. The posttransfusion survival needs to be determined by other methods, such as the nonagglutinable cell technique.

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	ACD BLOOD	5% LACTOSE ADDED	20% DEXTRAN ADDED	RESULTS AFTER FREEZING AND THAWING										
				1	2	3	4	5	6	7	8	9	AV.	S.D.
Hb. gm./100 ml.	15.6	14.78	9.3											
Hematocrit	48.2	36.5	24.6											
RBC x $10^6/mm^3$	4.92	4.66	2.85											
M.C.V.	98	78	86											
Free Hb. gm./100 ml.	.005	.055	.064	.26	.22	.26	.26	.24	.26	.24	.27	.24	.25	.15
Hb. lost, % of initial	.02	.23	.31	.95	.77	.97	.95	.84	.97	.85	1.02	.89	.91	.09
Recovery %	99.98	99.77	99.69	98.5	98.7	98.5	98.5	98.6	98.5	98.6	98.4	98.5	98.5	.1
Lactose gm./100 ml.		3.94	.28											
Dextran gm./100 ml.			18.8											

Table I. Recovery of red cells after modification with lactose, and freezing and thawing with the aid of dextran.

	ACD BLOOD	15% LACTOSE ADDED	24% ALBUMIN ADDED	RESULTS AFTER FREEZING AND THAWING										
				1	2	3	4	5	6	7	8	9	AV.	S.D.
Hb. gm./100 ml.	12.7	13.5	9.8											
Hematocrit	40.3	30.9	23.4											
RBC x $10^6/mm^3$	4.5	5.49	3.61											
M.C.V.	90	56	65											
Free Hb. gm./100 ml.	0	0	.018	.256	.256	.256	.237	.237	.219	.201	.237	.237	.237	.017
Hb. lost % of initial	0	0	.11	1.44	1.43	1.43	1.31	1.32	1.22	1.1	1.32	1.33	1.37	.11
Recovery %	100	100	99.89	98.45	98.46	98.46	98.58	98.57	98.67	98.79	98.57	98.56	98.57	.1
Lactose gm./100 ml.		10.85	1.15											
Albumin gm./100 ml.			21.57											

Table II. Recovery of red cells after modification with lactose and freezing and thawing with the aid of albumin.

	ACD BLOOD	5% LACTOSE 20% DEXTRAN ADDED	RESULTS AFTER FREEZING AND THAWING										
			1	2	3	4	5	6	7	8	9	AV.	S.D.
Hb., gm./100 ml.	13.0	7.1											
Hematocrit	41.2	17.5											
RBC x $10^6/mm^3$	4.56	2.52											
M.C.V.	90	69											
Free Hb. gm./100 ml.	0	0	.056	.130	.075	.038	.130	.038	.019	.038	.038	.062	.039
Hb. lost, % of initial	0	0	.31	.81	.47	.24	.82	.24	.12	.24	.24	.39	.25
Recovery %	100	100	99.69	99.19	99.53	99.76	99.18	99.76	99.88	99.76	99.76	99.61	.25
Lactose gm./100 ml.		4.0											
Dextran gm./100 ml.		15.38											

Table III. Recovery of red cells frozen with the aid of a mixture of lactose and dextran.

NO	PRE-FREEZING LOSSES %	LOSSES DUE TO F - T %	M.C.V. AFTER FREEZING	RECOVERY	SURVIVAL	DATA ON BLOOD TRANSFUSED					
						TOTAL Hb. gm.%	FREE Hb. gm.%	Hmct.	LACTOSE	DEXTRAN	K MEQ/L
87	.75	2.23	79	97.02	78.1	9.2	.40	25.1	.80	19.0	
88	.18	1.28	84	98.54	66.9	10.3	.23	26.5	.99	19.2	
89	.31	3.31	83	96.38	61.1	9.6	.46	24.6	.84	19.57	
90	.13	1.44	73	98.43	75.2	9.0	.198	24.4	.76	19.4	18.7
91	.20	2.18	86	97.62	75.6	10.2	.32	25.5	.62	19.71	17.6
92	.22	4.43	91	95.35	66.1	8.8	.54	21.1	.69	19.09	13.5
95	.33	2.47	100	97.2	72.9	10.7	.41	27.0	.88	19.18	14.8
99	.69	3.56	88	95.75	60.0	9.0	.58	22.8	.16	19.2	12.5
100	1.15	2.59	95	96.26	67.9	9.3	.46	24.0	.26	18.4	14.0
101	.25	4.14	84	95.61	69.9	9.5	.60	23.1	.36	19.1	14.5
103	.11	1.87	86	98.03	65.1	9.9	.29	25.7	.37	18.62	14.1
106	.07	1.44	72	98.49	70.9	9.75	.30	25.7	.89	19.12	14.0
Mean	.37	2.578	85	97.06	69.14	9.6	.40	24.6	.64	19.13	14.9
S.D.±	.314	1.036	7.2	1.13	5.45	.55	.13	1.6	.27	.34	1.8

F - T = Freezing and thawing
Lactose = % Lactose in supernatant
Dextran = % Dextran in supernatant

Table IV. Recovery and survival of 12 units of red cells frozen with the aid of lactose followed by the addition of dextran and autotransfused.

NO	PRE-FREEZING LOSSES %	LOSSES DUE TO F - T %	M.C.V. AFTER FREEZING	RECOVERY	SURVIVAL	DATA ON BLOOD TRANSFUSED					
						TOTAL Hb. gm.%	FREE Hb. gm.%	Hmct.	LACTOSE	ALBUMIN	K MEQ/L
96	.23	2.33	106	97.44	69.3	9.5	.40	24.9	.91	22.81	19.6
97	.22	2.75	84	97.03	57.2	10.4	.56	25.5	.91	22.3	19.9
98	.36	1.49	86	98.15	61.2	11.1	.36	29.3	.87	23.4	20.6
107	.008	2.1	78	97.89	51.2	10.5	.44	25.0	1.13	21.4	18.9
Mean	.20	2.17	89	97.63	62.2	10.4	.43	26.2	.96	22.5	19.8
S.D.	.13	.45	10	.43	4.4	.57	.075	1.8	.1	.73	.61

F - T = Freezing and thawing
Lactose = % Lactose in supernatant
Albumin = % Albumin in supernatant

Table V. Recovery and survival of 4 units of red cells frozen with the aid of lactose followed by the addition of albumin and autotransfused.

NO	ADDITIVE	RECOVERY	SURVIVAL	T 1/2
88	Dextran 20%	98.5	66.9	24
89	"	96.4	61.1	20
90	"	98.4	75.2	24
91	"	97.6	75.6	23
92	"	95.4	66.1	22
95	"	97.2	72.9	17
99	"	95.8	60.0	19
100	"	96.3	67.9	20
101	"	95.6	69.9	25
103	"	98.0	65.1	20
105	"	96.1	55.0	23
Mean		96.8	66.9	22.0
S.D.		1.09	6.3	5.3
96	Albumin 24%	97.4	69.3	31
97	"	97.0	57.2	32
98	"	98.2	61.2	31
Mean		97.5	62.6	31

Table VI. Recovery, 24 hour survival and T 1/2 of frozen red cells, transfused.

NO	ADDITIVE	RECOVERY	SURVIVAL
59A	Albumin 24%	95	42.5
59B	Albumin 20%	89	56.7
60	Albumin 24%	96	34.0
62	Dextran 20%	83	4.9
75	Dextran 20%	92.1	21.2

Table VII. Effect of freezing of blood with albumin or dextran alone on recovery and survival.

		UNCHANGED AFTER THAWING		RESUSPENDED			
				PLASMA		SALINE S.	
		AV.	S.D.	AV.	S.D.	AV.	S.D.
M.C.V.	IMMEDIATELY AFTER THAWING	85	2.6	99	7.5	94	6.8
	24 HOURS	83	3.1	88	6.1	89	5.2
RECOVERY	IMMEDIATELY AFTER THAWING	98.5	.088	87.8	0.82	77.7	1.3
	24 HOURS	97.4	.2	80.6	2.1	71.7	2.3

Table VIII. Mean corpuscular volume and recovery of 9 units of red cells frozen with the aid of lactose followed by dextran, maintained in the additive solution or resuspended in plasma and saline immediately after thawing and after 24 hours storage at $4^{\circ}C$.

	ANTI-A			ANTI-D SALINE		ANTI-D SLIDE SERUM			ANTI-D SLIDE SERUM CELLS WASHED 3X AUTOA	
	MANUAL	AUTOA		MANUAL	AUTOA	MANUAL CELLS WASHED 3X	AUTOA CELLS WASHED 1X			
		END	50% POINT				END	50% POINT	END	50% POINT
P.S. A_1 +										
N-F	256	256	64	-	-	128	-	-	2048 No end	1024
F	512	128	64	-	-	64	-	-	2048 No end	1024
H.M. A_2 +										
N-F	256	64	16	-	-	64	-	-	2048 No end	1024
F	256	32	16	-	-	64	-	-	2048 No end	1024
M.S. O +										
N-F	-	-	-	16	-	-	2048 512 No end		2048	2048
F	-	-	-	16	-	-	2048 No end		512	256
V.DeP O +										
N-F	-	-	-	16	-	-	2048 2048 No end		-	-
F	-	-	-	16	-	-	1024 512		-	-

N-F = Not frozen

F = Frozen

Table IX. Agglutinability of frozen and not frozen control cells.

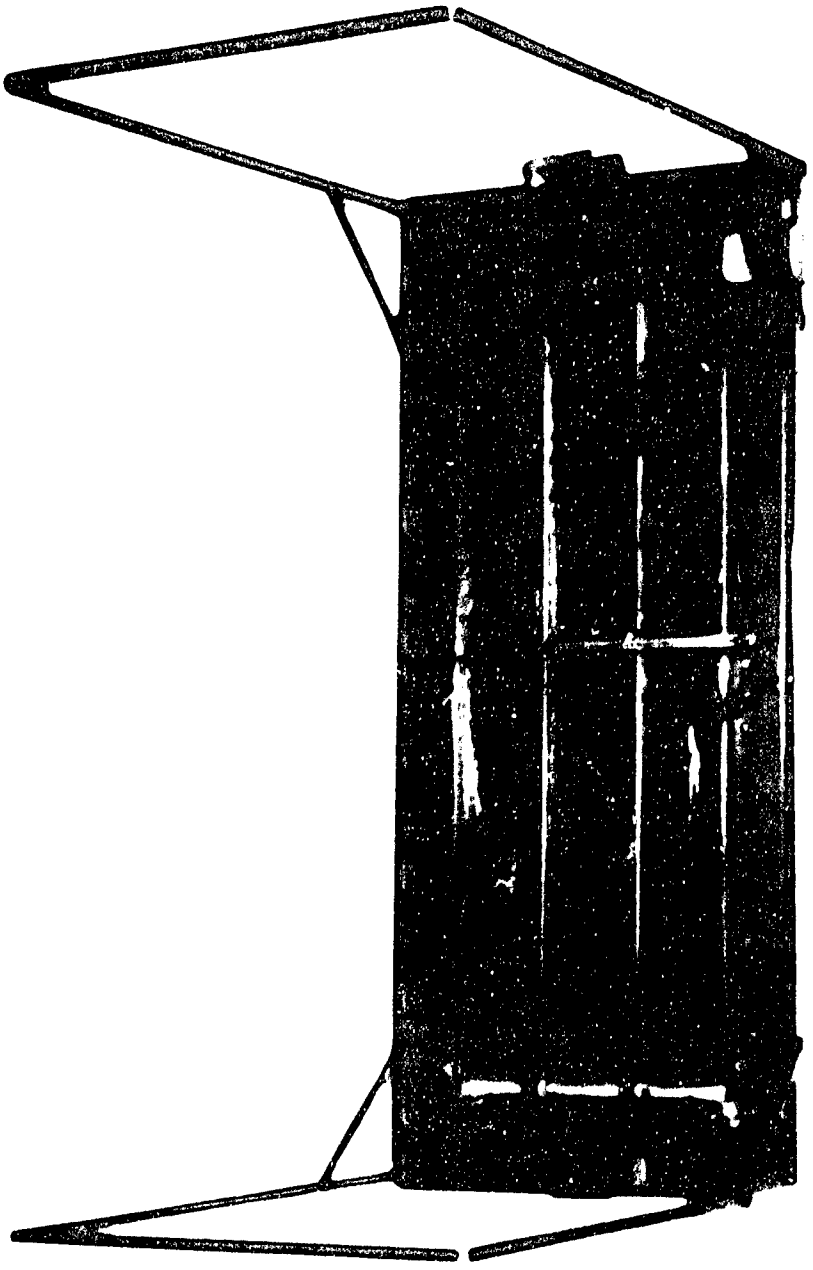
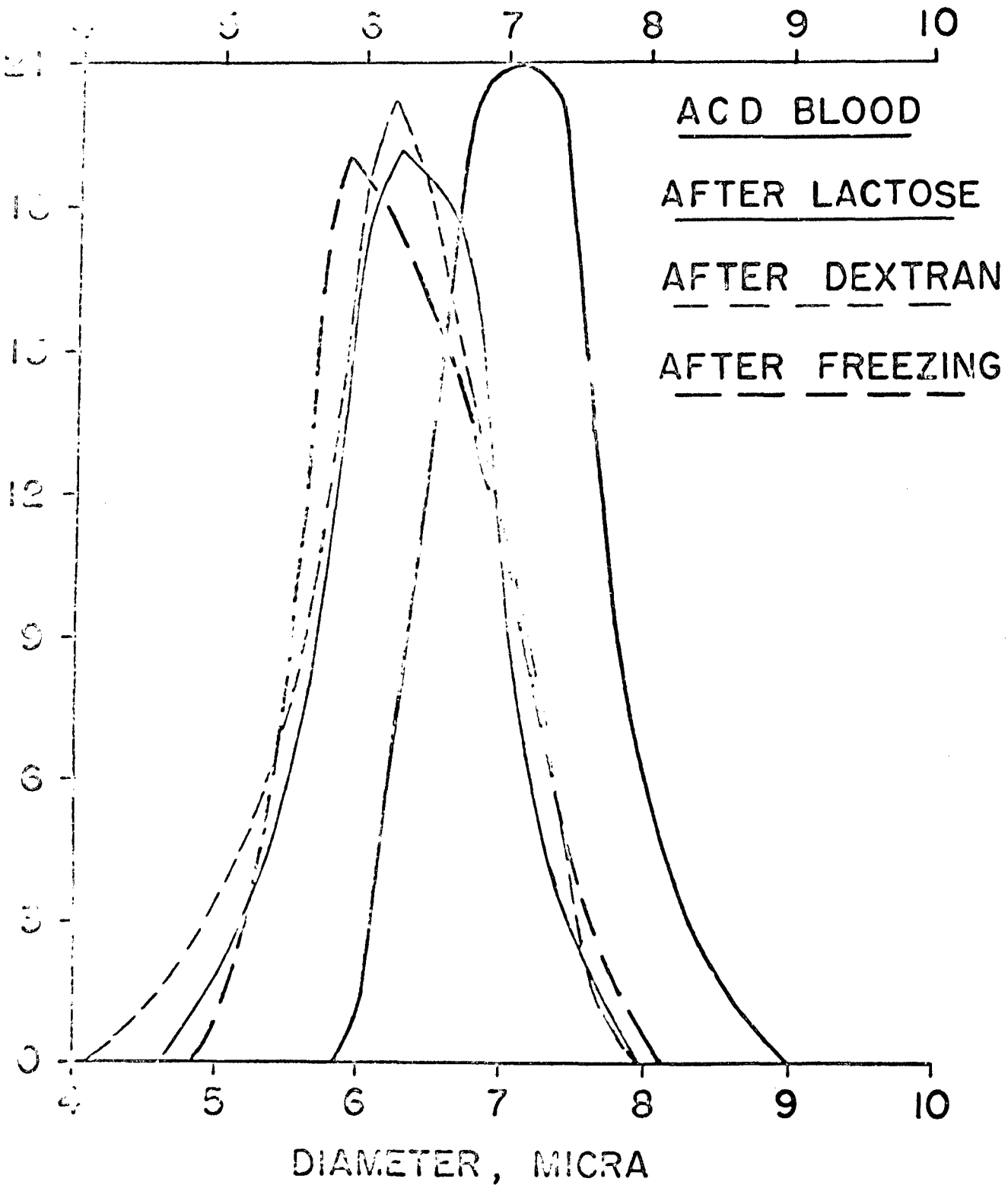
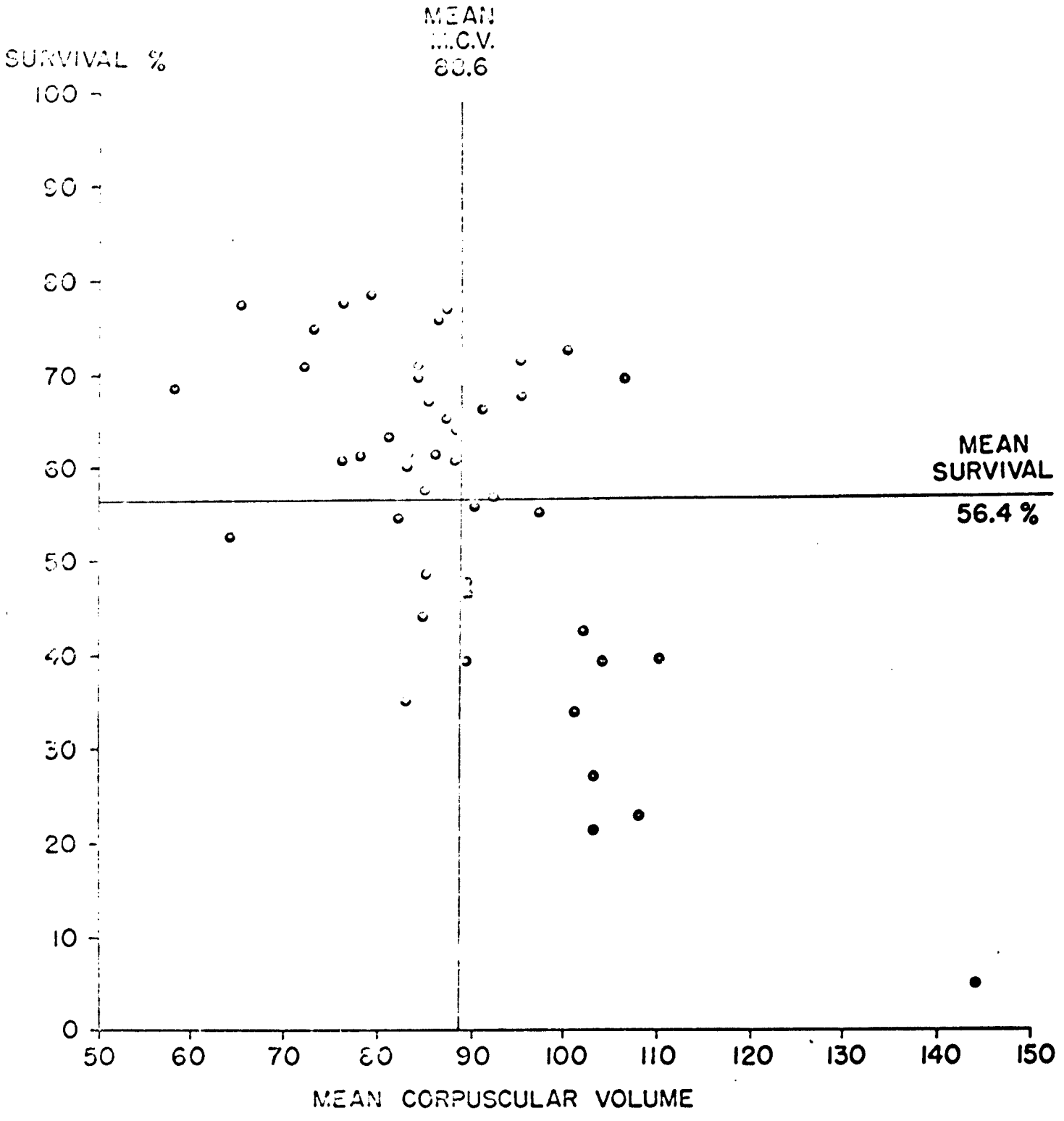
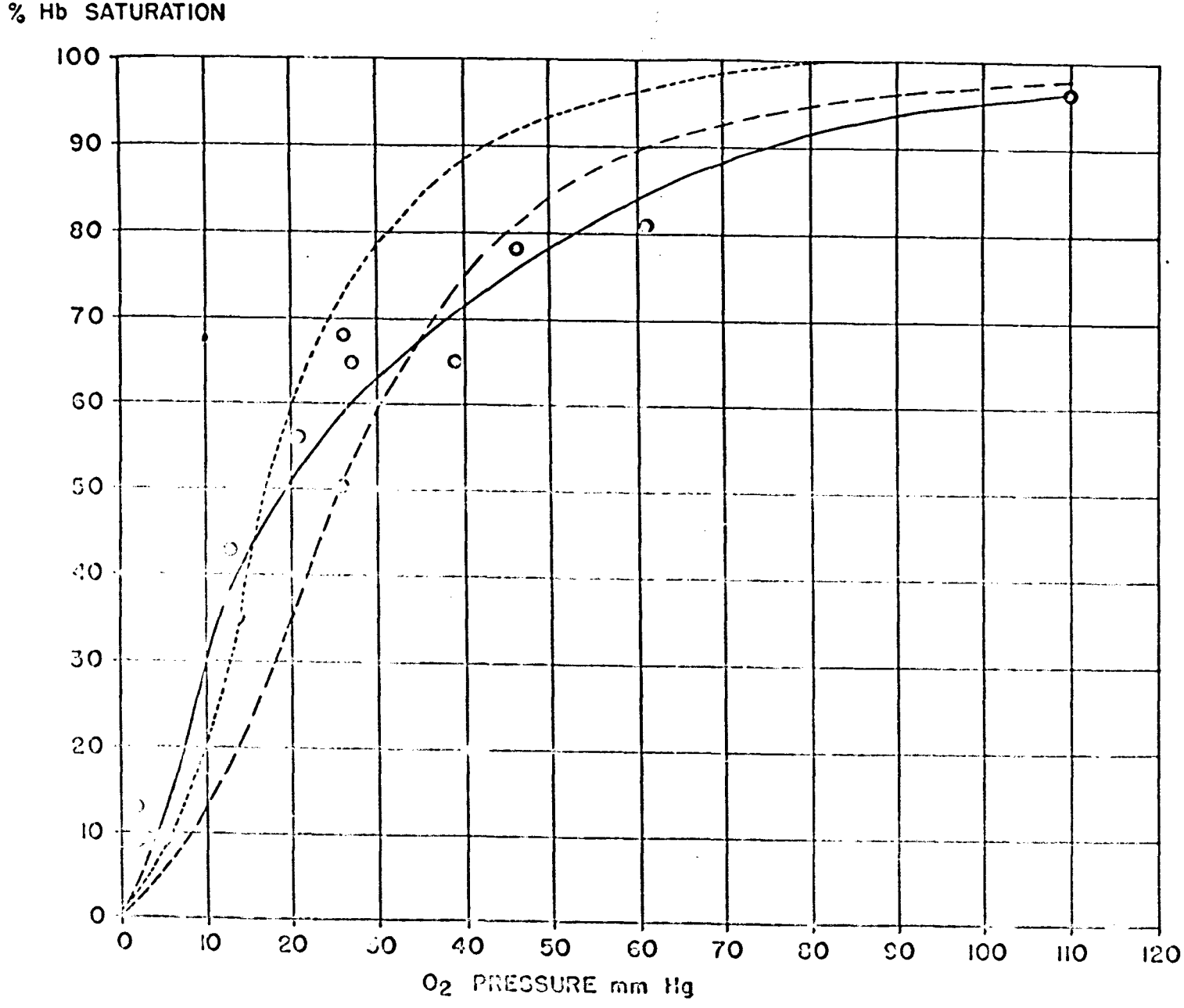


FIG. 1


ACD BLOOD
AFTER LACTOSE
AFTER DEXTRAN
AFTER FREEZING
4
5
6
7
8
9
10
DIAMETER, MICRA


MEAN
M.C.V.
88.6
SURVIVAL %
100
90
80
70
60
50
40
30
20
10
0
MEAN
SURVIVAL
56.4 %
50
60
70
80
90
100
110
120
130
140
150
MEAN CORPUSCULAR VOLUME

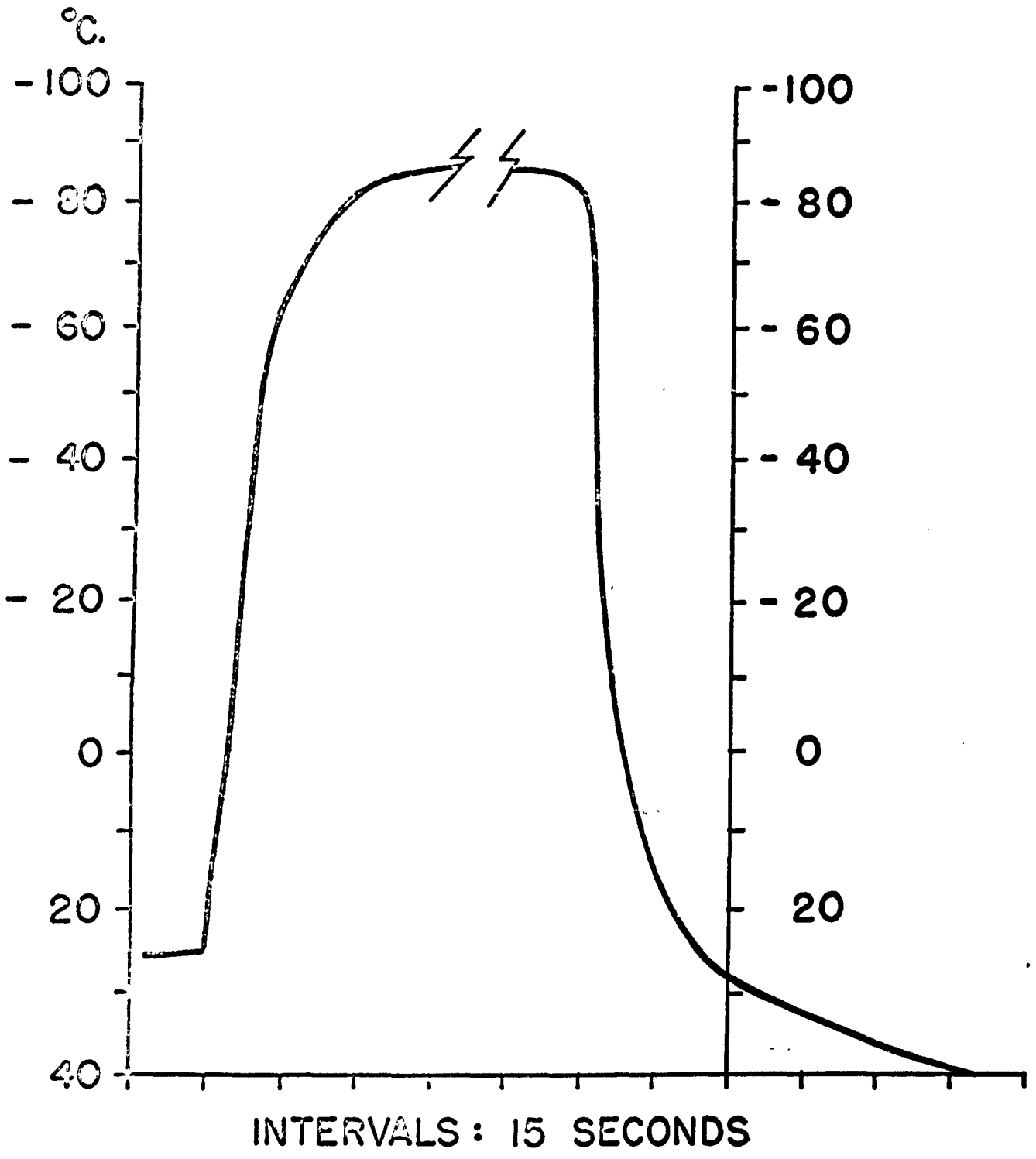


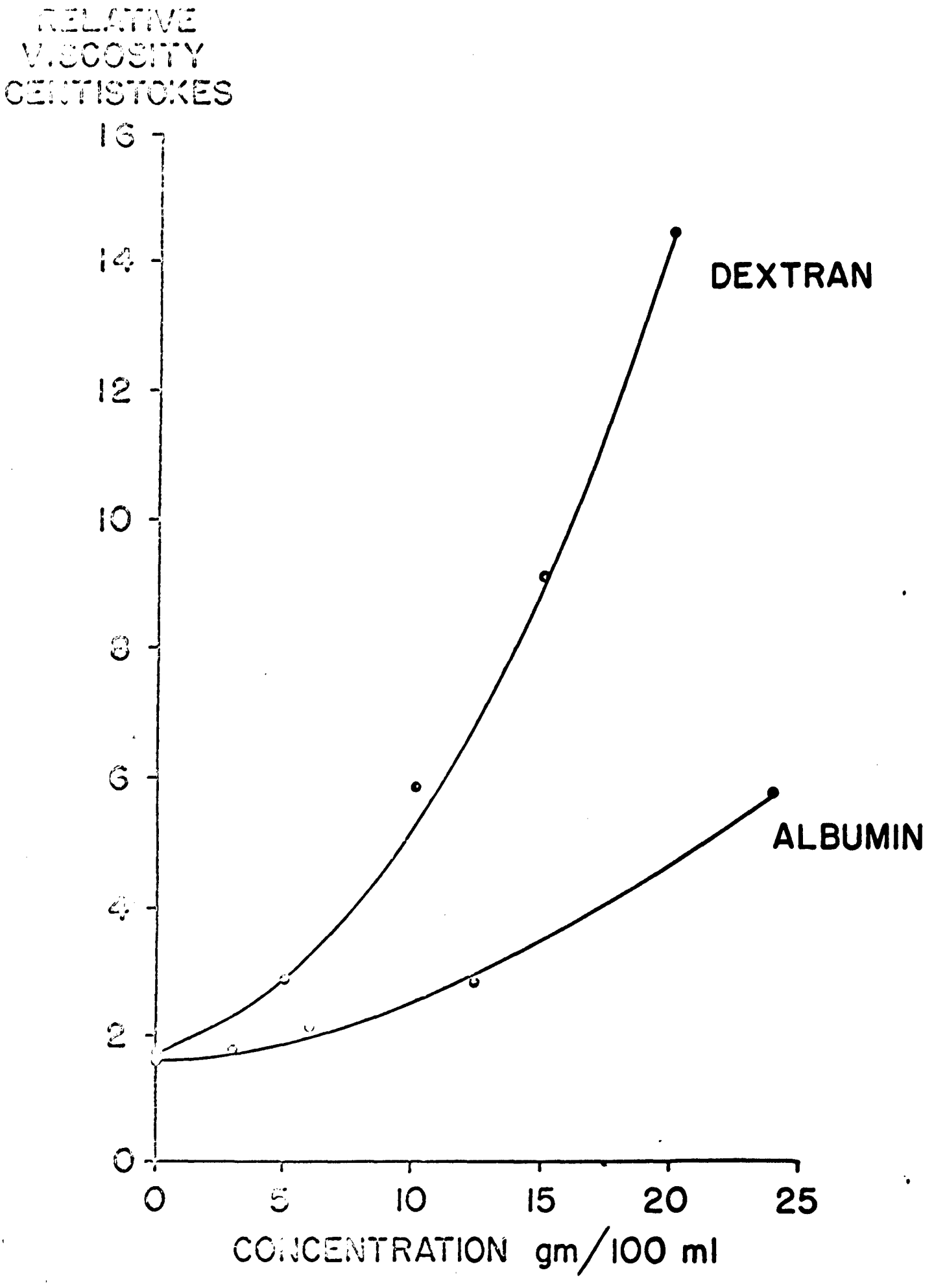
O_2 DISSOCIATION CURVE

— — — — FRESH, HEPARINIZED BLOOD

---------- ACD BLOOD, STORED 20 DAYS

———— FROZEN BLOOD


°C.
-100
- 80
- 60
- 40
- 20
0
20
40
-100
- 80
- 60
- 40
- 20
0
20
INTERVALS: 15 SECONDS


RELATIVE
VISCOSITY
CENTISTOKES
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
CONCENTRATION gm/100 ml
DEXTRAN
ALBUMIN