Studies on the Metabolism of Adenosine and Adenine in Stored and Fresh Human Erythrocytes

By Bertram A. Lowy and Marjorie K. Williams

The mature human erythrocyte, which is incapable of de novo purine nucleotide synthesis, can utilize preformed purines and purine nucleosides for the formation of purine nucleotides, in vitro.1,2 Studies in this laboratory have demonstrated that adenine is an effective precursor of the ATP of the human erythrocyte and that hypoxanthine, guanine, and xanthine lead to the formation of GTP.1 The aglycones of the purine nucleosides, inosine and guanosine, serve as precursors of GTP after being formed by phosphorylolytic cleavage of the ribosyl compounds by the purine nucleoside phosphorylase of the erythrocyte.3

The failure of hypoxanthine and inosine to serve as precursors of the ATP in the human erythrocyte, although they are efficiently utilized for IMP formation, has served to demonstrate a difference in a metabolic capacity of rabbit and human erythrocytes.4 This difference has been localized as a failure of the human cell to convert inosine 5'-phosphate to AMP.1

The metabolism of adenosine has also been investigated and its extensive conversion to IMP has been demonstrated, a metabolic fate that is compatible with the known rapid rate of deamination of adenosine to inosine in the human erythrocyte.1,3

Several reports have indicated that the presence of adenosine in blood storage media leads to an increased concentration of ATP and to an enhanced post-transfusion survival in stored cells.5–10 Overgaard-Hansen et al.8 have reported that adenosine is more effective than inosine in increasing the concentration of ATP in fresh cells, and they suggested that a portion of the adenosine may be phosphorylated directly. The elevation of ATP levels was especially pronounced at relatively high concentrations of adenosine.

This paper is concerned with some aspects of the utilization of adenosine-8-C14 and adenine-8-C14 for the formation of purine nucleotides in freshly obtained erythrocytes and in erythrocytes that have been stored for prolonged periods of time.

Materials and Methods

Incubation in Vitro

Blood from normal adult donors was collected in 0.2 volume of acid-citrate-dextrose solu-
Table 1.—The Effect of Adenosine-8-C\(^{14}\) Concentration on the Utilization for ATP Formation in Fresh and in Acid-Citrate-Dextrose (ACD) Stored Human Erythrocytes

<table>
<thead>
<tr>
<th>Incubated</th>
<th>Fresh</th>
<th>6-week ACD*</th>
<th>9 week ACD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>uM/mL</td>
<td>Adenosine 8-C(^{14})</td>
<td>cpm/uM</td>
<td>Adenine of ATP</td>
</tr>
<tr>
<td>.36</td>
<td>5.0</td>
<td>1.02 x 10(^5)</td>
<td>1.1</td>
</tr>
<tr>
<td>3.6</td>
<td>10.0</td>
<td>2.04 x 10(^4)</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Sixty ml. washed erythrocytes incubated with adenosine-8-C\(^{14}\) and glucose (150, \(\mu\)moles) for 2 hr. at 37 C.

*Carrier ATP (3.0 mg) added to extracts of stored blood at end of incubation.

**RSA = relative specific activity = \(\frac{\text{cpm per } \mu\text{M in product}}{\text{cpm per } \mu\text{M in precursor}}\times 100\)

Results

The incubation of freshly obtained human erythrocytes with adenosine-8-C\(^{14}\) at a low concentration resulted in a significant extent of labeling of the cellular ATP (Table 1). When the specific activity of the adenosine-8-C\(^{14}\) was decreased five-fold as a result of a ten-fold increase in the adenosine concentration and a doubling of the number of microcuries added, the relative specific activity of the ATP was 12 times larger, indicating a considerably greater utilization of labeled precursor for ATP formation. The most likely explanation for this observation is that direct phosphorylation of adenosine to AMP occurred. Direct phosphorylation of a portion of the adenosine may have been a consequence of its relatively high concentration and the resultant incomplete deamination of the nucleoside. The presence of adenosine kinase in human erythrocytes is inferred.

In cells stored for periods of 6 and 9 weeks, similar results were obtained. Although the relative specific activities of the adenine cannot be compared at the different storage periods because of the addition of carrier ATP to the stored samples at the end of the incubation, a marked increase in the relative specific activity of each sample was observed at the high adenosine concentration. The actual RSA values for the ATP of the stored blood samples are reflections of the low concentrations of ATP in the stored cells and of the dilution due to the addition of carrier adenine.

In order to provide information on the extent to which hypoxanthine, derived metabolically from the adenosine, may be utilized for adenine nucleotide formation, aliquots of washed erythrocytes were incubated with a high
Table 2.—The Effect of Inosine on the Utilization of Adenosine-8-C\textsuperscript{14} for Purine Nucleotide Synthesis

<table>
<thead>
<tr>
<th>Adenosine-8-C\textsuperscript{14}</th>
<th>Inosine</th>
<th>Adenine of ATP</th>
<th>Hypoxanthine of IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>in M/ml. cells</td>
<td>cpm, uM</td>
<td>RSA</td>
<td>RSA</td>
</tr>
<tr>
<td>3.6</td>
<td>1.62 x 10\textsuperscript{4}</td>
<td>0</td>
<td>13.6</td>
</tr>
<tr>
<td>3.6</td>
<td>1.62 x 10\textsuperscript{4}</td>
<td>1.8</td>
<td>14.1</td>
</tr>
<tr>
<td>3.6</td>
<td>1.62 x 10\textsuperscript{4}</td>
<td>3.6</td>
<td>14.3</td>
</tr>
<tr>
<td>3.6</td>
<td>1.62 x 10\textsuperscript{4}</td>
<td>7.2</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Sixty ml. washed human erythrocytes, freshly collected in ACD, added to 60 ml. isotonic phosphate buffer (pH 7.2) containing adenosine-8-C\textsuperscript{14} (10 μc, 216 μM) glucose (150 μM), and the indicated quantity of inosine. Incubated at 37 C. for 2 hours.

Concentration of adenosine-8-C\textsuperscript{14} and with different concentrations of unlabeled inosine. If hypoxanthine were an intermediate in the utilization of adenosine-8-C\textsuperscript{14} for ATP formation, a differential dilution effect would be observed in the ATP samples prepared from cell suspensions supplemented with inosine. Since no dilution was observed (Table 2), it may be concluded that neither hypoxanthine nor inosine is an intermediate in the conversion of adenosine to ATP. The conversion of adenosine-8-C\textsuperscript{14} to hypoxanthine-8-C\textsuperscript{14} and a subsequent dilution effect of the inosine are reflected in the specific activities of the hypoxanthine of IMP.

In order to evaluate the possibility that adenosine may be cleaved to adenine which is then utilized in the nucleotide pyrophosphorylase reaction, the possible presence of labeled adenine in the erythrocyte extracts was sought. When unlabeled carrier adenine was added to the trichloroacetic acid extracts, and reisolated by ion exchange and paper chromatographic methods, no significant radioactivity was found in the reisolated adenine. The absence of appreciable labeled adenine in the extracts suggests that cleavage of adenosine, per se, does not occur in the human erythrocyte. Although Korn and Buchanan\textsuperscript{15} have reported the isolation of a purine nucleoside phosphorylase from beef liver which will cleave adenosine to adenine and ribose 1-phosphate, the enzymes isolated from the human erythrocyte\textsuperscript{13} and rat liver\textsuperscript{14} exhibit a marked specificity for inosine, and will not cleave adenosine. The results of this experiment strongly suggest that adenosine utilization in the human erythrocyte occurs by direct phosphorylation and that adenosine utilization for ATP formation may be significant only at relatively high concentration, under conditions in which complete deamination of adenosine cannot occur immediately.

An additional experiment was carried out to determine the effect of adding a relatively large amount of unlabeled inosine to cells incubated with adenosine-8-C\textsuperscript{14} (Table 3). In fresh human erythrocytes, the presence of a large amount of inosine had little effect on the extent of conversion of adenosine-8-C\textsuperscript{14} to ATP. The presence of unlabeled inosine in incubation suspensions containing cells prepared from blood that had been stored for prolonged periods led to a marked enhancement of adenosine-8-C\textsuperscript{14} utilization. This may be explained by the fact that the utilization of adenine for ATP formation in aged cells is dependent upon a source of phosphoribosyl pyrophosphate, which in turn is
Table 3.—The Effect of Storage of Human Erythrocytes in ACD on Utilization of Adenine-8-C\(^{14}\) for ATP Formation in Presence and Absence of Unlabeled Inosine

<table>
<thead>
<tr>
<th></th>
<th>Incubated</th>
<th>Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenine-8-C(^{14})</td>
<td>cpm/(\mu)M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>.38</td>
<td>1.16 x 10(^5)</td>
<td>27.2</td>
</tr>
<tr>
<td>.38</td>
<td>1.16 x 10(^5)</td>
<td>31.0</td>
</tr>
<tr>
<td>+ 3.33 inosine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sixty ml. washed erythrocytes incubated with adenine-8-C\(^{14}\) and glucose (150, \(\mu\)moles) for 2 hr. at 37°C.

+Carrier ATP (3.0 mg.) added to extract of stored blood at end of incubation.

formed by the reaction of ribose 5-phosphate and ATP. In cells depleted of ATP or in which glycolysis occurs to a minimal extent, the addition of adenine will not lead to appreciable nucleotide formation. By the addition of inosine, a source of potential ribose phosphate is provided. A portion of the ribose 1-phosphate is metabolized to lactic acid with the concomitant replenishment of ATP from ADP. Another portion of ribose 1-phosphate may be converted to ribose 5-phosphate and, by reaction with ATP, to PRPP, which together with adenine will lead to the net formation of AMP. By subsequent phosphorylation reactions, the newly formed AMP will lead to increased cellular levels of ATP. It is thus clear that although adenine serves effectively as a precursor of ATP in fresh cells or in stored cells in which active glycolysis occurs, it is dependent upon products of glycolysis (namely ribose 5-phosphate and ATP) if it is to serve for net adenylate formation. The addition of inosine satisfies these requirements, and in cells stored for many weeks the presence of adenine and inosine proves to be far more effective for ATP formation than adenine alone.

DISCUSSION

The data presented here bear upon two aspects of the problem of ACD supplementation for improving blood storage media—namely, the relationship between adenosine concentration and its metabolism, and the glycolitic requirements of the cell for the formation of ATP from adenine. Additional substantiation is also provided for the inability of the human erythrocyte to convert IMP to AMP.

Although the supplementation of ACD media with adenosine does not seem feasible because of its marked vasodepressor activity, several investigators have studied the ATP levels and post-transfusion survival of human cells stored in media supplemented with adenosine. The results of the studies have varied considerably and these quantitative variations may relate to the adenosine concentrations as well as to the experimental design. It is of interest to note that a very high concentration of adenosine was employed in the studies of Mollison and Robinson, who reported marked improvement in the viability of stored erythrocytes.

The experiments concerned with the formation of ATP from adenine-8-C\(^{14}\) illustrate an important consideration in the potential use of an adenine supple-
mented storage medium. Although adenine serves efficiently as an adenylic precursor in fresh cells in which the formation of PRPP occurs readily, this is not the case in the cells aged over a prolonged period. The limiting factor in the formation of ATP is readily apparent since the addition of inosine markedly stimulates the utilization of adenine by providing a pentose phosphate which results in a regeneration of ATP from ADP during metabolism to lactic acid, and by subsequent utilization of the ATP for PRPP formation. With the availability of PRPP, the nucleotide pyrophosphorylase reaction can occur, with a net formation of AMP. Since most of the ribose phosphate is converted to lactic acid, considerable regeneration of ATP from ADP can occur. The importance of inosine supplementation together with the adenine is thus demonstrated.

Summary

The metabolism of adenosine-8-C\(^{14}\), at relatively high and low concentrations, was studied in human erythrocytes freshly obtained and after 6 and 9 weeks of storage in ACD. At high adenosine concentration (3.6 \(\mu\)M/ml. cells), considerable conversion to ATP was found in fresh and stored cells, suggesting that direct phosphorylation of adenosine occurred, a reaction that is minimal at low (0.36 \(\mu\)M/ml. cells) adenosine concentration because of extensive rapid deamination. Incorporation of the label via hypoxanthine or adenine is unlikely, since at high adenosine concentration no dilution of ATP labeling in the presence of unlabeled inosine (hypoxanthine) was found, nor was free labeled adenine detected in erythrocyte extracts.

A study of the metabolism of adenine-8-C\(^{14}\) in fresh and stored erythrocytes suggests that the presence of a suitable nucleoside (e.g., inosine) is required for efficient utilization of adenine-8-C\(^{14}\) for ATP formation in the erythrocytes of blood stored for prolonged periods.

Summario in Interlingua

Le metabolismo de adenosina-8-C\(^{14}\), a relativemente alte e basse concentrationes, esseva studiate in erythrocytos human frescamente obtenite e post 6 e 9 septimanas de magazinage in ACD. A alte concentrationes de adenosina (3.6 \(\mu\)M per ml de cellulas) un considerabile grado de conversion ad in triphosphato de adenosina esseva constatate in cellulas fresc e magazinate, lo que suggere que un phosphorylation directe de adenosina habeva occurrile. Iste reaction es minime a basse concentrationes de adenosina (0,36 \(\mu\)M per ml de cellulas) in consequentia del occurrentia de un extense deamination rapide. Le incorporation del marca via hypoxanthina o adenina es pauso probabile, viste que a alte concentrationes de adenosina nulle dilution del marcage de triphosphato de adenosina esseva constatate in le presentia de nonmarcate inosina (hypoxanthina) e viste etiam que nulle libere adenina con marca esseva detegite in extractos (le erythrocytos).

Un studio del metabolismo de adenina-8-C\(^{14}\) in erythrocytos fresc e magazinate suggere que le presentia de un appropriate nucleosida (per exemplo inosina) es requirite pro le efficace utilisation de adenina-8-C\(^{14}\) pro le forma-
tion de triphosphato de adenosina in le erythrocytos de sanguine magazinate durante prolongate periodos de tempore.

REFERENCES

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