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Research Article

STORAGE OF BLOOD BELOW 0° C. IN LIQUID STATE[1]

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INTRODUCTION

In their original observations in 1916, Rous and Turner (1) recognized the importance of refrigeration in the preservation of blood. In recent years Parpart and his co-workers (2) studied the effect of different temperatures. The temperatures most suitable for survival of human red cells were found to lie between 4° C. and 9° C. with an optimum at about 7° C. This has been confirmed by the *in vivo* studies of Gibson and his co-workers (3). Rapoport (4) studied the survival rate of red cells during storage at 4° C. and 25° C. and found that storage for two days at 25° C. produced changes equivalent to those of two weeks storage at 4° C. Temperatures above 10° C. favor more rapid deterioration of red cells. Storage temperatures below 0° C. could not be evaluated because blood freezes and then hemolyzes completely upon melting.

Such a study is possible only if blood can be kept liquid below 0° C. by some physical or chemical means, and the procedure itself is not deleterious to red cells. The present investigation attempts this by the use of high pressures or of alcohol to depress the freezing point of the whole blood.

Water can be prevented from freezing by application of hydraulic pressure. The phenomenon is valid with 2,045 atmospheres to − 22° C. For greater pressures the freezing points are not lowered as the water-ice phase diagram indicates. Bridgman (5) studied the phase changes for the full range of 10,000 atmospheres (Figure 1). At higher pressures than 2,045 atmospheres water freezes at increasingly higher temperatures. In the present investigation pressure was kept below 2,045 atmospheres. Thus a pressure of 590 atm. will lower the freezing point of water to − 5° C.; 1,090 atm. to − 10° C.; 1,540 atm. to − 15° C.; and 1910 atm. to − 20° C.

The effect of pressure on red cells was studied by Callery and Portier (6) as early as 1910. It was found by these authors that 10 atm. pressure did not cause hemolysis and that hemolysis occurs when the pressure is greater than 300 atm. Fontaine (7) found that defibrinated blood, kept for seven hours at 500 atm. pressure, showed only slight hemolysis. Red cells suspended in saline hemolyzed more rapidly. Haubrich (8) and Ebecke (9) found that pressure changed the physical form of the red cells, giving them a more spherical shape. Spherocytes are considered more susceptible to hemolysis. These early investigations indicated that pressure is by no means innocuous to red cells.

The storage of blood under pressure was considered worthy of further investigation because earlier studies were limited to only a few hours of storage of defibrinated blood or red cells suspended in saline, and were all carried out at room temperature. Since this study was completed, our attention has been called to the favorable report by Jessiman and Walter (10) using 22,500 lbs. per sq. inch and low temperatures.

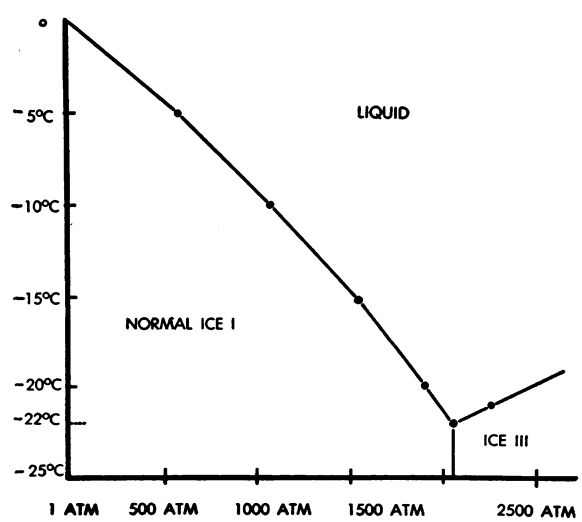

FIG. 1. WATER-ICE PHASE DIAGRAM AT TEMPERATURES AND PRESSURES CORRESPONDING TO THIS INVESTIGATION

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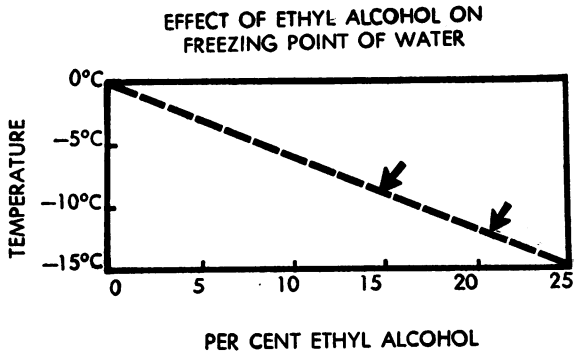

FIG. 2. EFFECT OF ETHYL ALCOHOL IN FREEZING POINT OF WATER

Alcohol lowers the freezing point of water, and binary mixtures freeze at temperatures between 0° and − 115° C. depending on composition. Figure 2 shows the depression of freezing point of water by the addition of alcohol in concentrations corresponding to the ranges in this investigation. The effect on the depression of the freezing point by an equivalent amount of alcohol is greater in blood, due to the presence of salts and proteins. The toxicity of alcohol is low. Up to 100 ml. of alcohol have been given intravenously daily. Alcohol can be removed quite readily from blood.

Alcohol is hemolytic to red cells, and its use to lower the freezing point of blood has, thus far, not been considered. Alcohol diluted with distilled water and added to blood causes a prompt and complete hemolysis. Alcohol is freely permeable through the red cell membrane, and isotonic saline is necessary for osmotic equilibrium. If osmotic hemolysis is prevented, alcohol hemolysis is only moderate at room temperature and is much retarded at 4° C. Below 4° C., alcohol is not hemolytic in a wide range of concentrations, providing isotonicity is maintained.

In view of this observation, it was decided to study the storage stability of whole blood containing various concentrations of alcohol. It was found practical to limit the study to the storage of blood at − 7.5° C. and − 12.5° C. by the addition of 15 per cent and 21 per cent alcohol, respectively.

MATERIAL AND METHODS

Whole blood was obtained from the Blood Bank of the Queens General Hospital in standard A-C-D solution. The A-C-D solution contained 1.33 per cent sodium citrate, 0.4 per cent citric acid, and 3 per cent glucose. Twenty-five cc. of such A-C-D solution were added to 100 cc. of whole blood. Two commercial low temperature storage cabinets were converted to constant temperature units operating in any pre-set range between 0° to − 25° C. by the use of a fan within the cabinet and adequate controls.

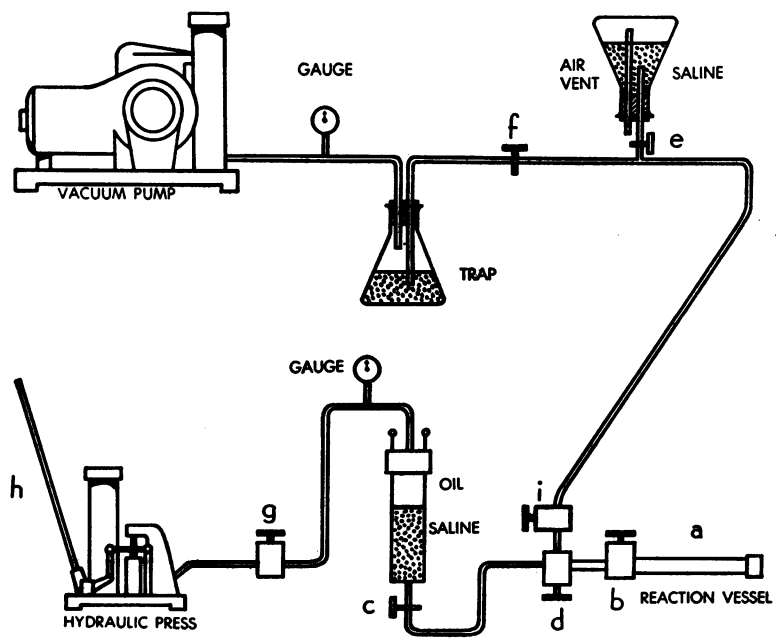

FIG. 3. SYSTEM FOR APPLICATION AND RELEASE OF HIGH PRESSURE

The initial study aimed to determine the effect of high pressure (Figure 3). The procedure for applying the pressure was as follows: 15 ml. of blood were introduced to fill completely the high pressure reaction vessel (a). The vessel was closed by valve (b) on the reaction vessel, and valve (b) was then attached to pressure system. To evacuate tubing leading from hydraulic press to the reaction vessel (a), valves (d, i, f) were opened and system evacuated by vacuum pump. Following evacuation, valve (f) was closed, valve (e) opened to allow saline solution to fill tubing. Now for pressure application, valves (e, i) were closed and, to prevent reflux, a slight pressure was applied to the system by manually pumping handle (h) with valve (b) still closed and valve (c) opened.

Now the valve (b) was opened and the desired pressure applied to the blood in reaction vessel (a). Valve (b) was closed and the pressure vessel was removed for storage.

To release pressure in the vessel, it was first warmed to 4° C. then reconnected to the system filled with saline. With valve (c) closed, the tubing was filled with saline. Now valve (i) was closed, and valves (c, d) were opened and a pressure applied to the system which was equal to that in the container. Valve (b) was opened and the pressure was matched. The pressure was then released rapidly or slowly, as desired, through valve (g). This ordinarily was accomplished in a few minutes.

For a study of the effects of alcohol, a commercial 95 per cent preparation was diluted by saline in a proportion of 65 parts alcohol to 35 parts of physiological saline. Larger amounts of alcohol precipitate blood proteins. Alcohol at 21 per cent concentration was the maximum used because hemolysis occurred at − 12° C. with higher levels. Dextrose was added to give a 500 mg. per cent concentration. The addition of dextrose to the alcohol seemed to prevent hemolysis during storage. One part of this (alcohol, dextrose, saline) or A-D-S solution containing 65 cc. of 95 per cent alcohol, 35 cc. of saline, and 500 mg. of glucose was added to two parts of whole A-C-D blood to obtain an approximate 21 per cent alcohol concentration. Also, one part of another A-D-S solution containing 42 parts of 95 per cent alcohol, 58 cc. of saline and 500 mg. of glucose, was added to one part of A-C-D blood to produce a similar approximate 21 per cent alcohol concentration in a more diluted blood. As alcohol was found hemolytic to red cells at room temperature, the A-D-S solution and all the syringes and containers used were cooled to − 12° C. In some samples only half of the calculated amount of A-D-S solution was added to the A-C-D blood at first and the diluted blood was stored for one and one-half hours at − 12° C. before the second half of the A-D-S solution was added.

The osmotic resistance of the red blood cells to 0.6 per cent saline was determined by the method of Parpart (11) and was expressed in per cent of total red cells hemolyzed. Samples taken from cold storage were tested without delay because blood diluted with alcohol hemolyzes rapidly at room temperature.

For each sample the degree of hemolysis was determined by the ratio of the hemoglobin content of the supernatant after centrifuging for five minutes at 1500 r.p.m. over the hemoglobin content of the whole blood, and is referred to as *percentage hemolysis.* The hemoglobin was determined by the standard HCl method in a Fischer Electrohemometer.

RESULTS

Pressure

Blood undergoes complete hemolysis at atmospheric pressure if the temperature is lowered from 4° C. to − 8° C., while blood under 680 atmospheres pressure shows about the same degree of hemolysis at 4° C. as at − 8° C. with percentage hemolysis of 54 and 59, respectively, after 14 days of storage. It can be assumed that freezing was prevented by the application of pressure. It is

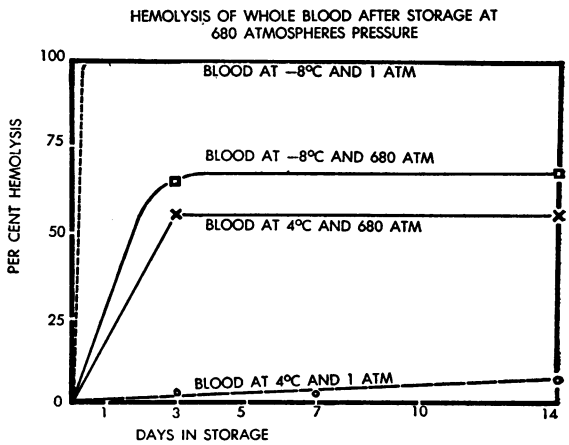

FIG. 4. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 680 ATMOSPHERES PRESSURE

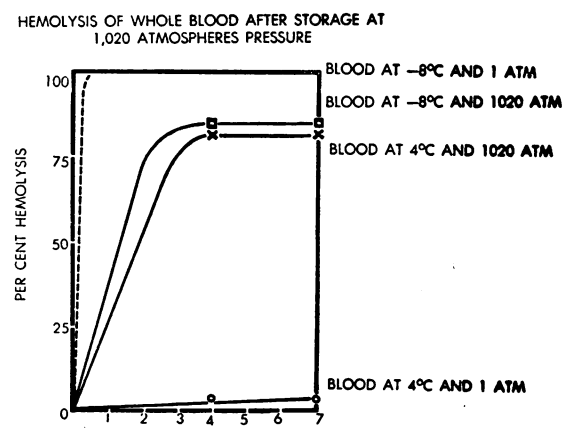

FIG. 5. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 1020 ATMOSPHERES PRESSURE

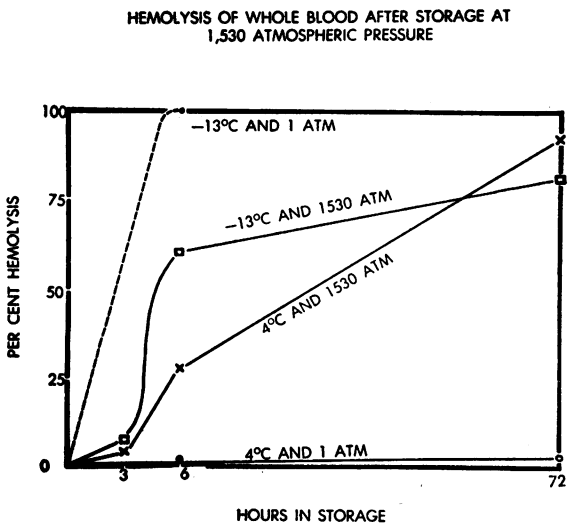

FIG. 6. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 1530 ATMOSPHERES PRESSURE

also evident that blood under pressure develops considerable hemolysis, irrespective of temperature. It appears that pressure accelerates the reactions responsible for the breakdown of blood in storage. The harmful effects of pressure were found to increase with the amount of pressure applied and of the duration of its application. Figures 4, 5, and 6 illustrate these effects for 680, 1,000, and 1,530 atmospheres of pressure. Figure 4 represents the lowest pressure at which a significant lowering of the freezing point of blood could be obtained, and Figure 6, the pressure corresponding to the maximum lowering of the freezing-point attainable on the basis of the ice-water phase diagram. It is interesting to note that the harmful effects of pressure are not instantaneous but develop with time in storage, the hemolysis increasing with higher pressures (Table I).

Clotting of blood in A-C-D solution, which occurred in storage under pressure, merits further study. It may be the result of a physico-chemical alteration of ionic equilibrium of the blood in the anticoagulant mixture.

Table I

Percent Hemolysis* of Whole Blood Stored at Various Pressures and Temperatures and Duration of Storage

	Duration of Storage		
Treatment	3 Hrs.	6 Hrs.	72 Hrs.
1530 atm. and 4°C	3.5	26	91 89 74
1530 atm. and -13°C	6.7	58	83
1 atm. and 4°C	—	0.8	1.1
1 atm. and -13°C		98	100
	4 days	7 days	
1020 atm. and 4°C	75 89 71	84 80 81	
1020 atm. and -8°C	79	92	
1 atm. and 4°C	4.5	2.0	
1 atm. and -8°C	100	100	
	3 days	7 days	14 days
680 atm. and 4°C	54 51 76	—	58 49 59
680 atm. and -8°C	72		59
1 atm. and 4°C	4.7	2.5	5.1
1 atm. and -8°C	100	100	98

* Defined as hemoglobin in supernatant after 1500 r.p.m. for 5 min. over hemoglobin of whole blood.

Alcohol

Figure 7 illustrates the grade of spontaneous hemolysis which occurs in A-C-D blood stored at − 12° C. in A-D-S solution as compared with blood stored at 4° C. in A-C-D solution alone. An increase of the spontaneous hemolysis occurred during storage in both at about the same rate.

Figure 8 illustrates the degree of osmotic resistance found in 0.6 per cent solution of sodium chloride, of blood stored at − 12° C. in A-D-S solution as compared with the same stored at 4° C. to which A-C-D solution was added. The difference is significant. A decrease of the resistance of the red blood cells is manifest in both during storage, less marked in the blood stored at − 12° C., provided that blood is not diluted to less than 54 per cent of its original volume. If blood is diluted to 27 per cent or 40 per cent of its original volume, the osmotic resistance is greatly diminished. Blood collected in a blood bank is already diluted to 80 per cent by the addition of A-C-D solution. Using one part of A-D-S solution to one part of A-C-D blood brings the blood volume to 40 per cent. By using two parts of A-C-D blood to one part of A-D-S solution, the blood volume is 54 per cent and by using two parts of A-D-S solution to one part of A-C-D blood, the volume drops to 27 per cent. Parpart (11) found a rapid decrease of osmotic resistance in blood stored at 4° C. if the volume of blood in the final diluent dropped below 40 per cent.

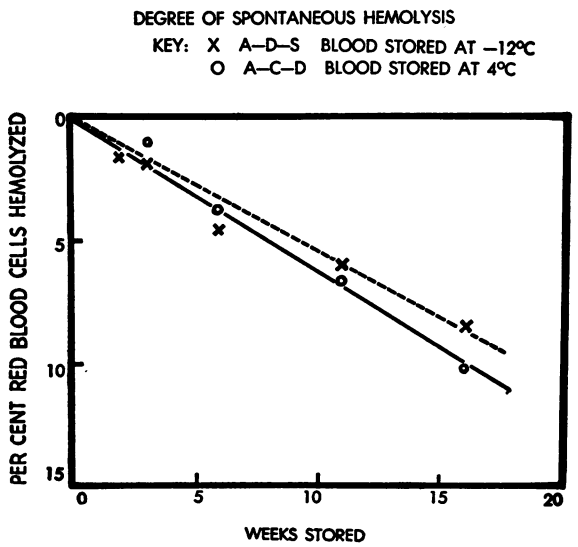

FIG. 7. DEGREE OF SPONTANEOUS HEMOLYSIS AT − 12° C. IN ALCOHOL-DEXTROSE SALINE

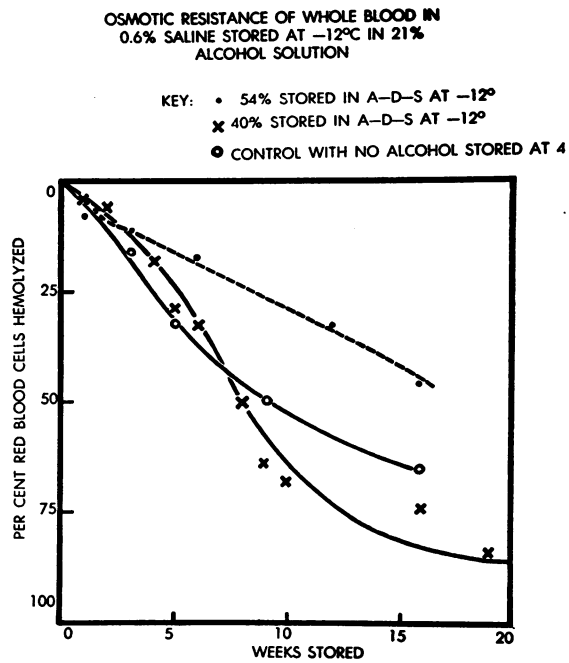

FIG. 8. OSMOTIC RESISTANCE OF WHOLE BLOOD TO 0.6 PER CENT NaCl STORED AT − 12° C. IN 21 PER CENT ALCOHOL-DEXTROSE SALINE

DISCUSSION

Attempts were made to lessen the hemolytic effect of pressure, by control of several variables. Rapid application and rapid release of pressure (1,500 atm.) to a sample of blood caused some immediate hemolysis, but the amount of damage was not considerable, being 0.28 Gm. hemoglobin per 100 ml. in the treated sample, as compared with 0.18 Gm. in the control, measured in the plasma. Blood held under 340 atmospheres for seven days showed no hemolysis, while another sample held under 680 atmospheres for the same period presented considerable hemolysis. A third sample had 340 atmospheres pressure applied for four days, and then an additional 340 atmospheres for three more days. The sample was as hemolyzed as if 680 atmospheres had been applied at once. The slow application and the slow release of pressure (up to 1,800 atm.) was tried at the rate of 10 atmospheres per minute. Again the harmful effects were of the same general order of magnitude

at the different pressures in storage as that found in the controls with single pressure applications.

The question whether the presence of air under pressure affected the red cells was considered. Pressure of 1,500 atmospheres was applied to two samples of blood, and air was not evacuated in one of them. The pressure was maintained for thirty minutes. In the control sample where the vacuum pump emptied the reaction vessel and pressure system as usual, the hemoglobin value of the overlying plasma was 0.09 Gm. hemoglobin per 100 ml. The test sample, without preliminary evacuation of the system before pressure application, had a hemoglobin value in the plasma of 0.11 Gm. The presence of air in the system did not increase hemolysis.

The application of silicone coating to the walls of the high-pressure reaction vessel did not reduce the extent of hemolysis. Samples of blood stored seven days at 4° C. under 340 atmospheres pressure showed a 0.3 Gm. hemoglobin in the plasma of the uncoated container and 0.5 Gm. hemoglobin in the blood sample in the silicone coated container.

Changes in the acidity, alkalinity, and osmotic pressure were studied. The addition of $NaHCO_3$ in amounts of 0.2 ml. normal $NaHCO_3$ to 20 ml. blood, prior to the application of pressure, did not prevent hemolysis. Adding HCl in amounts of 0.2 ml. normal HCl solution per 20 ml. blood was also tested. Treated samples were stored at − 7.5° C. under 545 atmospheres pressure. When tested, the sample treated with HCl contained 4.1 Gm. per 100 ml. and the control 3.7 Gm. per 100 ml. hemoglobin in the plasma. The addition of identical acid and alkaline solutions to blood without pressure did not show equivalent hemolysis during identical periods. The addition of distilled water in amounts of 10 per cent of original volume to one sample, and the addition of 0.2 Gm. NaCl to 20 ml. of blood, did not reduce but increased the degree of hemolysis at 545 atmospheres pressure.

The effects of instantaneous or rapid freezing and melting were studied. Samples of blood were subjected to 1,020 atmospheres pressure and cooled to − 11° C. The pressure was released suddenly so that instantaneous freezing would occur. After storage, the samples were defrosted by initially applying 1,020 atmospheres pressure instantaneously and then warming to 4° C. This did not prevent cell rupture, and complete hemolysis resulted.

The effect of pressure on red cell mass without plasma was found to be the same as on whole blood.

As noted, blood in A-C-D solution, if held under 1,020 to 1,530 atmospheres pressure for a few days, showed complete clotting. The addition of heparin or sodium oxalate to the A-C-D blood prevented clotting caused by such pressure. Since sodium oxalate prevented such clotting it may be that the original clotting was related to some shift in the ionization of calcium under pressure.

The rate of application and release of pressure, presence or absence of air under pressure, silicone coating of reaction vessel, addition of acid or alkali, change in osmotic pressure, and rapid freezing and melting were investigated. Varying each of the factors did not diminish the hemolysis.

The damage to the red cell caused by pressure may result from an altered metabolism of the cell. In most physical and chemical reactions, volume change occurs. Pressure opposes any change that increases volume and promotes a change that decreases volume. It is reasonable to assume that during storage under high pressures some metabolic reactions are promoted while others are retarded, depending on the volume changes in the individual chemical reactions. The sum of the effects of pressure on the red cell seems to favor hemolysis. Generally, cold retards metabolic activities of cells, and it was hoped that any damage caused by pressure would be compensated for by low temperature storage at − 13° C. It was disappointing to find that considerable hemolysis occured under pressure regardless of the temperature investigated.

In the experiments described, whole blood was successfuly stored at − 13° C. in the liquid state under pressure. Considerable change occurred in the blood with the increased pressure at any temperature investigated, as evidenced by the hemolysis, the extent of damage being related to the amount, and duration of pressure applied. Deterioration increased during storage. This paralleled the results of earlier investigations at room temperature on defibrinated blood or red cells suspended in saline. Our results do not parallel the recent favorable report of Jessiman and Walter (10) who subjected blood to 22,500 lbs. pressure per square inch and preserved it at − 15° C.

A few observations were made on animals. From each of five rabbits 20 ml. of blood were collected in 4 ml. A-C-D solution and cooled to 4° C. in a refrigerator. One part of blood was diluted by one part A-D-S solution containing 42 per cent alcohol, and the samples were stored at − 12° C. for four months. Following four months storage the supernatant fluid of the sample was syphoned off, replaced by saline and reinjected into the same rabbit. Unfortunately three rabbits died immediately following reinjection. The presence of fibrin clots accumulated during the long storage may have been instrumental in these deaths. Blood of the last two rabbits was filtered through cheese cloth and the rabbits survived reinjection well. The grade of spontaneous hemolysis of the blood, reinjected to one rabbit which survived was 6 per cent and the test of osmotic resistance against 0.6 per cent sodium chloride solution showed 78 per cent hemolysis.

Although blood stored at − 12° C. in the liquid state in alcohol and tested for spontaneous hemolysis and osmotic resistance did not show a striking improvement as compared with blood stored at 4° C., the possibility still exists that the addition of alcohol may be developed for long range preservation of blood. Storage of blood is affected by many factors, and only a few were evaluated. The evaluation test procedures performed dealt with the structural and osmotic state of the cells only. These are of primary interest but many other changes, which are related to metabolic activities of the cells, may be directly affected by low temperature. These changes are not revealed by the tests used. The rate of enzyme deterioration, glycolysis, lactic acid formation, changes of pH and potassium content of red cells were all found much reduced at 4° C. Below 0° C. such studies are not possible until the problem of prevention of hemolysis by freezing is solved. Such studies could not be undertaken in the present investigation. Determinations of spontaneous hemolysis and osmotic resistance of red cells do not give information concerning more subtle changes. Ability of the red cells to survive blood transfusion *in vivo* (as determined by the radioactive tagging with phosphorus, iron, etc.), is a more acceptable criterion of the value of preservation of blood below 0° C. in liquid state in A-D-S solution.

SUMMARY AND CONCLUSIONS

1. The preservation of blood at temperatures below freezing, kept liquid by the use of high pressures and by ethyl alcohol, was investigated.

2. The damage caused by pressure in the range investigated was considerable. High pressure low temperature preservation of whole blood does not seem to be a promising procedure for long term mass preservation of whole blood.

3. The presence of 21 per cent ethyl alcohol in the blood permits storage of whole blood at − 12° C. in liquid state. At this temperature 21 per cent alcohol does not hemolyze red cells.

4. The spontaneous hemolysis in blood, kept from freezing by alcohol and dextrose after four months storage at − 12° C., was equal to, or slightly less than, in blood stored at 4° C. in A-C-D solution alone.

5. Osmotic resistance of the red cells showed a definite but not remarkable improvement in the blood stored at − 12° C. in alcohol.

6. It is urged that the use of alcohol for the low temperature long range storage of blood and other tissues be studied.

REFERENCES

1. Rous, P., and Turner, J. R., The preservation of living red blood cells in vitro. I. Methods of preservation. J. Exper. Med., 1916, **23**, 219.
2. Parpart, A. K., Gregg, J. R., Lorenz, P. B., Parpart, E. R., and Chase, A. M., Whole blood preservation; a problem in general physiology. An 'in vitro' analysis of the problem of blood storage. J. Clin. Invest., 1947, **26**, 641.
3. Gibson, J. G., 2nd, Evans, R. D., Aub, J. C., Sack, T., and Peacock, W. C., The post-transfusion survival of preserved human erythrocytes stored as whole blood or in resuspension, after removal of plasma, by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, **26**, 715.
4. Rapoport, S., Dimensional, osmotic, and chemical changes of erythrocytes in stored blood. II. Evaluation of several acid and neutral preservation mixtures. Effect of storage at 25° C. in Alsever's solution. J. Clin. Invest., 1947, **26**, 616.
5. Bridgman, P. W., Thermo-dynamic properties of liquid water to 80° and 1200 kgm. Proc. Am. Academy of Arts and Sciences, 1912, **48**, 307.
6. Callery, G., and Portier, P., Influence des pressions élevées sur les phénomènes osmotiques. Compt. rend. soc. de biol., 1910, **69**, 245.

7. Fontaine, M., Recherches expérimentales sur les réactions des êtres vivants aux fortes pressions. Ann. Inst. Océanograph., 1930, **8**, 1.
8. Haubrich R., Über die Druckresistenz der Erythrocyten. Pflüger's Arch. f. d. ges. Physiol., 1937, **239**, 304.
9. Ebbecke, U., Über sphärocytenbildung and submikroskopische Feinstruktur der roten Blutkörperchen. Pflüger's Arch. f. d. ges. Physiol., 1937, **239**, 533.
10. Jessiman, A. G., and Walter, C. W., Observations on low temperature and high pressure as a means toward better red blood cell preservation. Surg. Forum, 1950, **36**, 529.
11. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M., The osmotic resistance (fragility) of human red cells. J. Clin. Invest., 1947, **26**, 636.