Oxygen Capacity of Stored Frozen Blood.* (25662)

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Principles of rapid freezing and thawing, with especial reference to red blood cells, have been outlined by Luyet(1,2). Following his work, Meryman and Kafig(3,4) prepared, by quick freezing and rapid thawing shortly thereafter, samples of dog and human blood which were, respectively, satisfactory in regard to cell recovery (percentage hemolysis) in the one case, and post-transfusion survival in the other. No storage experiments (with lapse of time between freezing and thawing) were done. The foregoing criteria are ones usually used in evaluating the merits of blood preservation technics. However, the most important function of the red blood cell, namely transport of oxygen to the tissues, is frequently ignored as a measure of the merit of such methods. The following gives results of observations of serial samples of frozen blood stored at low temperatures for periods up to one year. Analyses were done to determine resultant changes, especially in oxygen capacity, which had occurred in the red cell or its hemoglobin (Hb) content.

Materials and methods. The procedure was that described by Meryman and Kafig(4), with recent refinements noted below. Human or ox blood with added ACD solution A (15 ml/100 ml blood) was used. To this mixture (I), was added a 50% *d*-glucose solution (20 ml/100 ml of ACD blood). The final blood mixture (II) was frozen in individual portions of approximately 50 ml delivered into liquid nitrogen from a horizontally mounted syringe, the plunger of which was slowly moved by a screw mechanism. The fluid was forced through vinyl tubing leading to a capillary tube (or 29-gauge hypodermic needle) vertically mounted on a disc rotating rapidly over a liquid nitrogen bath. The frozen particles of the separate portions were stored in individual glass containers (stoppered with gauze plugs) immersed in liquid nitrogen. For reconstitution, liquid nitrogen in the plugged containers was discarded; the frozen particles were thawed by gradual sprinkling from a sieve for 30-45 minutes, into a solution mixture of 12.5 ml 0.9% NaCl and 2.5 ml 50% *d*-glucose, at 40°C. Final volume of the reconstituted blood (mixture III) varied from 52-61 ml. Reconstituted samples were analyzed for O_2 capacity according to Sendroy(5) for both active and total Hb content by the CO capacity method of Van Slyke, *et al.*(6), for cell volume by hematocrit, and for hemolysis by plasma Hb (oxy- and total) content as outlined by Hunter(7). All hemoglobin values were measured as, or converted to equivalent units of, bound O_2 (or CO). Serum potassium was determined by flame photometry (8). Mechanical loss of blood (mixture II) in the freezing and thawing makes quantitatively impossible a controlled, reproducible composition of the final, reconstituted mixture (III). Hence, changes taking place in storage of the samples were evaluated not from absolute values, but from percentage distributions of Hb, as total, carboxy-, and oxy-(6), in "plasma" and cell fractions of each blood mixture (III).† These values formed the basis of comparisons of extent to which physical integrity and Hb content of the erythrocytes, and hence their ability to transport oxygen, had been affected.‡ For comparative

* The authors are indebted to J. Bronson, G. W. Pearse, and E. Kafig for design and construction of the modified form of apparatus used, and to the latter for aid in preliminary trials of freezing and thawing technic. Determination of hemolysis values by H. A. Collison is gratefully acknowledged. Human and ox blood were obtained through courtesy of the Naval Hospital Blood Bank, Bethesda, and of Dr. A. H. Frank, Agri. Research Service, U. S. Dept. of Agri., Beltsville, Md., respectively.

† "Plasma" refers to non-cell portion of diluted plasma of the final, reconstituted blood (mixture III).

‡ Hemoglobin undergoing denaturation or degradation to a point beyond which it could no longer bind CO upon reduction with $Na_2S_2O_4$(6) would not be measured. However, the results given in the following would seem to make rather small, the likelihood of such an occurrence *i.e.*, any significant absolute reduction in amount of total Hb.

TABLE I. Effect of Rapid Freezing and Storage at −195°C, and Reconstitution of Blood.*

Sample mixture	Reconstitution after freezing Days	Hematocrit cell vol %	"Plasma" $\frac{}{(Hb)_{pl}}$ Vol %	Blood — $(Hb)_{bl}$ Vol %	Blood — $\frac{(CO\ cap)_{bl}}{(Hb)_{bl}} \times 100$ %	Blood — $\frac{(O_2\ cap)_{bl}}{(Hb)_{bl}} \times 100$ %	Hemolysis $\frac{Hb_{pl}}{Hb_{bl}} \times 100$ %	Cells — $\frac{CO\ cap_c}{Hb_{bl}} \times 100$ %	Cells — $\frac{O_2\ cap_c}{Hb_{bl}} \times 100$ %	"Plasma" — Potassium $(K)_{pl}$ mE/l	"Plasma" — $\frac{K_{pl}}{Hb_{bl}} \times 100$
Human II †		39.1	.45	16.86	99.0		1.6	97.4			
III	0	22.9	1.0	10.85	99.0		7.1	92.0			
"	21	26.1	1.4	14.06	98.3		7.4	91.0		28.8	15.1
"	35	29.7	1.6	14.05	98.4		8.0	90.5		28.0	14.0
Ox II †		26.7	.08	13.37	100.0	100.0	.44	99.6	99.6	7.56	4.14
III	14	18.7	.54	9.55	100.0	100.1	4.6	95.4	95.5	4.28	3.58
"	28	20.6	.63	10.06	99.6	99.7	5.0	94.6	94.7	3.96	3.13
"	112	17.9	.55	9.61	100.0	100.0	4.7	95.3	95.3	3.86	3.30
"	175	18.9	.84	10.48	99.0	99.3	6.5	92.6	92.8		
"	224	21.4	.58	11.15	99.4	97.7	4.1	95.3	93.7	4.57	3.22
"	287	17.9	.80	9.57	99.4	97.7	6.9	92.5	91.0		
"	329	18.2	1.00	10.24	99.2	96.8	8.0	91.3	89.1	4.29	3.43
"	364	18.7	1.37	10.61	99.6	97.4	10.5	89.1	87.2	4.25	3.26

* Values in parentheses refer to concentrations in components designated by subscripts; open values denote amounts in component fractions (concentration × component fraction).

† Not frozen.

purposes, loss of potassium from cells to plasma was indicated by the ratio (X 100) of meq of K to total hemoglobin (as ml of O_2 or CO) per unit volume of each reconstituted blood sample.

Results are shown in Table I. The first experiment was done on outdated (22 days old) human bank blood. Hemolysis, or relationship of amount of hemoglobin in "plasma" fraction to total in the whole blood, increased from an initial value of 1.6% (mixture II) to 7% on immediate freezing and reconstitution (mixture III). The value of 7-8 ± 0.4 (analytical probable error) % was found on successive observations of samples removed from storage at intervals of 4-5 days, to a terminal time (in storage) of 35 days. By that time, Hb in the active form (by CO capacity) in the blood had fallen from a level of 99.0 to 98.4 ± 0.7 (analytical P.E.) % of the total. As a result of hemolysis and conversion of active to inactive hemoglobin, the relationship of amount of active Hb in the cell fraction to total Hb in the blood fell from 97.4 to 90.5%.

In the second experiment, changes prior to freezing were avoided by use of freshly drawn defibrinated ox blood. Eight samples containing ACD and glucose (mixture II) were frozen and serially removed from storage for reconstitution and analysis at intervals of lapsed time to one year. Table I indicates increased hemolysis from an initial value of 0.44% before freezing to 4.6% for a sample promptly frozen, stored 2 weeks, then thawed and reconstituted. With longer storage, this value gradually increased to 10.5 ± 0.5 (analytical P.E.)% after 1 year. Hemoglobin in active form in the blood fell from the 100% level to 99.5 or 97.5 ± 0.7 (analytical P.E.)% by CO or O_2 capacity, respectively. Corresponding percentages of active Hb remaining in the cell fraction were 89.1 and 87.2%, respectively. As a result of hemolysis and migration of ions from cells to plasma, ratios of plasma K to total Hb decreased to a small extent from an initial value of 4.14 in the unfrozen mixture II, to values varying between 3.58 and 3.13 ± 0.12 (analytical P.E.) for samples stored from 2 weeks to 1 year.

Although no pH values were obtained, it may safely be assumed that the samples studied were at physiological neutrality. Glass electrode measurements on other samples showed human ACD bank blood (mixture I) 8 weeks old to have a pH of 6.52; however, reconstituted (ox) blood (mixture III) freshly prepared, or after freezing and storage for 4 months, had pH values of 7.35 and 7.41, respectively.

Discussion. Since freezing of blood results in a withdrawal of water from solution, and a concentration of salts, it is, in a sense, a de-

hydration. From the results of Keilin and Hartree(9), Haurowitz(10), Farr, Hiller and Van Slyke(11), and others, changes in the hemoglobin molecule occur on drying. Thus, when more or less purified preparations of hemoglobin are dried from the frozen or liquid state, with oxygen present, intra- or extra-molecularly, the molecule is rendered vulnerable to oxidation as methemoglobin.

As proposed by Meryman and Kafig(3) blood is quickly oxygenated by air during freezing, is stored and reconstituted, with hemoglobin in the form of HbO_2. Nevertheless, as shown by the excellent agreement of CO capacity with total Hb values for ox blood in Table I, little methemoglobin formation takes place. These results agree with the finding that merely freezing and thawing caused no change in activity of oxyhemoglobin solutions(11). It would seem that freezing, *per se*, is not a dehydration in the sense of a physical withdrawal of water. Furthermore, from the long range point of view, the continued presence of reducing enzyme systems within the cell, instantly available on thawing blood from solid to liquid, would be favorable for preventing oxidative formation of methemoglobin.

On the other hand, the gradually increasing discrepancy between CO and O_2 capacities, possibly indicates formation of a hypothetical heme derivative combining with CO but not with O_2(6). Although the loss of 2.6% (of which 0.5% is apparently in the form of methemoglobin) of O_2 capacity far exceeds the probable error of analysis, it represents a relatively unimportant diminution in functional activity of the reconstituted blood.

These changes in CO and O_2 capacity are reflected in corresponding values for cell fractions, and include the larger loss, by hemolysis, of Hb found in "plasma" to the extent of 10.5% (for ox blood) after 1 year's storage at −195°C.

The results for human blood confirm the occurrence of a small, gradual change in CO capacity (methemoglobin formation) as a result of freezing, storage, and thawing. They also show directly, as do values for ox blood inferentially, that hemolysis takes place immediately upon freezing and thawing. The resultant loss in ox blood, on this account, approximately doubles as storage is extended to one year.§

Potassium content of the "plasma" fraction represents the sum of a moiety escaping through the cell membrane from intact erythrocytes, and another, released from hemolyzed cells, as physical properties of the lipoprotein cell membrane are altered by low temperature and concomitant withdrawal of water as ice. Ox blood ratios of total K in the "plasma" to total Hb in the blood, all 2 or 3 times higher than normal, would be of little importance in transfusion practice. These ratios for human blood, approximately 12 times the values in normal, fresh samples, indicate the usual increase of plasma K for blood stored 21 days in the liquid state. There is no reason to attribute any significant change in plasma K to the effect of storage or ageing at −195°C. Indeed, the results for ox blood indicate, with passage of time in the solid state, as in the liquid, an increasing reversal on addition of glucose to mixture II(12), of the initially rapid migration of potassium from cells to plasma(13), in this case offsetting an increasing amount released from hemolyzed cells.

Summary. 1. Blood diluted to increased volume of approximately 40%, has been rapidly frozen and stored for one year, in liquid nitrogen. 2. Periodic examination of serial samples showed a gradual loss of O_2 capacity of 2.5%, of which 0.5% represented conversion to methemoglobin. 3. As a result of freezing and thawing, an immediate hemolysis of 5-6% took place, which was slowly approximately doubled during the year. 4. Accumulation of potassium in plasma as a result of escape from intact cells and liberation from hemolyzed erythrocytes was apparently arrested or nullified by reversed migration of K ions to the cells.

§ Obviously, the scatter of values for hemolysis at different points of storage indicates that these results (Table I) aside from relatively small analytical errors indicated, are not wholly attributable to age of sample, but reflect a procedural variability incurred in handling (preparation, freezing, storage, and thawing) of individual samples. However, an underlying general trend of increase in hemolysis with time, is quite clear.

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Received January 20, 1960. P.S.E.B.M., 1960, v103.

Stimulatory Effects of Glycine, L-Serine, Folic Acid and Related Compounds on Growth of Cell Cultures.* (25663)

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Twelve amino acids, L-glutamine, and infrequently L-asparagine are required for growth of cell cultures(1). Additionally, primary rhesus monkey testicular† and primary monkey kidney cultures(2) require glycine. Isolated HeLa and KB cells(3) and a strain of rabbit fibroblasts(4) require L-serine. Either glycine or L-serine is essential to the Novikoff hepatoma *in vitro*(5) and these amino acids stimulate growth of Walker carcinosarcoma 256(6) and Jensen sarcoma cultures(7).

In this communication the stimulatory effects of glycine and L-serine for a wide variety of both primary and stable line cultures are described and relationships of this requirement to folic and folinic acid are examined.

Methods and materials. Inocula for primary cultures were prepared by stirring the minced fresh tissue 2-6 hours with 0.2% trypsin (Difco 1:250) in Earle's balanced salt solution at 37°C. The cell suspension was filtered through gauze, collected by centrifugation and resuspended in whole medium. Stable line cells for inocula were prepared by trypsinization of stock cultures except Walker carcinosarcoma 256 cells which were readily shaken loose from the glass after chilling at 5°C. One ml of whole medium containing 100,000 fresh cells or 20,000 stable line cells was pipetted into 15 x 125 mm tubes which were placed in a slanted position in racks. The cultures were established (plated) by incubation for 24 hr at 37°C in an atmosphere of 8% carbon dioxide—92% air. The medium was removed and the tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graduated amounts of the test compounds. Incubation was continued for 3-5 days until the cells formed a sheet or attained maximal growth in presence of optimal amounts of the test compounds. The medium was not replenished. Growth was measured by the method of Oyama and Eagle(8). The whole medium in which the cells were established was similar to that previously described(9). It contained L-isomers of essential and nonessential amino acids as well as 0.2 mM pyruvate, 10 μg adenosine, and 5% bovine serum. No p-aminobenzoic acid was included in media used. The test medium differed in that it contained dialyzed serum, 0.2 mM each of pyruvate, alpha-ketoglutarate, and oxalacetate.

* This work was conducted under contract with Cancer Chemotherapy, Nat. Cancer Inst., NIH, U.S.P.H.S.

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