Studies on Human Erythrocyte Nucleotide Metabolism.  
I. Monisotopic Methodologies

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Methods were developed to assay the activities of ribosephosphate pyrophosphokinase (RPK, PRPP synthetase, E.C. 2.7.6.1) and adenine phosphoribosyltransferase (APRT, AMP pyrophosphorylase, E.C. 2.4.2.7) in hemolysates of human erythrocytes. RPK activity was determined by measuring the production of phosphoribosyl pyrophosphate (PRPP) and the formation of adenosine mononucleotide (AMP), the two products of the reaction. The $K_m$ for adenosine triphosphate (ATP) was 0.12 mM with the PRPP method and 0.1 mM with the AMP method. The $K_m$ for ribose-5-phosphate (R-5-P) was 0.14 mM with the former method and 0.033–0.042 mM with the latter. The pH optimum with the PRPP method was between 7.6 and 8.2. The mean RPK activity of hemolysates of normal mature erythrocytes was $30.5 \pm 3.78$ μmoles of PRPP or $30.6 \pm 3.82$ μmoles of AMP produced/hr per $10^{10}$ erythrocytes at $37^\circ$ C. Although RPK activity of normal mature erythrocytes did not appear to be markedly age dependent, activity was substantially increased in reticulocyte-rich blood. APRT activity was assayed by measuring the AMP produced in a system linked to the generation of oxidized nicotinamide adenine dinucleotide (NAD). The activity of normal mature erythrocytes was $3.38 \pm 0.37$ μmoles of AMP formed/hr per $10^{10}$ erythrocytes at $37^\circ$ C. Enzyme activity was significantly elevated in reticulocyte-rich blood and in the erythrocytes of a patient with the Lesch-Nyhan syndrome. These methods obviated the need for isotopic techniques and were readily applicable to small samples of blood.

The discovery of a wide variety of inborn errors of metabolism, many associated with hereditary hemolytic anemia, has fostered continuing interest in the red cell as biopsy material. The present report presents methodology...
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applicable to the study of erythrocyte nucleotide metabolism that does not require isotopic reactants. It, nevertheless, utilizes conveniently obtained amounts of blood and is applicable to investigation of aberrations in red cell metabolism of adenine nucleotides.1

MATERIALS AND METHODS

Ribosephosphate pyrophosphokinase (RPK, PRPP synthetase, E.C. 2.7.6.1) catalyzes the reaction:

\[ R-5-P + ATP \rightarrow \text{RPK} \rightarrow \text{PRPP} + \text{AMP} \]

One of the products, PRPP2 is an important nucleotide precursor in purine, pyridine, and pyrimidine metabolism.3-5 In the human erythrocyte, PRPP may react with adenine, hypoxanthine, and guanine via known reactions to give rise to AMP, IMP, and GMP, respectively,6 and its synthesis has been investigated chiefly in connection with certain disorders of purine metabolism.7-14 In the present study, RPK was assayed by separate measurement of the two products, PRPP and AMP.

A. The RPK Reaction as Measured by PRPP Synthesis

5-Fluoroorotic acid is convertible to 5-fluorouridine 5'-phosphate by a commercially available, partially purified yeast enzyme15,16

\[ 5\text{-fluoroorotic acid} + \text{PRPP} \rightarrow \text{orotidylic pyrophosphorylase} \rightarrow 5\text{-fluoroorotidine-5'}-\text{phosphate} \rightarrow \text{orotidylic decarboxylase} \rightarrow 5\text{-fluoro UMP} + \text{CO}_2 \]

The marked decrease in absorbancy at 295 m\(\mu\) that occurs as fluoroorotate is converted to fluoro-UMP offers a sensitive spectrophotometric method for following the reaction. The conversion of 1 \(\mu\)moles/ml of fluoroorotate results in an absorbance change (\(\Delta A\)) of 3.8 when cuvettes with a 1 cm light path are employed. PRPP synthesis in a human hemolysate system may thus be quantitated in the presence of fluoroorotate and the yeast enzyme preparation.

The "complete system" contained the following reagents expressed as \(\mu\)moles/cuvette (final volume 3 ml): NaCl-KH\(_2\)PO\(_4\) buffer, pH 8.0, 115; 5-fluoroorotic acid, 0.787; ATP, 4.0; KF, 10.0; MgCl\(_2\), 6.0; yeast enzyme, 100 \(\mu\)l; R-5-P, 2.0; and hemolysate approximately equivalent to 7.5 \(\times\) 10\(^7\) red cells, 250 \(\mu\)l. (Orotodine-5'-phosphate pyrophosphorylase, PRPP, and 5-fluoroorotic acid were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Enzyme listed as P-L Cat. No. 0456, PRPP (Mg salt) Cat. No. 4500, and 5-fluoroorotic acid Cat. No. 4558. Reference Catalogue 102-1970.) Decrease in absorbance (\(\Delta A\)) at 37\(^\circ\)C was monitored employing a Gilford Multiple Sample Recorder linked to a Du spectrophotometer. Saline-washed erythrocytes, approximately 3.0 \(\times\) 10\(^8\)/ml and separated from leukocytes by sedimentation, were diluted with nine volumes of water containing mercaptoethanol (MCE), 0.005% (v/v) and frozen and thawed three times.

Although RPK contains sulfhydryl groups necessary for activity, no observable losses ordinarily accompanied omission of MCE, and no potentiation was observed with manyfold greater concentrations of either MCE or GSH. Yeast enzyme was prepared fresh daily by dissolving the contents of one vial in 1.0 ml of 0.15 M Tris buffer, pH 7.6. Additions of fluoroorotate greater than those routinely employed failed to influence reaction rates initiated by addition of R-5-P or hemolysate. After 15 min of equilibration, \(\Delta A\) was continuously monitored over the subsequent 60 min during which the reaction remained linear.

Results are expressed as \(\mu\)moles of PRPP produced (or \(\mu\)moles of fluoroorotate converted to 5-fluoro-UMP)/10\(^9\) erythrocytes per hr at 37\(^\circ\)C. Fluoride (KF) was employed to inhibit phosphatases as recommended for crude extract assays. KF, up to 15 times the amount employed, did not influence assay results. In some experiments, additions of PEP, commercial crystalline AK, PK, and LDH (components of the companion assay for AMP) were added to the PRPP assay. These altered the results in no discernible way.
Table 1 indicates dependence of the assay system upon each of the following reactants: ATP, R-5-P, fluoroorotate, yeast orotidyl pyrophosphorylase, and hemolysate. No PRPP synthesis was observed with heat-inactivated hemolysate. The blank routinely employed was that lacking R-5-P.

**Michaelis Constants and pH Optimum:** With R-5-P constant at 2.0 μmoles/cuvette, the $K_m$ for ATP of normal erythrocytes was 0.12 mM. Seven points between 0 and 0.9 mM ATP were measured. With ATP constant at 4.0 μmoles/cuvette (1.33 mM ATP), the $K_m$ for R-5-P was 0.14 mM. The pH optimum for the assay system lay between pH 7.6-8.2. Activity decreased rapidly as pH was lowered below 7.2.

**Linearity of Activity With Hemolysate Concentration:** RPK activity was linear with hemolysates in amounts ranging from 100–300 μl/cuvette. Greater than 300 μl could not be conveniently measured because of optical limitations of the system. In routine assays, 250 μl of hemolysate were employed.

**Density Gradients:** When the top and bottom cells separated by discontinuous, buoyant density gradients were assayed, only slightly less RPK activity was observed in the older, denser cells of normal blood. When reticulocyte-rich blood was employed, however, differences were substantial, indicating that RPK activity was clearly increased in the reticulocyte.

**Stability of Erythrocyte RPK:** Hemolysates should not be prepared until shortly before use. Hemolysates stored at $-20^\circ$C likewise appeared more active when substrate R-5-P was present during freezing and thawing of erythrocytes and subsequent storage.

**Precautions:** Fluoroorotate was dissolved routinely in distilled H$_2$O. Stored at $-20^\circ$C, there was slow loss of absorbance at 295 μm, amounting to about 10% over an 8-day period. Fluoroorotate solutions should, therefore, be prepared at fairly frequent, convenient intervals and assayed periodically. The yeast enzyme preparation has contaminating enzymatic activities, such as enolase, that are irrelevant in the assay system employed.

An alternative assay of PRPP synthesis by human erythrocytes may be conducted employing a series of tubes containing the complete system at $37^\circ$C assayed individually at time zero and at any selected interval thereafter following addition of perchloric acid (PCA). The absorbance of an aliquot of the protein-free, acidic filtrate is measured at 295 μm. With appropriate blanks, a time-course graph of PRPP synthesis (fluoroorotate disappearance) may be plotted.

**B. The RPK Reaction as Measured by AMP Production**

The second product of the RPK reaction is AMP, which may be assayed employing a linked indicator system fortified with purified AK, PK, and LDH. Two moles of NADH are oxidized for each mole of AMP produced, and this must be recognized in calculating and comparing PRPP and AMP production by Methods “A” and “B.”

\[
\text{R-5-P} + \text{ATP} \rightarrow \text{RPK} \rightarrow \text{PRPP} + \text{AMP} \\
\text{AMP} + \text{ATP} \rightarrow \text{AK} \rightarrow 2 \text{ADP} \\
\text{PEP} + \text{ADP} \rightarrow \text{PK} \rightarrow \text{Pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} \rightarrow \text{LDH} \rightarrow \text{Lactate} + \text{NAD} 
\]

The complete assay system contained the following constituents in μmoles/final 3 ml volume: NaCl-KH$_2$PO$_4$ buffer, pH 8.0, 62.5; MgCl$_2$, 6.0; PEP, 6.0; NADH, 0.71; ATP, 4.0; KF, 10.0; AK, PK, LDH pool (enzymes in buffer), 10 μl (5 U of each enzyme present); R-5-P, 2.0; and hemolysate approximately equivalent to 7.5 × 10$^7$ erythrocytes 250 μl. The reaction was initiated by addition of either R-5-P or hemolysate and after 4-6 min equilibration at 37°C was monitored for 12 min at 340 μm, during which time it was linear. Blanks were subtracted, and results were converted to μmoles of NADH oxidized/hr.
Table 1. Assay of RPK by Measurement of PRPP Synthesis

<table>
<thead>
<tr>
<th>System</th>
<th>Δ A 295 μm/hr Per Cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.334</td>
</tr>
<tr>
<td>No fluoroorotate</td>
<td>0.004</td>
</tr>
<tr>
<td>No orotidyllic-PPase</td>
<td>0.002</td>
</tr>
<tr>
<td>No R-5-P</td>
<td>0.020</td>
</tr>
<tr>
<td>No ATP</td>
<td>0.014</td>
</tr>
<tr>
<td>No hemolysate</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*Representative experiment. Normal cells. Hemolysate equivalent to 8 × 10^7 erythrocytes was employed. Values cited for R-5-P were characteristic of blanks from which R-5-P was omitted but were from a separate experiment.

per 10^10 erythrocytes. Heating of hemolysate to 100°C for 3 min resulted in total RPK inactivation.

RESULTS

For the indicator system to measure validly the RPK reaction it must record only AMP (or ADP) generation and, hence, be PEP-dependent, and it must be dependent on R-5-P, ATP, Mg^{2+}, and hemolysate. Table 2 indicates that such was the case. A small amount of endogenous ATP accounted for the slight activity observed in the absence of ATP additions. Aside from this, the only blank activity appeared to be minimal loss of absorbance due to slow NADH degradation with time. In general, assay values obtained by measurement of AMP and PRPP production showed good correlation, though assay constituents and the reaction product removed were not identical for the two methods. In method B, additions of KF were necessary to inhibit enolase. With PEP and ATP present, reversal of glycolysis otherwise can lead to ADP generation mediated by hemolysate phosphoglycerokinase (PGK). In the absence of KF such a reaction was found to occur, but this was completely blocked by KF at assay concentrations. ADP formed as a result of ATPase activity also will record, but this reaction was exceedingly weak under the conditions employed. Any contribution to apparent activity, dependent on either reversal of glycolysis or dephosphorylation of ATP, was eliminated in each assay by subtracting blank activities in a system where R-5-P was the sole deletion.

Table 2. Assay of RPK by Measurement of AMP Production

<table>
<thead>
<tr>
<th>System</th>
<th>Δ A 340 μm /Hr Per Cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.30</td>
</tr>
<tr>
<td>No MgCl₂</td>
<td>0.12</td>
</tr>
<tr>
<td>No PEP</td>
<td>0.06</td>
</tr>
<tr>
<td>No R-5-P</td>
<td>0.07</td>
</tr>
<tr>
<td>No ATP</td>
<td>0.42</td>
</tr>
<tr>
<td>Nr hemolysate</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Above data are composite of values obtained from several experiments and are entirely representative. Value given for “complete system” is that of subject with RPK activity very close to normal mean. Activity noted in absence of ATP is due to small amounts of endogenous adenine nucleotides in hemolysate.
Neither the PGK nor ATPase reactions is R-5-P dependent. RPK activity appeared essentially the same by either method, irrespective of whether the subject was normal, had the high RPK activity characteristic of reticulocyte-rich blood, or, as will be shown in the subsequent report, had hemolytic anemia and marked reticulocytosis but was RPK-deficient.

In the dilute hemolysate assay system employed, conversion of R-5-P to G-3-P and F-6-P via transketolase and transaldolase did not occur to a measurable degree. In experiments deleting ATP, which is not required for either the epimerization of isomerization of pentose or for transketolase or transaldolase activity, no generation of triose was demonstrable in terms of NADH oxidation in the presence of additions of triosephosphate isomerase and α-glycerophosphate dehydrogenase. Further, under the same assay conditions, no F-6-P formation was measurable when GPI, G6PD, and NADP were added as an indicator system. In these circumstances, no measurable conversion of NADP to NADPH could be detected. With the method B assay, Kₘ ATP (R-5-P constant at 2 μmoles/cuvette) was 0.1 mM. The Kₘ R-5-P (ATP constant at 4 μmoles/cuvette) was 0.033–0.042 mM.

Comparison of Sulfhydryl Inhibition of RPK Assay by Methods A and B

Human erythrocyte RPK, like that of other tissues,² appears to possess a reactive sulfhydryl group necessary for activity. Table 3 records the effects induced by the sulfhydryl inhibitor p-chloromercuribenzoate (PCMB). By either method A or B, essentially complete inhibition is achieved at PCMB concentrations of 5 × 10⁻⁶ M. Further, the degree of inhibition in either assay is essentially identical at each inhibitor concentration tested.

RPK Activity in Normal and Reticulocyte-Rich Blood

Table 4 records our experience with the assays. Normal subjects were ostensibly free of disease and without anemia or reticulocytosis. All subjects with reticulocytosis had a variety of chronic hemolytic syndromes. Patients with reticulocytosis in response to hematinc therapy or who had diseases such as leukemia have been studied but are not included in Table 4. It was clear from all subjects studied, however, that RPK is substantially increased in reticulocyte-rich blood due to any cause. While perfect correlation of RPK activity with reticulocytosis was not expected and did not occur, highest enzyme values were observed in subjects with the most marked reticulocytoses.

Table 3. Inhibition of RPK by p-Chloromercuribenzoate*

<table>
<thead>
<tr>
<th>Final Inhibitor Concentration</th>
<th>Per Cent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Method A</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>9.0</td>
</tr>
<tr>
<td>5 × 10⁻⁶ M</td>
<td>20.0</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>55.0</td>
</tr>
<tr>
<td>5 × 10⁻⁵ M</td>
<td>94.0</td>
</tr>
</tbody>
</table>

*Complete system as described in text. Method A assays PRPP and Method B AMP production.
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Table 4. RPK Activity in Normal and Reticulocyte-Rich Blood

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Assay</th>
<th>RPK Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>A</td>
<td>Mean ± SD, Range</td>
</tr>
<tr>
<td>(26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retics. 3-15%</td>
<td>A</td>
<td>30.5 ± 3.78, 24.9-40.3</td>
</tr>
<tr>
<td>(10)</td>
<td>B</td>
<td>30.6 ± 3.82, 25.3-40.8</td>
</tr>
<tr>
<td>Retics. 15-35%</td>
<td>B</td>
<td>51.6 ± 4.28, 37.8-78.2</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retics. 35-53%</td>
<td>A</td>
<td>57.2 ± 5.39, 49.5-63.4</td>
</tr>
<tr>
<td>(3)</td>
<td>B</td>
<td>60.6 ± 5.51, 41.1-80.8</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results expressed as μmoles of PRPP (method A) or AMP (method B) produced/hr per 10^10 erythrocytes at 37°C. Numbers in parentheses indicate number of subjects studied.

The activity increase was large and consistent, and RPK activity must always be evaluated in this context.

RPK, with its highly reactive sulfhydryl group, is more susceptible to the vagaries in hemolysate preparation than more stable enzymes. While the results obtained by methods A and B usually agree closely, occasional unexplained variations occur. In one series of experiments not shown in Table 4, the measurement of PRPP production was conducted with additions of PEP and purified AK, PK, and LDH in amounts equal to those utilized in method B. On the identical hemolysate and at the same time, AMP production was assayed in the usual manner, but with fluoroorotate added, a constituent of the method A assay. Buffer was increased to 115 μmoles/cuvette in order to maintain pH precisely. In seven of eight successive assays by both methods, agreement was extremely close. In a single instance and for unexplained reasons, considerable variation was observed. As expected, closest agreement of assays performed by the two methods was achieved when a single hemolysate was assayed by both essentially simultaneously.

Nonisotopic Assay of Adenine Phosphoribosyltransferase (AMP Pyrophosphorylase, APRT, E.C. 2.4.2.7)

APRT catalyzes the reaction:

\[
\text{Adenine} + \text{PRPP} \xrightarrow{\text{APRT}} \text{AMP} + \text{PP}
\]

The AMP produced was assayed in a linked indicator system fortified with PEP, AK, PK, LDH, and NADH. AMP produced is converted to ADP and PEP to lactate with the simultaneous oxidation of 2 moles of NADH for each mole of AMP generated. The reaction was monitored as in method B for the RPK assay. The complete system contained the following reactants in μmoles/cuvette (final volume 3 ml, light path 1 cm, 37°C): NaCl-KH₂PO₄ buffer, pH 8.0, 115; PEP, 6.0; NADH, 0.71; MgCl₂, 6.0; KF, 10.0; adenine, 0.5; PRPP, 0.65–1.0; enzyme pool of AK, PK, and LDH (5 U of each), 15 μl; hemolysate prepared as for the RPK reaction equivalent to approximately 9.0 × 10^7 erythrocytes, 250 μl. PRPP was prepared as a 3.06 mg/ml solution (theoretic-
ally 6.5 μmoles/ml or 0.0065 M) and assayed as needed by the fluoroorotate-orotidine-5-phosphate pyrophosphorylase method. PRPP so prepared was stable in the frozen state over many days. The APRT reaction was followed after an initial equilibration period for 60 min, during which time it was linear. In the assay system, $K_m$ PRPP with adenine 0.5 μmoles/3 ml cuvette was 0.027 mM. Endogenous adenine nucleotides, all of which are converted immediately to ATP by the indicator system, were optimally adequate for the AK-mediated reaction. No addition of ATP was found necessary. ATP was constantly regenerated by the linked indicator system in the course of the assay. The demonstration of APRT activity was dependent upon adenine, PRPP, the indicator system, and hemolysate. Expressed as μmoles of AMP formed/hr per $10^{10}$ red cells (this equals 0.5 × μmoles of NADH oxidized), the mean ± SD of 16 normal bloods was 3.38 ± 0.37. Enzyme activity was substantially elevated in reticulocyte-rich blood. For example, in seven subjects with reticulocytosis of 4–25%, mean APRT was 6.69. In five additional cases where high-grade reticulocytosis of 25–54% was present, the mean was still higher, 11.34. Large increases in activity were also observed when cells from a subject with the Lesch-Nyhan7,8 syndrome and without reticulocytosis were studied. The increase in red cell APRT in the Lesch-Nyhan syndrome has been well documented,19 and in our subject APRT activity was three to four times normal.

In summary, erythrocyte RPK and APRT activities are readily definable in human erythrocytes employing nonisotopic methodology and small, conveniently obtained amounts of blood.

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REFERENCES

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