Storage of Platelet Concentrates Using Ion Exchange Resin Charged With Dibasic Phosphate

By Barbara Kotelba-Witkowska, Denise M. Harmening-Pittiglio, and Charles A. Schiffer

Platelet concentrates were prepared at twice the normal concentration and stored at room temperature for 7 days in either standard bags (controls) or bags to which 1 or 2 g of Amberlite resin beads charged with dibasic phosphate had been added. The resin beads served as a buffer system by providing a "slow release" form of phosphate ions as well as by binding CO₂ produced during platelet metabolism. Control platelets demonstrated rapid falls in pH, ATP content, morphology score, and thrombin-induced nucleotide release after 24 hr of storage with a fall in pH to less than 6.0 by day 3. Profound ultrastructural changes and a rise in pO₂, suggesting loss of platelet viability, accompanied these changes. In contrast, the resin-stored platelets remained near normal after 24 hr of storage, with preservation of discoid morphology, 95% of ATP levels, excellent ultrastructural appearance, and evidence of continued oxygen consumption after 3 days of storage. Even after 7 days of storage, ATP levels remained greater than 50% of baseline and ultrastructurally intact platelets were seen. In the 1-g resin bags the pH remained at baseline levels (6.9–7.0), while there was a rise in pH in the 2-g resin bags. These results demonstrate the beneficial effects of maintaining a higher pH during platelet storage and provide a new approach to studying the metabolic changes that occur during longer term storage.

AT THE PRESENT TIME, preservation of platelet concentrates is optimal at storage temperatures of 20–22°C with constant agitation and platelet concentrations between 0.8 and 1.6 × 10¹²/liter. Nonetheless, a substantial number of platelets stored in these conditions for 48–72 hr lose their discoid shape, ATP, ADP, as well as in vitro functions such as aggregation and the release reaction.⁹ Lactate produced via glycolysis as well as CO₂ produced from the Krebs cycle accumulate, which is associated with a decrease of the pH and a loss of platelet viability at pHs of 6.0 or below.⁴⁻⁷ Although recently described polyethylene containers that permit more rapid gas exchange can eliminate pH fall during storage of platelet concentrates, it is necessary to maintain an external 10% CO₂ atmosphere thereby decreasing the practicality of this approach.⁹

An alternative approach would be to maintain a more constant energy supply for the platelets by maintenance of intracellular ATP levels. The successful results obtained with Amberlite ion exchange resins charged with dibasic phosphate in the storage of whole blood suggested the use of the same resin system for the storage of platelet concentrates in that blood stored with the resin for up to 28 days maintains high pH, 2,3-DPG levels, and adequate levels of ATP.¹⁰ The Amberlite ion exchange resins provide a “slow release” source of inorganic phosphate that acts as a buffering system by combining with hydrogen ions in solution. In addition, the resins themselves can bind CO₂ and possibly organic anions thereby further increasing the buffering capacity of the system.¹⁰ In this article we will describe preliminary results utilizing the Amberlite exchange resin in the storage of platelet concentrate that demonstrate that normal pH and only moderate decreases in ATP can be achieved in concentrates stored for up to 7 days in the presence of resin.

MATERIALS AND METHODS

Platelet Concentrates (PC)

PC from ABO and Rh identical random donors were prepared by a manual double platelepheresis method using ACD-A as an anticoagulant. The PC were prepared in final volumes of 20 ml (i.e., half the usual volume resulting in twice the usual concentration) in an effort to maximally “stress” the preservative capability of the resin. Units of PC from three to four donors were pooled and centrifuged at 180 g for 3 min to remove residual leukocytes and erythrocytes. The supernatant platelet suspension was then divided in equal portions (40 ml each) and transferred to plastic blood bags with or without (control) ion exchange resin.

Platelet Bags

Both 300-ml Fenwal (PL-146 Plastic) and Cutter Transfer Bags (CL-3000) were utilized. The bags were initially opened, filled with 1 or 2 g of Amerlite IR-45 ion exchange resin, sealed, and heat sterilized. The resin had been charged with dibasic phosphate as previously described.¹⁰ Bags with or without resin were stored during 7 days of storage at room temperature (20%–24°C) with constant horizontal agitation at approximately 70–80 cycles/min. Thus, platelets and the inert resin beads, which are approximately 400μm in diameter were continually mixed in free suspension with each other.

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buffered with 0.1 s-collidine). The samples were post-fixed in 2% osmium tetroxide. The samples were washed in sucrose buffer (0.2 M 50%, 70%, 95%, absolute (2 times) and propylene oxide (2 times) for 30 days of storage in l-g resin bags and controls and fixed in 4% formaldehyde, 1% gluteraldehyde, in a 1:1 ratio at 4°C for 4-6 hr.

Platelet counts. Platelet counts were performed electronically in duplicate using a Coulter Thrombo Counter (Coulter Electronics Inc., Hialeah, Fla.).

Morphology. Morphological evaluation by phase microscopy was done using the scoring method described by Kunicki et al. In this method 200 individual platelets are evaluated morphologically, scored, and then totaled, with a maximum score of 400 being accorded to a preparation in which all of the platelets are normal and discoid in shape with progressively lower scores for spherical, dendritic, or ballooned platelets.

ATP content. Total ATP content was determined as described by Bucher using a phosphoglycerate kinase-glyceraldehyde phosphate dehydrogenase enzyme coupled system (Sigma Chemicals, St. Louis, Mo.).

Thrombin-induced release. Aliquots of the platelet concentrate were washed twice with 0.077 M EDTA-Tris saline buffer (pH 7.4), and their total adenine nucleotide release after stimulation with 0.06 NIH units of thrombin/ml (Sigma Chemicals) was assayed as previously described by Murer.

Estimation of pH, pO2, pCO2, and HCO3. These estimations were done using a Corning 165 Blood Gas Analyzer. The pH was measured at 37°C and temperature conversion to 22°C was calculated by the method of Rosenthal.

Electron microscopy. Platelets were obtained after 0, 1, 3, and 7 days of storage in 1 g resin bags and controls and fixed in 4% formaldehyde, 1% gluteraldehyde, in a 1:1 ratio at 4°C for 4-6 hr. The samples were washed in sucrose buffer (0.2 M sucrose in 0.1 M s-collidine). The samples were post-fixed in 2% osmium tetroxide buffered with 0.1 M s-collidine for 90 min. Prior to dehydration, tissues were stained en bloc with uranyl acetate for 1 hr. Platelets were dehydrated through a graded series of ethanol mixtures [35%, 70%, 95%, absolute (2 times) and propylene oxide (2 times)] for 30 min each time. The platelets were then infiltrated with a mixture of 50% propylene oxide and Epon for 1 hr and infiltrated into a mixture of 70% Epon: 30% propylene oxide for 24 hr. Platelets were embedded and polymerized at a temperature of 60°C for 48 hr. Toluidine-blue-stained 0.5μm Epon sections were prepared for light microscopy to select the areas for cutting thin sections for electron microscopy. Thin sections were double-stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope.

Statistical analysis. Statistical comparisons were done using a Student's t test for paired observations.

RESULTS

The results using 1 g of resin in either Fenwal or Cutter bags are compared to Fenwal bag controls in Table 1. Except for slight discrepancies in the changes in platelet count, there are no important differences between the Fenwal and Cutter resin bags and hereafter results will be referred together as “resin” results. Table 2 compares results using 2 g of resin in a Fenwal bag compared to Fenwal control. Because the 1 and 2 g resin experiments were done at different times, separate controls are included in each table. It should be noted that the control values were highly consistent in these sets of studies.

Platelet Count

During the 7 days of study, a decrease in platelet count was observed with a drop in the control bags to approximately 90% of baseline. The initial high baseline platelet counts achieved by halving the initial volume of the platelet concentrates should be noted. A more profound drop in platelet count was noted in both the 1 and 2 g resin bags that was statistically significant from controls by day 3 of storage in the 2-g resin experiment (p < 0.05). As can be seen in Table 1, the

<table>
<thead>
<tr>
<th>Day of Storage</th>
<th>Platelet Count (x 10^11/Liter)</th>
<th>pH</th>
<th>ATP (μmole/10^11 Platelets)</th>
<th>Thrombin-Induced Nucleotide Release (μmole/10^11 Platelets)</th>
<th>Morphology Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fenwal</td>
<td>Cutter</td>
<td>Control</td>
<td>Fenwal</td>
</tr>
<tr>
<td>0 (Baseline)</td>
<td>3.6 ± .3</td>
<td>—</td>
<td>—</td>
<td>6.68 ± .1</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>4.1 ± .2</td>
<td>3.8 ± .3</td>
<td>6.35 ± .3</td>
<td>7.35 ± .1</td>
<td>4.5 ± .4</td>
</tr>
<tr>
<td>3</td>
<td>3.1 ± .06</td>
<td>2.0 ± .2</td>
<td>5.73 ± .07</td>
<td>7.32 ± .08</td>
<td>1.2 ± .03</td>
</tr>
<tr>
<td>7</td>
<td>3.4 ± .3</td>
<td>1.6 ± .06</td>
<td>5.82 ± .01</td>
<td>7.4 ± .05</td>
<td>.6 ± .06</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM of 3 experiments, except on day 7 where n = 2.
fall in platelet count was not quite as rapid in the Cutter bags. Examination of the resin beads under phase microscopy did not demonstrate platelets coating the beads.

**pH**

Within 24 hr of storage at high concentration, the pH in the control bags had decreased significantly and was less than 6.0 by day 3. In contrast, the 1-g resin bags maintained a pH that did not differ significantly from baseline even after 7 days of storage. A statistically significant increase in pH was observed using 2 g of resin (Table 2) beginning as early as 24 hr after storage ($p < 0.005$).

**ATP**

The amount of total ATP decreased to approximately 80% after 24 hr, to 19% after 3 days, and to 10% of baseline after 7 days of storage in control preparations. There was no significant fall in the ATP levels in the 1-g resin bags until 3 days of storage, and in these bags the total ATP content was still approximately 50%-60% that of baseline after 7 days. The results using 2 g of resin were similar to the 1-g resin results.

**Thrombin-Induced Release of Adenine Nucleotide**

After 24 hr of preservation of control platelets, only 45% of baseline thrombin-induced release was noted. Thrombin-induced release was also decreased in all of the resin bags by 24 hr, but to a lesser extent. No release was observed after 3 days of storage in either the control or resin bags.

**Morphology Score**

There was a profound change in the morphology of the control cells by the third day of storage with the overwhelming majority of the cells being either ballooned or rounded with large numbers of dendritic projections. Although the morphology scores fell in the resin bags as well, the score remained statistically significantly better than controls at 3 and 7 days. In addition, approximately 30% of the platelets retained a discoid configuration in the resin bags at 7 days.

**$pO_2$, $pCO_2$, Bicarbonate**

The $pO_2$ in the control bags was 0 during the first 24 hr of storage and then rose after the third and seventh day of platelet preservation. The addition of 1 or 2 g of resin maintained the low $pO_2$ level during 3 days of storage with a rise occurring only on the seventh day (Table 3), when the $pO_2$ reached approximately 50% of the amount observed in control bags in both the 1 and 2 g resin bags.

The $pCO_2$ rose during the first 24 hr of storage in the control bags and then began to decrease, almost reaching 0 mm Hg by day 7. No rise in $pCO_2$ was seen in any of the resin experiments. Rather, the $pCO_2$ fell during the first day. Continued falls similar to the control bags occurred during the third and seventh day of storage.

$HCO_3$ was gradually depleted to zero during 7 days of storage in control bags. In contrast, $HCO_3$ levels remained between 30% and 60% of controls at day 7 in the 1 and 2 g resin bags, respectively.

**Electron Microscopy**

The control cells were normal ultrastructurally and were almost entirely discoid in shape at the initiation of the experiment (not shown). After 24 hr of storage, most control platelets had lost their discoid appearance and were rounded in shape with multiple cytoplasmic projections (Fig. 1). By day 3 of storage, the control platelets were grossly abnormal with evidence of marked degranulation, absence of microtubules, and multiple broad cytoplasmic projections (Fig. 2). Many platelets were disintegrated or ballooned, and large amounts of platelet debris were visible. By day 7, there was total degranulation of all the control platelets with obvious membrane breakage (Fig. 3).

### Table 3. $pO_2$, $pCO_2$, $HCO_3$: 1 and 2 Gram Resin Studies

<table>
<thead>
<tr>
<th>Days of Storage</th>
<th>$pO_2$ (mm Hg)</th>
<th>$pCO_2$ (mm Hg)</th>
<th>$HCO_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fenwal</td>
<td>Cutter</td>
</tr>
<tr>
<td>1 g of resin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>68 ± 4</td>
<td>1.3</td>
<td>.3</td>
</tr>
<tr>
<td>7</td>
<td>135</td>
<td>82</td>
<td>59</td>
</tr>
<tr>
<td>2 g of resin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>34.3 ± 19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>137 ± 1.5</td>
<td>56 ± 3</td>
<td>2 ± .5</td>
</tr>
</tbody>
</table>

Results are means ± SEM of 3 experiments except on day 7 with 1 g of resin where $n = 1$. 
In contrast, the overall morphology of the resin-preserved platelets remained relatively normal after 3 days of storage. In general, the Cutter-resin stored platelets (Figs. 4–6) had somewhat better morphological preservation than the Fenwal-resin platelets. After 24 hr, most of the resin platelets remained discoid with preservation of normal numbers of granules (Fig. 4). Discoid shape was maintained after 72 hr of storage, and definite microtubules were seen in many of the platelets in both the Fenwall and Cutter bags (Fig. 5). By day 7, the number of apparently nonviable platelets had increased and most of the remaining platelets were rounded, without obvious microtubules and with dilated canalicular systems (Fig. 6). Nonetheless, the morphology of the “resin” platelets was clearly better preserved than the control platelets at 7 days.
PLATELET STORAGE

Fig. 5. Platelets stored with resin for 3 days remained discoid with near-normal numbers of granules in most platelets when compared to baseline. Some apparently damaged platelets (center and upper left) were noted (x3960).

Fig. 6. Platelets stored with resin for 7 days exhibited considerable degranulation and loss of discoid shape, although a number of intact platelets remain (x3840).

DISCUSSION

During 7 days of storage of platelet concentrates at double the usual concentration of platelets in standard Fenwal polyvinyl chloride containers, a marked decrease in pH from baseline was observed presumably as a result of the production of lactic acid through the glycolytic pathway as well as CO₂ production through the Krebs cycle. After 3 days, the pH was 5.8, a level at which platelets are felt to be nonviable. The greatest O₂ consumption, CO₂ production, and pH decreases were observed during the first 24 hr. These are similar to observations made by others during preservation of platelet concentrates with high platelet counts. The extremely low bag pH, even after short periods of storage, which is compatible with previous estimates of the rate of platelet oxygen consumption, is evidence of the increased metabolic activity of platelets stored in high concentrations. It is presumed that the increased platelet concentration results in an increased lactic acid production by the Pasteur effect. High CO₂ production, which exceeds the capacity for its diffusion across the standard plastic containers, also contributes to the fall in pH. By the third day when the pH had fallen below 6.0, a decrease in the consumption of O₂ and production of CO₂ was noted due to the decrease in viability of the stored platelets. Simultaneously, striking changes in the morphology of the platelets were observed, which after 3 days of preservation reached less than 50% of the initial morphology score values with almost complete loss of discoid shape accompanied by marked degranulation.

In association with the decrease of pH after 24 hr and 3 days, a striking decrease in the total amount of ATP/platelet to 80% and 19% was observed, respectively. From previously published observations of platelet concentrates stored at +4°C and +22°C, it is known that the reduction of the total ATP level is secondary to the degradation of metabolic ATP to hypoxanthine. It has previously been shown that with a total ATP decrease of only 10% during 24 hr of storage, the metabolically active ATP is reduced by 40%–50%. It is likely therefore that close to total depletion of metabolically available ATP occurred in the control platelets. This decreased “energy source” might well account for the observed deterioration of in vitro platelet function. The present results are consistent with this hypothesis because the amount of adenine nucleotides released by thrombin decreased to 45% after 24 hr and to 0% after 3 days of preservation. Changes in membrane receptors for thrombin, which have been demonstrated in platelets that have been altered by storage, could also have contributed to the decreased responsiveness to thrombin. The ultrastructural changes seen on day 3 in the resin-stored platelets are consistent with the latter possibility.

The addition of the resin markedly enhanced the buffering capacity of the system even with the high concentration of platelets used. The use of 1 g resin kept the pH of platelet concentrates at 7.0 during the 7 days, comparable to baseline levels. The use of resin
was associated with a low pH2O in the bag till the third day, reflecting the continued presence of metabolically active platelets. No striking increase of CO2 was observed because the produced CO2 was probably combined directly by the resin particles. The lactic acid produced could also have attached to the resin molecules in exchange for the released phosphate anions, although these experiments provide no direct evidence for this hypothesis.

The maintenance of the pH at 7.0 by the resin also resulted in improved morphology of preserved platelets with maintenance of discoid shape and microtubules for 3 days at double platelet concentrations. Even after 7 days of storage, the majority of platelets were granulated and morphologically intact. Whether platelets stored under these conditions for 7 days could circulate posttransfusion is speculative, although there does appear to be a good relationship between posttransfusion recovery and platelet morphology in other experimental conditions.5

Contrary to the values obtained in control platelets, the total amount of ATP during the first 24 hr of preservation with the resin system was maintained at baseline levels. This could be accounted for by an incorporation of phosphate anions released from the resin via R-5-P (ribose-5-phosphate) to PRPP (phosphoribosyl-1-pyrophosphate). However, according to Holmsen, AMP and R-5-P incorporate p32-orthophosphate at a very slow rate, indicating very low or absent de novo synthesis of adenine nucleotides by platelets.18,19 Another explanation for the maintenance of the ATP levels is maintenance of adequate pH by the resin, which by enhancing the enzyme activity of the glycolytic pathway, especially hexokinase and phosphofructokinase, allowed greater glucose utilization during the storage period.10 Although the pH did not change appreciably during the whole 7-day period of storage with 1 g of resin, the total ATP level after the third and seventh day of preservation was only 77% and 56% of the baseline level, respectively. Platelets produce ATP not only by glycolysis but through mitochondrial oxidative phosphorylation as well.20 From the pH2O data obtained on the seventh day, it seems however that the oxidative ATP production was diminished as evidenced by the pH2O increase in both the 1-g and 2-g resin platelets.

White et al. have also recently presented data suggesting a salutary effect of higher pH on platelet function, nucleotide content, and morphology during long-term storage.21 By initially collecting platelet-rich plasma at a high pH (7.7–7.8) with maintenance of pH between 7.4 and 7.6 by the daily addition of NaOH or HCl, these investigators were able to preserve platelets in special plastic flasks for 14–21 days. Discoid shape in 5%–20% of platelets, serotonin and adenine nucleotide levels that were 50%–70% of fresh platelets, and the ability to synthesize adenine nucleotides were noted after 21 days of storage. In comparison to the present resin study, however, lower platelet concentrations (~380,000/µl) and a more cumbersome method of pH correction were used, and it is unclear whether this approach could be easily incorporated into blood banking practice.

Two grams of resin produced higher pH values (7.32–7.4) during the 7 days of storage than did 1 g. This was probably the effect of greater combining of CO2 by resin molecules that resulted in lower pCO2 during the whole preservation period. This binding of CO2 by the resin is also illustrated by the marked differences in bag pCO2 between the control and resin bags during the first 24 hr of storage, which is the period of time that the platelets were most metabolically active and produced the greatest amounts of CO2. The HCO3 level was also highest after 7 days of platelet preservation when 2 g of resin was used. This may reflect the increased buffering by the resin with relative “sparing” of the HCO3 buffer. However, no improvement was observed in platelet count, the total ATP level, release of adenine nucleotides or pH2O tension when 2 g of resin were used in comparison to the results obtained with 1 g of resin. Disc to sphere transformation, degranulation possibly due to aggregation, and the release reaction have been noted in platelets stored at higher pHs.14 In the present experiments, the morphology, as judged by phase microscopy, was similar in the 1 and 2 g resin bags at 3 and 7 days. Electron micrographs were not done in the 2-g preparations, and it is possible that more subtle changes due to the higher pH were not detected by phase microscopy.

The use of Cutter bags with 1 g of resin gave similar results to Fenwal bags with either 1 g or 2 g of resin with the exception of statistically significantly higher platelet counts in the Cutter bag. No obvious explanation is available for the fall in platelet count, as microaggregates occurred infrequently and the platelets did not seem to bind to the resin. The apparently “stable” platelet counts in the control bags may in part be due to counting of some of the platelet fragments (Figs. 2 and 3) with the electronic particle counter. It is also possible that repeated contact with the resin beads during agitation produced increased shear forces resulting in increased platelet destruction. Placement of the beads in a small dialysis membrane pouch within the bags could help to sort out this question. A similar fall in platelet count was noted by White et al.21 after 5–10 days of storage in their system.
In conclusion, this study on the effect of the resin on the metabolism and morphology of platelets during long-term storage at ambient temperature showed the importance and benefit of maintenance of higher pH due to the removal of lactic acid and CO₂ metabolites from the plasma that was associated with a maintenance of the ATP level of these cells. Although the mechanism of the preservation of ATP levels is not clear, the use of the resin-charged phosphate anion appears to open a number of new options in the study of platelet storage and metabolism.

REFERENCES

Storage of platelet concentrates using ion exchange resin charged with dibasic phosphate.

B Kotelba-Witkowska, DM Harmening-Pittiglio and CA Schiffer