Cation Flux and Electrolyte Composition of Frozen-Deglycerolized Blood

By Stanley Wallach, John W. Zemp, John A. Cavins, Lawrence J. Jenkins Jr., Monroe Bethea, Lawrence Freshette, Lewis L. Haynes and James L. Tullis

The brief storage time of blood at 4 C. limits its availability for mass casualty catastrophes, extensive surgical procedures and replacement therapy of massive hemorrhage. Recently, Haynes, Tullis and their associates, utilizing the glycerolization technic devised by Tullis et al., have administered 1000 units of blood that had been stored in the frozen state from three months to three years. This blood provides effective red cell and intravascular volume replacement, is free of an excessive adverse reaction rate, survives normally in vivo and is suitable for priming and volume replacement in procedures utilizing extracorporeal circulation. In support of the clinical efficacy of frozen-deglycerolized blood, studies of its gas exchange, composition, and metabolism have been instituted. O'Brien and his associates have shown the oxyhemoglobin dissociation curve of reconstituted frozen-deglycerolized blood to be comparable to that of blood collected in ACD anticoagulant. In the present report, the electrolyte composition and cation flux of reconstituted frozen-deglycerolized blood, freshly collected blood and blood stored at 4 C. have been compared. These data provide additional evidence that erythrocyte function is preserved after glycerolization and prolonged freezing.

Methods

Preparation

Fresh blood was collected from healthy donors utilizing four anticoagulant solutions:

1. Citric acid-sodium citrate-dextrose (ACD) solution, National Institutes of Health Formula A, 75 ml. of anticoagulant solution per 475 ml. donor blood. Units were studied immediately after collection and after storage for 12 or more days at 4 C.

2. Sodium heparin, 2000 U. dissolved in sodium phosphate buffer (pH 7.4), 30 ml. of anticoagulant solution per 500 ml. donor blood.

3. Fenwal resin collection bags in which the blood passes through a cation exchange column before entering the bag.

4. As controls, blood was collected by direct venepuncture into heparinized syringes.

Frozen-deglycerolized blood one day to 32 months old was obtained by either the previously described technic or a more recent modification. The deglycerolized units resuspended in isotonic 5 per cent albumin solution and the stored units of ACD blood were preincubated at 37 C. for three hours prior to study. All samples of fresh blood were analyzed within 60 minutes of collection.
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Electrolyte Studies

The red cells and plasma of the fresh, stored and frozen-deglycerolized bloods were separated quantitatively in waisted Butler-Cushman type tubes as outlined by Keitel. The water content of the cells was determined by differential weight before and after drying at 105°C for 48 hours. Chemical analyses were performed on plasma and trichloracetic acid extracts of weighed samples of cells. Sodium and potassium were determined by flame photometry, calcium by the method of Berger and magnesium by titration of protein and calcium free extracts with disodium ethylenediaminetetra-acetate using eriochrome black T as an indicator. Chloride was measured in a potentiometric titration apparatus using the appearance of excess Ag⁺ ion as the endpoint. Inorganic and total acid soluble phosphorus were determined by the method of Fiske and Subbarow.

Erythrocyte electrolyte concentrations (EEC) were expressed as mEq. or mM per kg dry weight to correct for variations in water content and cell size. These values may be converted to other parameters by the following formulae:

$$\text{EEC (mEq./Kg. cells)} = \text{EEC (mEq./Kg. cell solids)} \times \frac{\% \text{ dry weight}}{100}$$

$$\text{EEC (mEq./L. cells)} = \text{EEC (mEq./Kg. cell solids)} \times \frac{\% \text{ dry weight}}{100} \times 1.095$$

where 1.095 is assumed to be the specific gravity of red cells.

Cation Flux Studies

Potassium flux studies were performed by a modification of the method of Solomon utilizing a stationary water bath and room air as the gaseous phase. The incubation flasks were agitated manually every 20 minutes to prevent sedimentation of cells. At 30, 60, 120 and 180 minutes after addition of K⁴², aliquots of blood were withdrawn, immediately centrifuged, and the plasma removed for determination of K⁴² content. Blood pH (aerobic), hematocrit, and plasma potassium concentration were determined at zero and 180 minutes. All samples were analyzed in a scintillation well detector within a 30-minute period to obviate decay correction. The per cent K⁴² remaining in the plasma in each sample was calculated as follows:

$$\% \text{ K⁴² remaining in the plasma} = \frac{\text{plasma counts} \times (1-\text{hematocrit}) \times 100}{\text{whole blood counts}}$$

The per cent K⁴² remaining in the plasma was plotted semilogarithmically against time (fig. 1) and the slope of the line determined. Potassium flux was calculated as follows:

$$\text{Potassium flux (mEq./L. erythrocytes/hour)} = \text{Slope} \times \text{plasma potassium concentration (mEq./L.)} \times \frac{(1-\text{hematocrit})}{\text{hematocrit}}$$

In 10 representative instances in which the intracellular potassium concentration was known, the flux values were comparable to those obtained by Solomon's method of computation (fig. 1).

Sodium flux was measured by a modification of Harris and Maizels' method utilizing a stationary water bath, room air as the gaseous phase and three hours of preincubation with Na²² prior to study. After preincubation the cells were quickly washed twice with warm saline and restored to original volume by resuspension in glucose enriched nonradioactive plasma obtained from a separate aliquot of the same blood. Whole blood and plasma specimens were obtained immediately after resuspension. The resuspended blood was then reincubated aerobically for 180 minutes and additional plasma specimens obtained at 30, 60, 120 and 180 minutes. Blood pH (aerobic), hematocrit, and plasma sodium con-
Fig. 1.—Representative calculations of potassium flux. The upper graph indicates the calculation employed as compared to the method of Solomon in the lower graph. \( P \): Plasma concentration of radiopotassium; \( P_e \): calculated plasma concentration of radiopotassium at equilibrium; \( k \): transfer coefficients.

Concentration were determined at zero and 180 minutes. The per cent \( Na^{22} \) remaining in the erythrocytes at each time interval was determined as follows:

\[
\text{erythrocytes} = \frac{\% \text{Na}^{22} \text{ in } [\text{whole blood counts} - \text{plasma counts} \times (1-\text{hematocrit})] \times 100}{\text{whole blood counts} - \text{plasma counts at 0 min.} \times (1-\text{hematocrit})}
\]

The per cent \( Na^{22} \) remaining in the erythrocytes was plotted semilogarithmically against time (fig. 2) and the slope determined. Sodium flux was calculated as follows:

\[
\text{Sodium flux (mEq./L. erythrocytes/hour)} = \text{slope} \times \text{erythrocyte sodium concentration (mEq./L. cells)}
\]

The three-hour exchangeability of erythrocyte sodium with \( Na^{22} \) was determined by quantitatively separating erythrocytes and plasma after a three-hour incubation with \( Na^{22} \) in Butler-Cushman type tubes.\(^9\) Approximately 2 ml. of cells was transferred to a counting tube and the weight and radioactivity of the sample determined. The sample was then diluted to 50 ml. and its sodium content determined by flame photometry. A 1 ml. sample of plasma was transferred to a counting tube and its radioactivity and sodium content determined. The per cent exchangeability was calculated as follows:

\[
\% \text{Exchangeability} = \frac{\text{specific activity of erythrocytes} \times 100}{\text{specific activity of plasma}}
\]
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Fig. 2.—Representative calculation of sodium flux.

Except for the studies of the effect of pH on potassium flux, the comparisons between fresh, stored, and deglycerolized bloods represent effects not only of method of anticoagulation but also of biologic variation among the donors used. All data were analyzed for statistical significance by Student’s t test.

RESULTS

Extracellular Electrolyte Composition (Table 1)

In fresh heparinized syringe blood, extracellular electrolyte concentrations were within the accepted normal ranges. The variations in extracellular electrolyte concentrations in the remaining fresh bloods were the result of the composition of the anticoagulant solutions employed. In stored ACD blood the extracellular electrolyte changes were similar to those previously reported. Incubation of stored ACD units for three hours at 37 C. prior to analysis failed to effect sufficient re-entry of potassium into the cells to decrease the extracellular concentration of potassium to normal.

In frozen-deglycerolized blood the extracellular concentration of sodium was generally higher than that of the resuspending medium, indicating egress of intracellular sodium during the preincubation period. In those units processed by the older technic, small amounts of hemolysis occurred and the extracellular concentrations of potassium, magnesium, and phosphorus were slightly higher than those of the resuspension medium. In units processed by the newer technic, hemolysis was negligible and potassium concentrations were lower than that of the resuspension medium. Small amounts of extracellular calcium were found in most samples. Extracellular concentrations of chloride
Table 1—Extracellular Electrolyte Composition of Fresh, Stored and Frozen-Deglycerolized Blood

<table>
<thead>
<tr>
<th>Blood</th>
<th>Number of Units</th>
<th>Mean Electrolyte Concentration (mEq./L.)</th>
<th>Mean Phosphorus Concentration (mM/L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Na</td>
<td>K</td>
</tr>
<tr>
<td>Heparin (syringe)</td>
<td>7</td>
<td>148</td>
<td>4.06</td>
</tr>
<tr>
<td>Heparin (bag)</td>
<td>6</td>
<td>161</td>
<td>3.42</td>
</tr>
<tr>
<td>Resin</td>
<td>6</td>
<td>169</td>
<td>1.33</td>
</tr>
<tr>
<td>ACD (fresh)</td>
<td>7</td>
<td>183</td>
<td>4.03</td>
</tr>
<tr>
<td>ACD (stored)</td>
<td>6</td>
<td>176</td>
<td>24.0</td>
</tr>
<tr>
<td>Frozen-deglyc.*</td>
<td>18</td>
<td>165</td>
<td>5.51</td>
</tr>
<tr>
<td>Frozen-deglyc.†</td>
<td>10</td>
<td>—</td>
<td>4.70</td>
</tr>
</tbody>
</table>

*Processed by method described in ref. 2.
†Processed by method described in ref. 8.

were lower than that of the resuspending medium, indicating a shift of chloride intracellularly.

Erythrocyte Electrolyte Composition (Table 2)

Mean erythrocyte electrolyte concentrations of all fresh bloods were comparable to previously reported values. Storage of ACD blood resulted in marked increases in erythrocyte sodium and chloride and a decrease in erythrocyte potassium and total acid soluble phosphorus. In frozen-deglycerolized erythrocytes, the concentrations of sodium were moderately elevated and the potassium concentrations were insignificantly decreased (p < .1). In units processed by the older technic, erythrocyte concentrations of chloride were increased, but in units processed by the newer technic they were normal. The erythrocyte concentration of total acid soluble phosphorus was normal in frozen-deglycerolized blood processed by either technic.

The mean erythrocyte concentration of calcium was 1.34 mEq. per Kg. solids in heparinized syringe blood and was decreased in units collected in heparin-sodium phosphate solution and ACD solution. Both these anticoagulant solutions contain substances that complex calcium, suggesting that a decrease in the extracellular concentration of ionized calcium results in a loss of cellular calcium. This effect was most evident in resin collected cells where the erythrocyte calcium concentration fell to one-tenth normal. Frozen-deglyc-
Table 3.—Potassium and Sodium Fluxes of Fresh, Stored and Frozen-Deglycerolized Blood (37 C.)

<table>
<thead>
<tr>
<th>Blood</th>
<th>Mean Potassium Flux (mEq./L. cells/hour)</th>
<th>Mean Sodium Flux (mEq./L. cells/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Units</td>
<td>Observed</td>
</tr>
<tr>
<td>Heparin</td>
<td>11</td>
<td>2.11</td>
</tr>
<tr>
<td>Resin</td>
<td>9</td>
<td>1.97</td>
</tr>
<tr>
<td>ACD (fresh)</td>
<td>10</td>
<td>1.58</td>
</tr>
<tr>
<td>ACD (stored)</td>
<td>7</td>
<td>2.30</td>
</tr>
<tr>
<td>Frozen-deglyc.</td>
<td>17</td>
<td>1.48</td>
</tr>
</tbody>
</table>

*Corrected to a mean cell volume of 90 μ3.

Table 4.—Effect of pH on Potassium Flux of Fresh Heparin and ACD Bloods (37 C.)

<table>
<thead>
<tr>
<th>Blood</th>
<th>Number of Units</th>
<th>pH</th>
<th>Hematocrit</th>
<th>Extracellular Potassium Conc. mEq./L.</th>
<th>Potassium Flux (mEq./L. cells/hour)</th>
<th>Corrected Potassium Flux* (mEq./L. cells/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>6</td>
<td>7.5</td>
<td>44.5</td>
<td>4.04</td>
<td>2.11</td>
<td>2.11</td>
</tr>
<tr>
<td>ACD</td>
<td>6.9</td>
<td>45</td>
<td>4.95</td>
<td>1.65</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>3</td>
<td>7.4</td>
<td>46.5</td>
<td>4.10</td>
<td>1.86</td>
<td>1.86</td>
</tr>
<tr>
<td>Acid heparin</td>
<td>6.7</td>
<td>47.5</td>
<td>7.08</td>
<td>1.89</td>
<td>1.94</td>
<td></td>
</tr>
<tr>
<td>ACD</td>
<td>5</td>
<td>6.9</td>
<td>43</td>
<td>4.79</td>
<td>1.56</td>
<td>1.78</td>
</tr>
<tr>
<td>Alkaline ACD</td>
<td>7.8</td>
<td>28</td>
<td>3.76</td>
<td>2.35</td>
<td>1.70</td>
<td></td>
</tr>
</tbody>
</table>

*Corrected to a mean cell volume of 90 μ3.

Deglycerolized erythrocytes resuspended in synthetic media also had low concentrations of calcium. Mean erythrocyte concentrations of magnesium were normal in all bloods studied.

**Potassium Flux (Table 3)**

In fresh blood anticoagulated with heparin, potassium flux, as measured by the influx of radiopotassium, ranged from 1.47 to 2.43 mEq./L. cells/hour with a mean value of 2.11 mEq./L. cells/hour. This mean value was higher than that observed by Solomon,13 possibly due to the modified conditions of the present experiments. There were no significant variations in pH, hematocrit or extracellular potassium concentration. The mean potassium flux of resin collected blood was similar to that of fresh heparinized blood. In contrast to these findings, the mean potassium flux of fresh ACD blood was decreased. This decrease was confirmed in experiments in which half-unit quantities of blood from the same donor were anticoagulated with heparin and ACD respectively (table 4). Reduction of the pH of heparinized blood by the addition of lactic acid resulted in no change in potassium flux (table 4), but elevation of the pH of ACD blood by the addition of NaHCO₃ resulted in a marked increase in potassium flux (table 4). Since there were wide variations in mean corpuscular volumes of the bloods studied (table 5) and flux is expressed per liter cells, the flux data in tables 3 and 4 were corrected to a mean cell volume of 90μ3. When this was done, the observed differences in flux di-
Table 5.—Mean Corpuscular Volumes (MCV) of Fresh, Stored and Frozen-Deglycerolized Erythrocytes

<table>
<thead>
<tr>
<th>Blood</th>
<th>Number of Units</th>
<th>MCV (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Resin</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>ACD (fresh)</td>
<td>7</td>
<td>103</td>
</tr>
<tr>
<td>ACD (stored)</td>
<td>5</td>
<td>108</td>
</tr>
<tr>
<td>Frozen-deglyc.</td>
<td>10</td>
<td>118</td>
</tr>
<tr>
<td>Frozen-deglyc.†</td>
<td>26</td>
<td>93</td>
</tr>
</tbody>
</table>

*Processed by method described in ref. 2.
†Processed by method described in ref. 8.

appeared. These corrected values are consistent with earlier studies in which changes in pH induced by altering the CO₂ content of the gaseous phase had no effect on potassium flux.¹⁷

In contrast to mean corrected potassium fluxes of 1.80–2.11 mEq./L. cells/hour in all fresh bloods, the mean corrected potassium flux of stored ACD blood was elevated to 2.76 mEq./L. cells/hour. Although this elevation might be due to increased inward diffusion occurring with increased extracellular potassium concentration, other studies have shown the addition of potassium to fresh blood does not cause an increase in potassium flux.¹³,¹⁷ It is likely that a major portion of the increase resulted from nondiffusional movement of potassium intracellularly during the experiment. Although measurements of extracellular potassium concentrations at the beginning and end of each experiment did not provide clear evidence of such movement, other studies have shown that the high extracellular potassium concentration of stored ACD blood decreases upon incubation.

The mean corrected potassium flux of frozen-deglycerolized cells was 1.94 mEq./L. cells/hours and no variation in potassium flux due to duration of freezing was observed.

**Sodium Flux (Table 3)**

The mean corrected sodium flux of fresh blood as measured by the efflux of radiosodium ranged from 4.89 to 5.50 mEq./L. cells/hour. Among individual units, corrected values ranged from 3.30 to 7.26 mEq./L. cells/hour. Consequently, the mean fluxes for heparinized, resin collected, and fresh ACD bloods listed in Table 3 were not statistically different (p < .3). These mean values were higher than those reported by Harris and Maizels.¹⁴ Although no significant changes in pH, hematocrit, or extracellular sodium concentration occurred during incubation, it is probable that the modifications introduced in the technic were responsible for this difference.

The mean corrected sodium fluxes of stored ACD and frozen-deglycerolized blood were 5.74 and 4.79 mEq./L. cells/hour. Because of the variation in individual values mentioned above, neither mean value was statistically different from those of fresh blood or each other (p < .2). Among units of frozen-deglycerolized blood, no correlation between sodium flux and duration of freezing was noted.
Table 6.—Radionuclide Exchangeability of Fresh, Stored ACD, and Frozen-Deglycerolized Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Number of Units</th>
<th>Mean Erythrocyte Sodium Concentration (mEq./L. cells)</th>
<th>Mean Per Cent Exchangeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>6</td>
<td>13.3</td>
<td>82</td>
</tr>
<tr>
<td>Resin</td>
<td>4</td>
<td>15.1</td>
<td>84</td>
</tr>
<tr>
<td>ACD (fresh)</td>
<td>4</td>
<td>13.8</td>
<td>77</td>
</tr>
<tr>
<td>ACD (stored)</td>
<td>6</td>
<td>32.3</td>
<td>44</td>
</tr>
<tr>
<td>Frozen-deglyc.</td>
<td>9</td>
<td>23.0</td>
<td>76</td>
</tr>
</tbody>
</table>

Sodium Exchangeability (Table 6)

Solomon has reported that the exchangeability of erythrocyte sodium with added radionuclide is not complete. In the present study, the mean three-hour exchangeability of fresh bloods ranged from 77 to 84 per cent and no statistically significant differences in mean exchangeability among the fresh bloods studied were observed. Stored ACD cells had a considerably higher sodium content and a lower exchangeability. Frozen-deglycerolized blood presented the unique situation of extreme variability of exchange. Individual values varied from 57 to 100 per cent and were not correlated with intracellular sodium content or duration of freezing. Despite their high sodium content, the mean exchangeability of frozen deglycerolized blood was comparable to that of fresh erythrocytes.

Discussion

The preservation of normal cation flux and electrolyte concentrations in frozen-deglycerolized erythrocytes provides strong evidence for their retention of the active processes involved in electrolyte transport. Since no decrease in cation flux of stored ACD erythrocytes occurred, it may be presumed that these cells are also capable of normal metabolic activity and their markedly abnormal electrolyte distribution is partially or totally reversible. The present data, combined with previous reports showing normal in vivo survival, oxygen carrying capacity and phosphate partition characteristics indicate that frozen-deglycerolized erythrocytes are metabolically active and are suitable for single and massive transfusions and for procedures requiring pump oxygenators.

In addition to long storage time and excellent retention of erythrocyte electrolyte concentrations, the normal extracellular potassium concentration of frozen-deglycerolized blood represents a distinct advantage in transfusing hyperkalemic patients and in massive blood volume replacement. The production procedure is versatile and it is theoretically possible, by modifications in technic, to produce frozen-deglycerolized blood with a wide range of electrolyte concentrations and pH. If this proves feasible, it should be possible to produce units that precisely meet the needs of anemic patients with a variety of disturbances in electrolyte and acid-base balance.

Summary

Cation flux and the extracellular and erythrocyte concentrations of sodium,
potassium, calcium, magnesium, chloride, and acid soluble phosphorus were measured in fresh blood collected in various anticoagulant solutions, ACD blood stored at 4 C, and in frozen-deglycerolized blood stored at −80 to −120 C. Considerable variations in concentrations of extracellular and erythrocyte electrolytes were encountered due to certain characteristics of the anticoagulants employed and to the effects of storage, temperature, pH, and extracellular electrolyte concentration on erythrocyte electrolyte concentration. In contrast to ACD blood stored at 4 C, frozen-deglycerolized blood 1 day to 32 months old had slightly elevated erythrocyte concentrations of sodium and chloride and normal erythrocyte concentrations of potassium and phosphorus. Both ACD and frozen-deglycerolized blood had normal sodium and potassium fluxes. These data show that prolonged freezing and glycerolization of erythrocytes do not significantly impair the active transport processes responsible for maintenance of cellular electrolyte concentration.

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