The Biosynthesis of Purine Nucleotides de Novo in the Rabbit Reticulocyte in Vitro*

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The mature rabbit erythrocyte has been shown to be incapable of synthesizing de novo the purine portions of adenosine triphosphate and of guanosine triphosphate, in vitro (2). It is able, however, to carry out the final steps of this pathway from sodium formate and 5-amino-1-ribosyl-4-imidazolecarboxamide, a finding which suggests that a portion of the over-all enzymatic sequence is lost during the maturation process (2, 3).

The study of the metabolic capacities of mammalian erythrocytes at various stages in their life span has now been extended to a consideration of the ability of rabbit reticulocytes to synthesize purine nucleotides, in vitro. The reticulocyte differs from the mature erythrocyte in several respects: it possesses a reticulum which in all likelihood is ribonucleoprotein and which is lost during maturation, it has an intact tricarboxylic acid cycle and electron transport system (4), and it can synthesize heme (5) and globin (6, 7).

The studies which are reported in this paper demonstrate that the rabbit reticulocyte, in contrast to the mature erythrocyte, is able to synthesize, de novo, the purine portions of adenosine triphosphate and guanosine triphosphate, in vitro.

EXPERIMENTAL PROCEDURE

Preparation of Reticulocytes—New Zealand white rabbits, weighing about 4 kg each, received daily subcutaneous injections of 30 mg of acetylphenylhydrazine in 2.5 ml of isotonic sodium phosphate buffer (pH 7.2). The administration of acetylphenylhydrazine was continued until the reticulocyte count had risen to about 70% or more, usually within 6 to 7 days. Reticulocyte counts were made on smears supravitally stained with phenylhydrazine was continued until the reticulocyte count had risen to about 70% or more, usually within 6 to 7 days. Reticulocyte counts were made on smears supravitally stained with Wright's stain (8). About 24 hours after the final injection, the animals were exsanguinated by cardiac puncture. The blood, collected in heparin, was centrifuged and the packed cells were washed twice by resuspension in isotonic sodium chloride solution. The buffy coat, containing white blood cells and some reticulocytes, was removed. Reticulocyte counts were performed on the pooled washed cells.

Incubation in Vitro—The incubations were carried out in isotonic sodium phosphate buffer (pH 7.2) or in plasma, in a Dubnoff metabolic shaker at 100 cycles per minute (9).

Isolation and Purification of Purines—ATP and GTP were isolated and hydrolyzed as described (9). The specific activities of the adenine and guanine were determined. Samples of adenine were degraded to 4-amino-5-imidazolecarboxamidine and the specific activities were determined (3, 10).

RESULTS AND DISCUSSION

The ability of the rabbit reticulocyte to synthesize purine nucleoside triphosphates de novo was investigated by incubating reticulocyte-rich erythrocyte preparations, obtained from acetylphenylhydrazine-treated animals, with sodium formate-C14 or glycine-2-C14, with the other precursors of the purine ring, and with glucose. The results, summarized in Table I, indicate that the reticulocyte, in contrast to the mature cell (2, 3), is capable of synthesizing purine nucleotides, de novo, and demonstrate that the relative specific activity of the adenine of ATP is greater than that of the guanine of GTP. Similar findings were obtained with reticulocyte preparations obtained from animals made anemic by repeated bleeding. The utilization of sodium formate-C14 for the synthesis of the purines of ATP and GTP in the reticulocyte was similar to that found for the purines of the RNA of rabbit bone marrow by Abrams and Goldinger (11) and by Totter (12). These investigators also found a considerably greater relative specific activity in the adenine as compared to the guanine. In a comparable study with reticulocytes, Kruh and Borsook (13) observed incorporation of glycine-2-C14 into the purine bases of RNA, in vitro, with somewhat greater incorporation into the guanylic acid than into the adenyllic acid.

In order to compare the utilization of sodium formate by the over-all pathway de novo with the incorporation of sodium formate into a partially formed purine precursor, aliquots of reticulocyte preparations were incubated for 3 hours with sodium formate-C14 and either the small molecule purine ring precursors, 4-amino-5-imidazolecarboxamide, or 5-amino-1-ribosyl-4-imidazolecarboxamide (Table II). Although considerable activity was found in the purines of the nucleoside triphosphates formed by the pathway de novo, greater activity was observed in the purines derived from the cells incubated with the imidazolecarboxamide or its ribosyl derivative. The incorporation of sodium formate into the latter compounds for ATP synthesis was very much greater in the reticulocyte than that previously found in the mature cell (2, 3). In the mature cell, however, the rela-
In the sodium formate-C\(^14\) experiment, the calculation is based upon equal incorporation into carbon atoms 2 and 8.

A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incubated</th>
<th>Concentration</th>
<th>RSA (\text{c.p.m./pmole isolated nucleotide (100)})</th>
<th>RSA (\text{c.p.m./pmole sodium formate-C}(^14) administered}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sodium formate-C(^14) (20 (\mu)c)</td>
<td>150</td>
<td>1.07</td>
<td>0.53</td>
</tr>
<tr>
<td>A</td>
<td>Glucose, L-aspartic acid, L-glutamine</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Glycine, sodium carbonate</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Glycine-2-C(^14) (20 (\mu)c)</td>
<td>75</td>
<td>0.75</td>
<td>0.32</td>
</tr>
<tr>
<td>B</td>
<td>Glucose, L-aspartic acid, L-glutamine, sodium formate</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Sodium carbonate</td>
<td>75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Erythrocyte preparation contained 89% reticulocytes, 0.3% leukocytes.
\(^b\) 25 ml of washed cells incubated in 25 ml of isotonic sodium phosphate buffer (pH 7.2) for 1 hour at 37\(^\circ\).
\(^c\) RSA = relative specific activity

\[ \text{RSA} = \frac{\text{c.p.m./pmole isolated purine (100)}}{\text{c.p.m./pmole sodium formate-C}\(^14\) administered} \]

As a consequence of the maturation of the reticulocyte, changes occur that are reflected in a reversal of the adenine to guanine specific activity ratio in the acid-soluble nucleotides of the two cell types. These changes may be associated with the relative rates of conversion of inosinic acid to adenylic and guanyllic acids (14, 15), or may reflect alterations in the metabolic pools of the purine nucleotides. Changes in metabolic pools perhaps may occur as a result of the breakdown of RNA during the final phase of maturation of the reticulocyte (16, 17).

The relationship of the incubation period to the specific activity of the adenine of isolated ATP was studied by incubating a reticulocyte preparation with sodium formate-C\(^14\), the purine ring precursors, and glucose. At several time intervals, aliquots were removed and adenine was prepared from the ATP. As indicated in Table III, the synthesis of ATP proceeded in a linear fashion during the 2-hour incubation period. The adenine samples were then degraded to 4-amino-5-imidazolecarboxamidine, by the elimination of carbon 2 of the original purine ring (10).

Since sodium formate is a precursor of both carbon atoms 2 and 8, synthesis by the biosynthetic sequence \(de novo\) should yield a degradation product with one-half the specific activity of the adenine. The specific activities of the degraded adenine samples are consistent with this mechanism (Table III). If appreciable utilization of sodium formate had occurred by exchange via the inosinic acid transformylase mechanism (18) or by incorporation into a preexisting pool of purine nucleotide precursors (e.g. 5-amino-1-ribosyl-4-imidazolecarboxamidine 5'-phosphate), the ratio of labeling in carbon 2:carbon 8 would have deviated markedly from unity. Since approximately equal labeling was found in carbons 2 and 8, it may be concluded that synthesis \(de novo\) of the purine ring occurred.

In view of the finding that erythrocyte preparations rich in reticulocytes possess the capacity for purine synthesis \(de novo\), the possibility arose that a young cell population, recently matured from these reticulocytes, might also possess this capacity. To test that possibility, reticulocytosis of 47% was produced in a rabbit by the administration of acetylphenylhydrazine, a level at which synthesis \(de novo\) of purine nucleotides can be demonstrated. Fifteen days after the termination of acetylphenylhydrazine administration, a preparation of washed red blood cells from this animal contained 4.7% reticulocytes and a population of relatively young erythrocytes, most of which were

\[ \text{TABLE II} \]

\(Biosynthesis of ATP and GTP in rabbit reticulocyte\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incubated</th>
<th>Concentration</th>
<th>RSA (\text{c.p.m./pmole isolated nucleotide (100)})</th>
<th>RSA (\text{c.p.m./pmole sodium formate-C}(^14) administered}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sodium formate-C(^14) (20 (\mu)c)</td>
<td>150</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>A</td>
<td>Glucose, L-aspartic acid, L-glutamine</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Glycine, sodium carbonate</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Sodium formate-C(^14) (20 (\mu)c)</td>
<td>75</td>
<td>26.4</td>
<td>11.1</td>
</tr>
<tr>
<td>B</td>
<td>Glucose</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4-Amino-5-imidazolecarboxamidine</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Sodium formate-C(^14) (20 (\mu)c)</td>
<td>75</td>
<td>66.5</td>
<td>13.9</td>
</tr>
<tr>
<td>C</td>
<td>Glucose</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Ribosyl AICA</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Erythrocyte preparation contained 89% reticulocytes, 0.3% leukocytes.
\(^b\) 25 ml of washed cells incubated in 25 ml of isotonic sodium phosphate buffer (pH 7.2) for 1 hour at 37\(^\circ\).
\(^c\) In Part A, the calculation is based upon equal incorporation into carbon atoms 2 and 8.

\[ 4\text{-Amino-1-ribosyl-4-imidazolecarboxamidine.} \]

\[ \text{TABLE III} \]

\(Effect of incubation time on } de novo synthesis of ATP in rabbit reticulocyte,^a in vitro \)

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Adenine of ATP</th>
<th>Degradation of adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>4-Amino-5-imidazolecarboxamidine</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2700</td>
<td>1100</td>
</tr>
<tr>
<td>1.0</td>
<td>3700</td>
<td>1900</td>
</tr>
<tr>
<td>2.0</td>
<td>5000</td>
<td>2200</td>
</tr>
</tbody>
</table>

\(^a\) Erythrocyte preparation contained 85% reticulocytes, 0.4% leukocytes.
\(^b\) 25 ml of washed cells incubated in 25 ml of isotonic sodium phosphate buffer (pH 7.2) at 37\(^\circ\) with sodium formate-C\(^14\) (20 \(\mu\)c), 150 \(\mu\)moles; glycine, 75 \(\mu\)moles; sodium carbonate, 75 \(\mu\)moles; L-aspartic acid, 150 \(\mu\)moles; L-glutamine, 150 \(\mu\)moles; and glucose, 150 \(\mu\)moles.

\[ \text{Glycine, sodium carbonate.} \]
the synthetic capacity of very young erythrocytes which are not capable of synthesizing purine nucleotides de novo, in response to acetylphenylhydrazine. Radioactivity was detected in the isolated ATP or GTP. The findings suggest that young erythrocytes which have matured with sodium formate-C$^{14}$ and the other small molecule precursors are capable of purine biosynthesis observed. The attempts to evaluate this capacity for de novo purine nucleotide biosynthesis in the mature rabbit erythrocyte has the capacity for the final reactions of purine nucleotide synthesis indicates that only a portion of the metabolic sequence is lost and that the loss occurs at a reaction before the formation of 5-amino-1-ribosyl-4-imidazole-carboxamide 5'-phosphate (2, 3). The finding that the mature rabbit erythrocyte can utilize preformed purines for nucleotide formation provides evidence for the formation of 5-phosphoribosylpyrophosphate from glucose within the cell and indicates that the missing step or steps are concerned with the formation of the purine ring itself (0, 21).

**SUMMARY**

The rabbit reticulocyte, prepared from animals made anemic by acetylphenylhydrazine administration or by bleeding, has the capacity for de novo purine nucleotide biosynthesis in vitro. This biosynthetic capacity is lost on maturation of the reticulocyte.

Adenosine triphosphate and guanosine triphosphate can also be synthesized in this cell from sodium formate-C$^{14}$ and 4-amino-5-imidazolecarboxamide or 5-amino-1-ribosyl-4-imidazolecarboxamide. The relative labeling of the purines of the nucleoside triphosphates is the reverse of that observed in the mature erythrocyte.

**REFERENCES**


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**Table IV**

<table>
<thead>
<tr>
<th>Incubateda</th>
<th>Cellular composition</th>
<th></th>
<th>Isolated</th>
<th>Adenine of ATP</th>
<th>Guanine of GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of cells</td>
<td>Leukocytes</td>
<td>Reticulocytes</td>
<td>%</td>
<td>0.2</td>
<td>42</td>
</tr>
<tr>
<td>A</td>
<td>Buffy coat removed preincubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Leukocytes added preincubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Buffy coat removed preincubation; erythrocytes lysed postincubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*25 ml of washed cells incubated in 25 ml of isotonic sodium phosphate buffer (pH 7.2) for 1.5 hours at 37° with sodium formate-C$^{14}$ (20 μc), 150 μmoles; glycine, 75 μmoles; sodium carbonate, 75 μmoles; L-aspartic acid, 150 μmoles; L-glutamine, 150 μmoles; and glucose, 150 μmoles.
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