Adenine and Guanine Nucleotide Metabolism During Platelet Storage at 22°C

By C.M. Edenbrandt and S. Murphy

PLATELETS are commonly stored for transfusion as 50-mL platelet concentrates (PCs) at 22 ± 2°C with constant agitation in autologous plasma. In the 1970s, platelets were stored in first-generation containers (such as Fenwal's PL-146; Deerfield, IL) constructed of plastics that had inadequate permeability to oxygen to meet the demands of the cells within. In such containers, lack of oxygen led to accelerated production of lactic acid, decreases in medium pH, and cell death when pH fell below 6.0. This often results in postinfusion viability in vivo.7 Further studies showed that an increase in plasma hypoxanthine.8 In 1962, Simon et al reported that the addition of adenine (750 μmol/L final concentration) at the initiation of storage increased ATP levels at the end of storage and improved postinfusion viability in vivo.7 Further studies showed that the added adenine was incorporated gradually into adenine nucleotides so that half of the original adenine remained at the end of storage, half of the radioactivity was transferred to hypoxanthine (45%) and GTP + GDP + xanthine (5%) by the time storage was completed. The isotopic data were consistent with the presence of a radioactive (metabolic) and a nonradioactive (storage) pool of ATP + ADP at the initiation of storage with each pool contributing approximately equally to the decline in ATP + ADP during storage. The results suggested a continuing synthesis of GTP + GDP from ATP + ADP, explaining the slower rate of fall of GTP + GDP relative to the rate of rise of plasma xanthine. Throughout storage, platelets were able to incorporate 14C-hypoxanthine into both adenine and guanine nucleotides but at a rate that was only one-fourth the rate of hypoxanthine accumulation. All of these data should be helpful in improving the function and viability of PC as currently stored for 5 days, in devising methods for storage beyond 5 days, and in the development of synthetic media for PC storage.

The major cause (or causes) of such deterioration during storage is not known. One possibility would be failure of energy metabolism with depletion of metabolic adenine triphosphate (ATP) and consequent deterioration of cellular function. Parallels could be drawn to storage of red cells. As early as 1947, Rapoport showed that red cell storage was accompanied by ATP breakdown to hypoxanthine.8 In 1962, Simon et al reported that the addition of adenine (750 μmol/L final concentration) at the initiation of storage increased ATP levels at the end of storage and improved postinfusion viability in vivo.7 Further studies showed that the added adenine was incorporated gradually into adenine nucleotides so that half of the original adenine remained after 6 weeks.4 With adenine supplementation, accumulation of hypoxanthine began earlier so that higher levels, approximately 200 μmol/L, were reached after 6 weeks of storage. Thus, there was evidence that ATP depletion played a role in loss of red blood cell viability during storage and that manipulation of the system by addition of adenine improved results.

There have been only a few studies of adenine nucleotide metabolism in PC storage and none of storage in “second generation” containers. Rao et al reported a fall in platelet ATP and ADP during 3 days of storage in “first generation” containers.9 Interpretation of such studies is complicated because Holmsen,10 Ireland,11 and Holmsen and Rozenberg2 have shown that there are at least two pools of adenine nucleotides in platelets. One pool, the cytoplasmic metabolic pool, rapidly incorporates 32P and 14C-adenine, whereas the storage pool, consisting of the granular content of ATP and ADP, is not labeled, at least in experiments performed over hours as opposed to days. The metabolic pool is thought to meet the platelet’s ongoing metabolic needs, whereas the storage pool resides in dense granules that are secreted when the cell is stimulated by agonists. The meta-
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that some of the depletion had to be from the storage pool. In this study, we have examined adenine nucleotide metabolism during PC storage for 7 days in second-generation containers. Using a high-pressure liquid chromatography (HPLC) technique, we have been able to measure adenosine monophosphate, adenine, and hypoxanthine, as well as ATP and ADP. The HPLC method also allowed us to measure guanosine triphosphate (GTP), guanosine diphosphate (GDP), and xanthine so that we could provide the first data on guanine nucleotide metabolism during PC storage as well.

MATERIALS AND METHODS

Material. Perchloric acid (70%), potassium carbonate, ammonium phosphate, and ammonium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). Cellulose filters, with a pore size of 0.45 μm, were from Millipore (Bedford, MA). Purine and pyrimidine bases, nucleosides, nucleotides, hexokinase (E.C.2.7.1.1.), myokinase (E.C.2.7.4.3.), xanthine oxidase (E.C.1.2.3.2.), purine nucleoside phosphorylase (E.C.2.4.2.1.), adenosine deaminase (E.C.3.5.4.4.), guanase (E.C.3.5.4.3.), 5'-nucleotidase (E.C.3.1.3.5.), and uricase (E.C.1.7.3.3.) were from Sigma (St Louis, MO). For the preparation of standard solutions, all purines and pyrimidines were individually dissolved in double-distilled water to a concentration of 1 mol/L (stock solution) and stored at −70°C.

[8-14C]adenine hydrochloride (53 mCi/mmol; 50 μCi/mL) was from New England Nuclear (Boston, MA) and [8-14C]hypoxanthine (54 mCi/mmol) was from Amersham (Arlington Heights, IL). The radiopharmaceutical purity of these compounds was checked by our HPLC system (see below) and found to be greater than 98% and 99%, respectively. Bio-HP, Scintiverse, liquid scintillation fluid was from Fisher Scientific.

Preparation of PC. Platelet-rich plasma (PRP) was prepared from blood obtained from human volunteers and anticoagulated with either citrate-phosphate-dextrose (CPD) or CPD-adenine (17.3 mg adenine/450 mL whole blood). PCs were prepared from PRP as previously described. The PC volume was so adjusted that the average volume during storage (considering sample volumes to be removed for study) would be 50 mL. PCs were stored in commercial available transfer packs made from either PL-146 or PL-732 chromographed. For quantitative measurements, peak heights were measured manually and compared with a standard curve for the corresponding compound. The standard curve (peak height versus absorbance) was found to be linear (r > 0.997) for all compounds over the concentration range of interest in this study.

Ammonia measurement. Ammonia was measured using the ammonia enzymatic determination kit (Sigma).

Radiolabeling studies. [8-14C]adenine hydrochloride, 0.5 mL (25 μCi), was added at the initiation of storage to each of four PCs in PL-732 with CPD as the primary anticoagulant. Samples were drawn from all four PCs immediately after mixing, after 14 and 24 hours, and after 3, 5, and 7 days of storage. For radioactivity measurements, the injection volume of sample extract had to be increased to 75 μL to obtain sufficient counts above background in each peak. The eluent of each chromatographic peak was manually collected and mixed with 10 mL liquid scintillation fluid and counted in a liquid scintillation counter (Liquid Scintillation Spectrometer, Packard Instruments, Downers Grove, IL). From the data obtained for both chemical amount and radioactivity, the specific activity of each purine compound was calculated. Unfortunately, the increased injection volume produced overlap between the three groups of PCs (LD 1.1 ± 0.1 × 1012/L, LN 1.1 ± 0.1 × 1012/L, and LE 1.2 ± 0.1 × 1012/L) or in erythrocyte count (LD 1.7 ± 0.6 × 1012/L, LN 2.0 ± 0.3 × 1012/L, LE 2.7 ± 0.6 × 1012/L).

**Purine extraction.** Purine compounds were extracted from samples of PC, PFP obtained by centrifugation of a PC sample at 12,000 g for 1 minute in an Eppendorf centrifuge (model 5414, Eppendorf GMBH, Hamburg, FRG), PFP that had been stored as a control, and PC platelets that had been washed by the method of Walsh. Sample, 0.9 mL, was extracted with 0.1 mL of 6.6 N HClO4 on ice. The protein precipitate was spun down at 12,000g for 3 minutes. The supernatant (0.5 mL) was neutralized with 65 μL of 2 mol/L potassium carbonate and the precipitate removed by centrifugation. All procedures were carried out on ice and the extracts were stored at −70°C until analyzed.

**HPLC.** Our HPLC system consisted of a model 6000A solvent delivery system, a model U6K universal injector, a model 450 variable wavelength detector (Waters, Milford, MA) and an Omnisccribe pen recorder (Houston Instruments, Houston, TX). The chromatographic separation was performed on a 30 cm × 3.9 mm internal diameter (ID) stainless steel column prepackaged with 10-μm silica particles to which octadecyl groups have been bonded (MBondapak C18, Waters). The 0.2 mol/L NH4H2PO4 eluent was made up in double-distilled water, adjusted to pH 5.3 with NH4OH, filtered, and degassed before use. Samples were analyzed with isocratic elution at a flow rate of 1.0 mL/min. Five microliters of sample or a standard solution were manually injected using Hamilton syringes (Hamilton, Reno, NV). Chromatographic peaks in experimental samples were detected by absorbance at 260 nm and were identified by comparison to known standards regarding retention times, ratios of absorbance at 260 nm versus 280 nm, and 260 nm versus 230 nm. Peak identification was also performed by addition to the sample of a known amount of standard to demonstrate a quantitative increase in the height of a specific peak (spiking). Furthermore, specific enzymes (see above) were added to an aliquot of the sample that was buffered to an appropriate pH. The sample was incubated at 37°C for the required time and was then rechromatographed. The disappearance of the substrate and the appearance of the appropriate reaction product confirmed both the identity and the purity of the chromatographic peak. Also, to identify a single peak in a sample, this peak was manually collected after chromatography, incubated with a specific enzyme, and rechromatographed. For quantitative measurements, peak heights were measured manually and compared with a standard curve for each compound. The standard curve (peak height versus concentration of purine compound) was constructed using commercial standards and was found to be linear (r > 0.997) for all compounds over the concentration range of interest in this study.

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**Radionuclide studies.** [8-14C]adenine hydrochloride, 0.5 mL (25 μCi), was added at the initiation of storage to each of four PCs in PL-732 with CPD as the primary anticoagulant. Samples were drawn from all four PCs immediately after mixing, after 14 and 24 hours, and after 3, 5, and 7 days of storage. For radioactivity measurements, the injection volume of sample extract had to be increased to 75 μL to obtain sufficient counts above background in each peak. The eluent of each chromatographic peak was manually collected and mixed with 10 mL liquid scintillation fluid and counted in a liquid scintillation counter (Liquid Scintillation Spectrometer, Packard Instruments, Downers Grove, IL). From the data obtained for both chemical amount and radioactivity, the specific activity of each purine compound was calculated. Unfortunately, the increased injection volume produced overlap between the

bolistic pool has an ATP:ADP ratio of 8:1, whereas the storage pool ratio is 2:3. Because the depletion of ADP was relatively greater than ATP in the study by Rao et al, it was concluded that some of the depletion had to be from the storage pool.
neighboring peaks, ATP and ADP, and GTP and GDP. Therefore, the radioactivity in ATP + ADP, and in GTP + GDP, are given as the sum of radioactivity of the two compounds.

To determine whether hypoxanthine could be incorporated into adenine and guanine nucleotides of platelets during storage (the salvage pathway), 3-mL samples were drawn from each of two PCs (PL-732, CPD) on days 0, 1, 3, 5, and 7 of storage. [8-14C]-hypoxanthine was added to each sample in an amount designed to achieve a specific activity of about 20 mCi/mmol of hypoxanthine immediately after mixing. The samples were incubated at 22°C for 4 hours in plastic tubes with intermittent agitation. Chemical amount and radioactivity of each purine compound were analyzed immediately after mixing and after 4 hours' incubation, as described above.

Statistical analysis. Rates of change during storage were determined as slopes of the "best fit" curve using simple linear regression. Rates of change were determined for both absolute concentrations and for concentrations expressed as percentage of the concentration at the start of storage. The latter procedure allowed greater sensitivity in detecting statistically significant differences, because for some variables such as adenine nucleotide content of fresh platelets there is considerable variation among normal individuals. The data were analyzed using unpaired and paired t-test and F-test for some variables such as adenine nucleotide content of fresh platelets. Within-day precision was (CV) ATP 1.8%, ADP 2.2%, AMP 6.5%, GTP 2.5%, GDP 7.5%, hypoxanthine 2.9%, and xanthine 8.6%. Between-day precision of the method was assessed by analyzing the same fresh PC on five different days (CV): ATP 2.0%, ADP 2.6%, AMP 3.7%, GTP 2.2%, GDP 5.4%, hypoxanthine 2.4%, and xanthine 16.1%.

Characteristics of PC at the start of storage. Day 0 values of adenine and guanine nucleotides in platelets are given in Table 1. There were no significant differences when the two plastics and anticoagulants were compared. Hypoxanthine and xanthine concentrations in PC–plasma are also shown in Table 1. There was significantly more hypoxanthine in plasma from PCs when adenine was included in the anticoagulant (P < .05) in both PL-732 and PL-146 containers. There was no significant difference in xanthine among the PCs.

Changes during storage. In the PCs studied, there was no significant change in platelet count during storage. pH did not fall below 6.9 during 7 days of storage in PL-732 or below 6.3 during 3 days of storage in PL-146. Mean platelet volume did not change during storage in PL-146 CPD-adenine or PL-732 with either anticoagulant, but increased by a mean of 20.5 ± 9.1% in PL-146 CPD. The latter is consistent with previous work, showing cellular swelling when pH fell to 6.5 to 6.7.

During PC storage, there was a steady decrease in platelet adenine and guanine nucleotides after the first 24 hours of storage (Fig 1). In CPD-adenine (Fig 1A), we found an increase of ATP (P < .05) from days 0 to 1 in both PL-146 and PL-732. This was not observed with CPD. Continuous accumulation of hypoxanthine and xanthine was found in the

<table>
<thead>
<tr>
<th>Table 1. Day 0 Values of Adenine and Guanine Nucleotides in Platelets (μmol/10^{11} platelets), and Hypoxanthine, Xanthine, and Adenine in Plasma (mol/L)</th>
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<td>ATP</td>
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<td>Hypoxanthine</td>
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<td>Xanthine</td>
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<td>Adenine</td>
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Values are means ± SD. Abbreviation: ND, not detectable.
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Fig 1. Platelet nucleotide metabolism during storage for 7 days in PL-732 containers. (A) Platelet ATP, ADP, and AMP. (B) Platelet GTP and GDP. (C) Hypoxanthine, xanthine, and adenine in plasma from PC. Closed and open symbols and uninterrupted and interrupted lines refer to results when primary anticoagulants were CPD and CPD-adenine, respectively. Values are given as mean ± SD.

To allow quantitative comparison between the rates of decline of adenine and guanine nucleotides in platelets, which are expressed in μmol/10¹¹ platelets, and the rates of increase of hypoxanthine and xanthine in plasma, which are expressed in μmol/L, we used the platelet concentration of each bag to convert the adenine and guanine nucleotide concentrations to μmol/L. Table 2 gives these rates of change and indicates which are significantly different from zero. When CPD was the primary anticoagulant, the rate of decrease of total adenine nucleotides was balanced quantitatively by the rate of increase in hypoxanthine. In CPD-adenine, adenine continuously decreased and the accumulation of hypoxanthine was augmented twofold to threefold relative to CPD (P < .01 for both PL-146 and PL-732). The sum of the decrease in adenine nucleotides and adenine balanced quantitatively the increase of hypoxanthine. Xanthine accumulated in all four PC groups with no statistically significant difference among them. In all four groups, the rate of decrease of total guanine nucleotides was somewhat less than the rate of increase in xanthine. Adenine consumption and hypoxanthine and xanthine accumulation were not found when PFP was stored as a control.

Table 2. Rates of Change per Day of Purine Compounds During PC Storage for Three Days in PL-146 and Seven Days in PL-732 Using Either CPD or CPDA as Anticoagulant

<table>
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<tr>
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<th>CPD</th>
<th>CPD-Adenine</th>
<th>CPD</th>
<th>CPD-Adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>-4.6 ± 2.4*</td>
<td>-1.6 ± 3.1†</td>
<td>-5.9 ± 1.4*</td>
<td>-3.0 ± 1.1*</td>
</tr>
<tr>
<td>ADP</td>
<td>-3.2 ± 3.3*</td>
<td>-3.7 ± 2.5*</td>
<td>-4.3 ± 0.7*</td>
<td>-3.0 ± 0.7*</td>
</tr>
<tr>
<td>AMP</td>
<td>0.0 ± 0.2t</td>
<td>0.0 ± 0.2t</td>
<td>-0.4 ± 0.1*</td>
<td>-0.3 ± 0.1*</td>
</tr>
<tr>
<td>Total AN</td>
<td>-7.8 ± 5.6*</td>
<td>-5.2 ± 5.3t</td>
<td>-10.6 ± 2.1*</td>
<td>-6.3 ± 1.7*</td>
</tr>
<tr>
<td>Adenine</td>
<td>ND</td>
<td>-13.9 ± 4.2*</td>
<td>ND</td>
<td>-21.7 ± 1.7*</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>9.0 ± 2.0‡</td>
<td>19.3 ± 2.4*</td>
<td>11.5 ± 2.6*</td>
<td>27.3 ± 2.2*</td>
</tr>
<tr>
<td>GTP</td>
<td>-1.2 ± 0.2*</td>
<td>-0.8 ± 0.4‡</td>
<td>-1.0 ± 0.1*</td>
<td>-0.6 ± 0.2*</td>
</tr>
<tr>
<td>GDP</td>
<td>-0.3 ± 0.3‡</td>
<td>-0.1 ± 0.5†</td>
<td>-0.4 ± 0.2*</td>
<td>-0.4 ± 0.2*</td>
</tr>
<tr>
<td>Total GN</td>
<td>-1.5 ± 0.5*</td>
<td>-0.9 ± 0.9§</td>
<td>-1.4 ± 0.2*</td>
<td>-1.0 ± 0.4*</td>
</tr>
<tr>
<td>Xanthine</td>
<td>2.2 ± 0.6‡</td>
<td>1.7 ± 0.6‡</td>
<td>2.7 ± 0.5*</td>
<td>2.9 ± 0.3*</td>
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</table>

Values of intracellular nucleotides are expressed as μmol/L of PC rather than on a per cell basis as in Table 1. This allows a direct, quantitative comparison between the rates of change of intracellular nucleotides and extracellular adenine, hypoxanthine, and xanthine, which are also expressed as μmol/L of PC. Values are means ± SD. P values reflect significance relative to a zero rate of change. n = 4 for all groups except PL-732-CPDA, where n = 5.

*P < .001.
†P > .05.
‡P < .01.
§P < .05.
The four groups of PCs were compared regarding the rates of change in concentration of adenine and guanine nucleotides during storage. We found no significant difference for any of the compounds when comparing PL-732 CPD with PL-146 CPD and PL-732 CPD-adenine with PL-146 CPD-adenine. However, in PL-732, significant differences were found between CPD and CPD-adenine. In CPD-adenine, there was a significantly lower rate of decrease of ATP, GTP, ATP + ADP + AMP, and GTP + GDP. These differences in PL-146 were not statistically significant.

Role of contaminating leukocytes. In the studies designed to determine the role of leukocytes in the changes observed, there was a significant difference on day 0 between LD PCs and LE PCs in ATP (LD 4.48 ± 0.40 μmol/10^11 platelets v LE 5.82 ± 0.47 μmol/10^11 platelets; P < .001); ADP (LD 2.63 ± 0.26 μmol/10^11 platelets v LE 3.16 ± 0.29 μmol/10^11 platelets; P < .01); and GTP (LD 0.58 ± 0.09 μmol/10^11 platelets v LE 0.72 ± 0.08 μmol/10^11 platelets; P < .05). The increased values for LE PC undoubtedly reflect the presence of ATP, ADP, and GTP in leukocytes. There were no significant differences on day 0 between LD PC and LN PC or between LN PC and LE PC. During 7 days of storage, there was no significant difference among the preparations in the rates of change of purine nucleotides or their metabolites, indicating that the changes described in the previous section were due to platelet metabolism.

Ammonia production. There was pronounced ammonia production during 7 days of storage. In PL-732 with CPD as anticoagulant, levels (mmol/L) were 0.31 ± 0.04, 0.63 ± 0.01, and 0.75 ± 0.03 on days 1, 4, and 7, respectively. There was no significant difference between LD PC and LE PC in the rate of ammonia accumulation. There was much less ammonia production when PFP was stored as a control with levels (mmol/L), 0.07 ± 0.01, 0.15 ± 0.03, and 0.22 ± 0.03 on days 1, 4, and 7, respectively.

Studies with 14C-adenine labeling. When 14C-adenine was added to fresh CPD PC, it was incorporated into purine nucleotides and their metabolites so that no 14C-adenine was detectable after 14 hours. Therefore, 14 hours was taken as the initial (day 0) measurement of radioactivity in these compounds. Separation of stored PC into platelets and plasma showed the cells to contain all the 14C-labeled adenine and guanine nucleotides and no radioactive nucleosides and bases, whereas the plasma contained radioactive hypoxanthine and xanthine. The final amount of adenine nucleotides formed from 14C-adenine was approximately 0.3 μmol/10^11 platelets, a level too low to produce a measurable increase of ATP + ADP. The sums of radioactivity in the samples investigated from days 0 to 7 did not differ significantly from the radioactivity of the 14C-adenine originally added, suggesting that the substances under study were the main purine metabolites and indicating that no radioactivity was unaccounted for during the time of investigation.

After the initial labeling at 14 hours, there was continuous decrease of radioactivity in ATP + ADP over the next 7 days accompanied by an increase of radioactivity in hypoxanthine (Fig 2A). The chemical amount of ATP + ADP decreased by about 35% during this period (−0.40 ± 0.06 μmol/10^11

![Graph](image_url)

Fig 2. Changes in percent of total radioactivity in (A) ATP + ADP (closed symbols, uninterrupted line) and hypoxanthine (hypox) (closed symbols, interrupted line), and (B) GTP + GDP (open symbols, uninterrupted line) and xanthine (xan) (open symbols, interrupted line) after labeling of ATP + ADP at the start of storage with 14C-adenine. (C) Changes in specific activities. On day 0, approximately 70% and 25% of radioactivity were in ATP + ADP and hypoxanthine, respectively. During storage, radioactivity moved from adenine nucleotides to hypoxanthine, GTP + GDP, and xanthine. The specific activities of ATP + ADP and hypoxanthine decreased, whereas that of GTP + GDP increased. The specific activity of xanthine increased slightly but not to a statistically significant degree. Values are given as means ± 1 SD.
platelets per day; \( P < .001 \)), whereas the radioactivity of ATP + ADP decreased by about 50% \((-2.263 \pm 270 \text{ counts per minute (cpm)/d}; P < .001)\). Thus, there was a decrease in specific activity of ATP + ADP during storage of about 23% \((-126 \pm 64 \text{ cpmmol}/d; P < .001)\), indicating that radioactive ATP + ADP was being preferentially degraded (Fig 2C). The specific activity of hypoxanthine was always higher than the specific activity of ATP + ADP and it too decreased during storage (Fig 2C).

Radioactivity was also incorporated into AMP, but it represented only 0.6 \pm 0.3\% of the total radioactivity. The specific activity of AMP was \(745 \pm 216 \text{ cpmmol at the start of storage, far less than that of ATP + ADP (3,669 \pm 744 \text{ cpmmol}). Radioactivity in AMP did not change significantly during storage. Because the chemical amount of AMP decreased slightly \((-0.019 \pm 0.004 \text{ mmol/10^11 platelets/d}; P < .001)\), its specific activity showed a minor increase \((+40 \pm 18 \text{ cpmmol/d}; P < .05)\).

Radioactivity was slowly distributed into guanine nucleotides increasing by 123 \pm 26 \text{ cpmmol/d} \((P < .001)\) (Fig 2B). However, this radioactivity was less than 5\% of the total. The specific activity of GTP + GDP increased continuously during storage \((351 \pm 36 \text{ cpmmol/d}; P < .001)\) and did not differ significantly from that of ATP + ADP on day 7. Total radioactivity in xanthine increased continuously as well during storage \((153 \pm 27 \text{ cpmmol/d}; P < .001)\), but the slight increase in its specific activity was not statistically significant. Because the specific activity of GTP + GDP was always higher and increased faster than that of xanthine, it is likely that the \(^{14}\text{C}-\text{adenine} \text{ was first incorporated into GTP + GDP and later degraded together with nonradioactive GTP + GDP to xanthine.}

Studies with \(^{14}\text{C}-\text{hypoxanthine.} \text{}^{14}\text{C}-\text{adenine was} \text{ added to 3-mL samples withdrawn on days 0, 1, 3, 5, and 7 from two PCs (PL-732, CPD). Taking into account the steady production of hypoxanthine in PC during storage, the amount of radiolabeled hypoxanthine added was adjusted to attempt to achieve as constant a specific activity of hypoxanthine as possible immediately after mixing. This approach was reasonably successful, because the mean specific activity of hypoxanthine for the five samples immediately after mixing was \(42.8 \pm 12.5\) and \(43.7 \pm 28 \text{ cpmmol/pmol in each of the two studies. During the 4-hour incubations of these aliquots, radioactivity was incorporated into adenine and guanine nucleotides and xanthine. The total amount of radioactivity in the peaks analyzed after the 4-hour incubations did not differ significantly from the amount of radioactivity initially added to the two PCs. Thus, no radioactivity was lost during the incubation, suggesting that the main \(^{14}\text{C}-\text{hypoxanthine metabolites were studied.}

The rates of incorporation of \(^{14}\text{C}-\text{hypoxanthine into adenine and guanine nucleotides were calculated from the radioactivity measured in ATP + ADP + AMP and GTP + GDP, respectively, and from the mean specific activity of hypoxanthine during the incubation time. The uptake rate into adenine nucleotides was \(41.3 \pm 7.0\) and \(51.5 \pm 14.0 \text{ nmol/10^{11} platelets per 4 hours in each of the two PCs, equivalent to a utilization of plasma hypoxanthine of 5.5 \pm 0.9\) and \(4.9 \pm 1.3 \text{ \mu mol/L/d. The mean hypoxanthine accumulations in these two PCs were 20.2 \text{ \mu mol/L/d and 21.4 \text{ \mu mol/L/d. Therefore, about 25\% of the net production of hypoxanthine was reincorporated. The rate of \(^{14}\text{C}-\text{hypoxanthine incorporation into guanine nucleotides was 5.1 \pm 1.6\) and 6.3 \pm 2.7 \text{ nmol/10^{11} platelets per 4 hours, respectively, i.e., about one tenth of the uptake rate into adenine nucleotides. There was no significant difference in uptake rate between the two PCs or between different days of storage for either guanine or adenine nucleotides. Radioactivity was also found in xanthine after the 4-hour incubation, demonstrating the further degradation of radioactive guanine nucleotides to that compound.}

DISCUSSION

Using our HPLC technique, we found results for adenine and guanine nucleotides in fresh PC (Table 1) that were similar to previous reports.\(^{11,17-22}\) Reported concentrations of hypoxanthine and xanthine in plasma vary widely \((0.4 \text{ to } 7 \text{ \mu mol/L}) and 0.4 \text{ to } 5 \text{ \mu mol/L}, respectively.\(^{24-33}\) It has been shown that if plasma and blood cells are not separated immediately after phlebotomy, hypoxanthine and xanthine will continue to be formed in vitro.\(^{31,33}\) Several hours are required for the preparation of fresh PC so that our plasma and cell samples were separated for analysis about 3 to 4 hours after blood collection. This, no doubt, explains our somewhat higher values compared with the literature. The higher values of hypoxanthine in PC with adenine are undoubtedly due to an increased rate of hypoxanthine formation during this 3- to 4-hour period, similar to that observed during the subsequent week of storage (Fig 1).

The studies of PC derived from blood anticoagulated with CPD demonstrate a continuing decline in adenine nucleotide content in both PL-146 and PL-732 containers. There was a 20\% reduction in both ATP and ADP after 3 days of storage. This is in contrast to the report of Rao et al,\(^{4}\) who found both a larger reduction of adenine nucleotides \((-27.1\% \text{ for ATP and } -39.1\% \text{ for ADP}) and a greater reduction in ADP than in ATP. We have no definite explanation for these differences, although Rao et al used different techniques: luciferin-luciferase assay of ethanol extracts of platelets. However, because 90\% or more of platelet ADP is in the storage pool, our results are consistent with their conclusion that some of the nucleotide depletion during PC storage was from the storage pool.

After 7 days of storage in PL-732 with CPD as primary anticoagulant, platelet adenine nucleotides had decreased to approximately 60\% of day 0 values. Plasma hypoxanthine levels rose at the same rate at which adenine nucleotides fell (Table 2), strongly suggesting that the latter were being converted to hypoxanthine quantitatively, either by deamination of AMP in the metabolic pool to IMP with further metabolism to hypoxanthine or by release from the storage pool with conversion to hypoxanthine by plasma enzymes. Figure 3 depicts these two possibilities schematically. When adenine was present in the primary anticoagulant, it was carried over into the PC so that its concentration at the start of storage was approximately 220 \text{ \mu mol/L}. During PC storage, the concentration of adenine decreased gradually so that approximately two thirds had been consumed after 7
The oval represents the cell wall. Amounts of ATP, ADP, GTP, and GDP are drawn to scale. Adenine nucleotides from both the metabolic and storage pools are continuously degraded to hypoxanthine. Approximately 25% of hypoxanthine formed is reincorporated into adenine and guanine nucleotides through the hypoxanthine phosphoribosyl-transferase reaction. GTP and GDP are formed from the adenine nucleotide pool, presumably through IMP, and subsequently degraded to xanthine. If adenine is present it can be readily incorporated into the adenine nucleotide pool.

There is currently no information concerning possible interchange of material between the metabolic and storage pools during storage.

Fig 3. Adenine and guanine nucleotide metabolism during PC storage. The oval represents the cell wall. Amounts of ATP, ADP, GTP, and GDP are drawn to scale. Adenine nucleotides from both the metabolic and storage pools are continuously degraded to hypoxanthine. Approximately 25% of hypoxanthine formed is reincorporated into adenine and guanine nucleotides through the hypoxanthine phosphoribosyl-transferase reaction. GTP and GDP are formed from the adenine nucleotide pool, presumably through IMP, and subsequently degraded to xanthine. If adenine is present it can be readily incorporated into the adenine nucleotide pool.

There is currently no information concerning possible interchange of material between the metabolic and storage pools during storage.

The presence of adenine was associated with a significant rise in ATP levels during the first 24 hours of storage (Fig 1) and a subsequent reduction in the rate of loss of adenine and guanine nucleotides during the following 6 days. With adenine present, there was a 2- to 2.5-fold increase in the rate of hypoxanthine accumulation compared with PC without adenine. The sum of the rates of decrease of adenine and adenine nucleotides equaled the rate of increase of hypoxanthine. An early increase in ATP, better ATP maintenance, and faster hypoxanthine production are also seen when adenine is added to red cells before storage. As mentioned in the introduction, this is associated with improved viability when the red cells are infused in vivo after storage. On the other hand, Holme et al have shown a strong correlation between platelet ATP levels and in vivo viability in a wide variety of circumstances. Therefore, adenine may be important in efforts to extend PC storage.

Finally, it is possible that platelet function is better preserved with adenine supplementation, although this question has not yet been studied in detail.

Ukrainski et al described ammonia accumulation of about 150 μmol/L/d during 3 days of PC storage at 22°C in a first-generation container. The rate of increase in our study in a second-generation container was similar. One source of ammonia would be deamination of AMP to IMP (Fig 3), which would increase ammonia levels by the same rate as hypoxanthine formation. Because net hypoxanthine production is about 10 μmol/L/d, either gross hypoxanthine formation is 15 times the net formation or other major sources of ammonia formation must be present. Our studies with 14C-hypoxanthine suggest that the rate of hypoxanthine utilization is only 25% of net formation and that therefore the latter possibility is more likely. Metabolism of amino acids is another possible source that we are currently investigating.

The interpretation of the 14C-adenine studies is complex. Fifty percent of the radioactivity present in platelet ATP + ADP on day 0 was found in hypoxanthine on day 7. There is considerable experimental support for the conventional concept that platelet ATP + ADP is divided into a granular storage pool and a cytoplasmic metabolic pool, each containing approximately two thirds and one third of total ATP + ADP, respectively. Over several hours of incubation, the storage pool is labeled poorly by radioactive precursors such as 14C-adenine. In our study, the specific activity of ATP + ADP decreased from days 1 to 7, suggesting preferential degradation of radiolabeled material throughout storage. Because both chemical and isotopic studies indicated that almost all of the degraded ATP + ADP was being metabolized to hypoxanthine, we were surprised that the specific activity of hypoxanthine also decreased from days 1 to 7. This can be explained by the high specific activity of hypoxanthine at the 24-hour measurement (8,130 ± 2,153 cpm/nmol) relative to the simultaneous specific activity of ATP + ADP (3,525 ± 791 cpm/nmol) (Fig 2). This labeled hypoxanthine had been diluted by the unlabeled hypoxanthine (6.3 ± 3.0 μmol/L), which was present at the time of addition of 14C-labeled adenine and amounted to about 30% of the total concentration of hypoxanthine at the 24-hour measurement. Correcting for this unlabeled hypoxanthine, the specific activity of hypoxanthine formed during the first 24 hours was about 11,234 cpm/nmol, three times the simultaneous specific activity of total ADP + ATP and therefore equivalent to that predicted for the metabolic pool. During storage from days 1 to 7, the specific activity of ATP + ADP degraded was continuously higher than that of the remaining ATP + ADP but lower than that of the hypoxanthine already formed. During the 7 days of these isotopic studies, chemical ATP + ADP decreased by about one third. The isotopic measurements were consistent with the initially labeled and unlabeled pools each contributing approximately equally to this decrease, i.e., degradation of half of the labeled pool and one fourth of the unlabeled pool. This would result in the 25% decrease in the specific activity of ATP + ADP that was observed.

The observation that the specific activity of AMP was only 20% of that of ATP + ADP in fresh platelets is new. Some authors have assumed that 14C-adenine distributes evenly among ATP, ADP, and AMP in the metabolic pool and that all AMP is in this pool. If this were true, the specific activity of AMP would be higher than that of ADP and ATP because of the nonradioactive ADP and ATP in the storage pool. Our findings point to a more complex pattern of distribution of 14C-adenine among the adenine nucleotides and suggest a
sizable pool of AMP that is not in isotopic equilibrium with metabolically active ATP and ADP. These are the first studies to examine guanine nucleotides during PC storage. They play a major role in many cell functions, such as the synthesis of adenine nucleotides from IMP by the purine salvage pathway and the initiation of platelet aggregation and release by the phosphoinositide pathway. Therefore, deficiencies may be important. As for adenine nucleotides, guanine nucleotides showed a gradual reduction to 60% of control values after 7 days. As GTP and GDP decreased, plasma xanthine increased, but at a greater rate. The steady increase of radioactivity in GTP, GDP, and xanthine found in the 14C-adenine labeling studies indicate a continuous flow of adenine nucleotides to AMP, IMP, and GDP with subsequent degradation to xanthine (Fig 3). Similar labeling was also demonstrated in the 14C-hypoxanthine experiments. This shuttle of purines from adenine nucleotides and hypoxanthine to guanine nucleotides and xanthine explains the greater increase in xanthine during storage relative to the decrease in total guanine nucleotides. This part of purine metabolism has not been shown for platelets previously.

Platelets are not able to synthesize purine nucleotides de novo. However, hypoxanthine may be salvaged by hypoxanthine phosphoribosyl-transferase to form IMP, which can then be converted to AMP and GMP (Fig 3). Jerushalmy et al and Rivard et al demonstrated hypoxanthine incorporation into purine nucleotides of fresh, washed platelets incubated at 37°C. In the present study, hypoxanthine was incorporated into purine nucleotides at a rate of approximately 50 nmol/4 h/10^{11} platelets. This pathway remained intact during 7 days of PC storage. In accord with the two earlier studies, 90% and 10% of the hypoxanthine were incorporated into adenine and guanine nucleotides, respectively. Because we measured both the rate of purine nucleotide degradation to hypoxanthine and the rate of hypoxanthine incorporation into purine nucleotides, we were able to show that as much as 25% of the net production of hypoxanthine could be reincorporated through the salvage pathway.

There are several possible causes for the adenine nucleotide degradation that occurs during PC storage. Our studies provide information about platelet adenine and guanine nucleotide metabolism over a period of time equivalent to the platelet life span. They may be providing insight into normal platelet physiology, suggesting that adenine nucleotide depletion occurs with in vivo aging. On the other hand, it may simply be that as ATP is used for the cell's baseline needs, it cannot be resynthesized rapidly enough because the conditions of storage are metabolically unfavorable. Under these circumstances, AMP would accumulate and be degraded to hypoxanthine. This metabolic depletion might permit leakage of storage pool nucleotides. On the other hand, during storage, platelets may be intermittently stimulated to secrete granule contents because of agitation and contact with the plastic walls of the container. We know that α-granular contents such as β-thromboglobulin appear in supernatant plasma during storage.

There is evidence that secretion by fresh platelets is accompanied by degradation of metabolic ATP to hypoxanthine. Recently, it was shown that addition of inhibitors of platelet function such as theophylline and prostaglandin E1 to PC during storage improved maintenance of platelet morphologic integrity and ATP levels. In these studies, there was strong correlation between ATP levels and measurements that reflect morphologic integrity, which in turn have correlated with in vivo viability in the past. Certainly further understanding of the mechanisms of nucleotide depletion and the relationship between this depletion and other measurements reflecting cellular integrity should allow us to improve the quality of platelets during and after storage.

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