The Effect of Reticulocytosis in the Rabbit on the Activities of Enzymes in Pyrimidine Biosynthesis

By MYRON LOTZ AND LLOYD H. SMITH, JR.

THE CIRCULATING erythrocyte offers a model system for the study of the relationship of enzyme activity to cell age. A number of enzymes decline in activity in the aging erythrocyte. A few enzymes have been found to maintain relatively constant activity during erythrocyte maturation or during aging of the mature cell. The mature mammalian erythrocyte contains both purine and pyrimidine nucleotides which are essential cofactors in many of its metabolic reactions. Recent studies indicate that the erythrocyte is unable to effect de novo synthesis of purines. Similarly there is a block in pyrimidine biosynthesis, reflecting the loss of dihydroorotic dehydrogenase activity. Information is not available on the effect of cell age on the activities of individual enzymes involved in the biosynthesis of purine and pyrimidine nucleotides. In the present work, five sequential enzymes leading to uridine-5'-phosphate have been assayed during aging of rabbit reticulocytes (fig. 1). Activities of all of these enzymes were found to be much higher in the reticulocyte than in the normocyte, and to decrease rapidly during the process of maturation.

METHODS

A reticulocytosis of approximately 75 per cent was produced in New Zealand rabbits (2 to 3 Kg.) by the daily injection of 1 ml. of 2.5 per cent acetylphenylhydrazine over a period of 6–8 days. At intervals of 4–6 days thereafter, erythrocytes were obtained by the dextran sedimentation method as previously described. Reticulocyte counts carried out before and after double sedimentation of erythrocytes did not differ significantly, making it improbable that this technic preferentially separated out either the younger or the older cells. The separated erythrocytes were hemolyzed by rapid freezing and thawing and aliquots were taken for enzyme assays. Leukocyte contamination was less than 1 per 10,000 erythrocytes, a level too low to contribute significantly to the enzyme activities measured.

Assay procedures were carried out as previously described. In brief, the methods were as follows: (1) aspartate carbamyltransferase—the formation of carbamylaspartic acid-C\(^14\) (CAR-C\(^14\)) from l-aspartic acid-C\(^14\) and carbamylphosphate; (2) dihydroorotate—the hydrolysis of dihydroorotic acid-C\(^14\) (DOH-C\(^14\)) to carbamylaspartic acid-C\(^14\); (3) dihydroorotic dehydrogenase—the oxidation of dihydroorotic acid-C\(^14\) to orotic acid-C\(^14\) (OA-C\(^14\)); (4) orotidylic pyrophosphorylase—the release of carbon dioxide-C\(^14\) from carboxyl-labeled OA-C\(^14\) in the presence of 5-phosphoribosylpyrophosphate, Mg\(^++\), and excess partially purified yeast orotidylic decarboxylase; (5) orotidylic decarboxylase—the release of carbon dioxide-C\(^14\) from biosynthetically prepared carboxyl-labeled orotidine-5'-phosphate (O5P-C\(^14\)). All activities have been expressed as \(\mu\) mole product formed per 10\(^9\) erythrocytes. The reproducibility of the enzyme assays as employed has been documented in the linear relationships obtained between enzyme activities and enzyme concentrations, as previously reported.

Intact reticulocytes or erythrocytes were incubated in Krebs-Ringers phosphate-glucose.
Fig. 1.—Pathway of Uridine-5'-Phosphate Biosynthesis. Abbreviations include: CAP, carbamyl phosphate; L-asp, l-aspartic acid; CAA, carbamylaspartic acid; DHO, dihydroorotic acid; OA, orotic acid; PRPP, 5-phosphoribosylpyrophosphate; OSP, orotidine-5'-phosphate (orotidylic acid); UMP, uridine-5'-phosphate.

containing carboxyl-labeled OA-C14 or carboxyl-labeled carbamylaspartic acid-1-C14 (CAA-1-C14), both at 2.5 x 10^-4 M. C14Oa was trapped in centerwell Hyamine base and counted as previously described in a tricarb liquid scintillation spectrometer.13 5-Fluoroorotic acid (5-F-OA) and 6-azauridine (6-Azur), obtained through the courtesy of Drs. T. C. Hall and Emil Frei, III, respectively, were used in concentrations of 10^-3 M. To prevent bacterial growth, penicillin was included in the incubation mixture in concentrations of 500 u./ml.

RESULTS

Enzyme activities of aspartate carbamyltransferase, dihydroorotase, dihydroorotic dehydrogenase, orotidylic pyrophosphorylase, and orotidylic decarboxylase in rabbit erythrocytes following completion of acetophenylhydrazine hemolysis are presented in figure 2. The decline in activities is charted as a function of time with subscribed reticulocyte counts. The figures presented are from representative studies on individual rabbits. The time sequence of erythrocyte enzyme activities was studied in a total of six rabbits. Control values for orotidylic pyrophosphorylase and orotidylic decarboxylase activities exhibited considerable variations from rabbit to rabbit, but with the production of reticulocytosis the activities were always increased by factors consistent with those summarized by the data of figure 2.
Fig. 2.—Enzymatic activities in rabbit reticulocytes invoked by phenylhydrazine. The graphs illustrate repeated assays in individual rabbits over the period of reticulocytosis. Abbreviations used are described in fig. 1.

An estimation of the absolute enzyme activity of reticulocytes was made by the method of Allison and Burn. These values are presented in table 1 together with the ratio of calculated enzyme activities in reticulocytes to those found in mature, control erythrocytes. Because of the assumptions required in such estimations, the absolute figures have limited validity. The orders of magnitude, however, allow for comparison with previous studies of other enzymes, as listed in table 2.

Pyrimidine metabolism in intact reticulocytes and normocytes was assayed by measuring C\textsuperscript{14}O\textsubscript{2} release from carboxyl-labeled OA-C\textsuperscript{14} and carboxyl-labeled CAA-1-C\textsuperscript{14}. The validity of using these decarboxylations as indices of uridine-5'-phosphate synthesis via orotidine-5'-phosphate (fig. 1) has been supported by data presented more extensively elsewhere, and can be briefly summarized as follows:

a. The release of C\textsuperscript{14}O\textsubscript{2} is more rapid following incubation with carboxyl-labeled precursors than following incubation with precursors labeled elsewhere in the molecule.

b. Antineoplastic agents which have been shown in vitro to inhibit pyrimidine nucleotide formation markedly inhibit the release of C\textsuperscript{14}O\textsubscript{2} from carboxyl-labeled precursors.

c. Antimetabolites active in the purine nucleotide synthetic sequence fail to exhibit such inhibition.

As shown in table 3, decarboxylation of carboxyl-labeled OA-C\textsuperscript{14} was more rapid in the reticulocytes than in the normocytes. Activities of both cell types
Table 1.—Enzyme Activities Found in Rabbit Reticulocytes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Calculated retic activity (mM/mole/10^6 cells)</th>
<th>Ratio of reticulocyte: normocyte activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate carbamyltransferase</td>
<td>6140</td>
<td>60:1</td>
</tr>
<tr>
<td>Dihydroorotase</td>
<td>1270</td>
<td>200:5</td>
</tr>
<tr>
<td>Dihydroorotic dehydrogenase</td>
<td>400</td>
<td>∞</td>
</tr>
<tr>
<td>Orotidylic pyrophosphorylase</td>
<td>1340</td>
<td>12:1</td>
</tr>
<tr>
<td>Orotidylic decarboxylase</td>
<td>700</td>
<td>10:1</td>
</tr>
</tbody>
</table>

Table 2.—Summary of Reported Enzyme Activities in the Reticulocyte as Compared with the Normocyte

<table>
<thead>
<tr>
<th>Calculated Reticulocyte Enzyme Activity</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>cathepsin</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>fumarase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>pyrophosphatase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>succinic dehydrogenase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>aconitase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>cytochrome oxidase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>acid inorganic pyrophosphatase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>carboxylic anhydrase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>hexokinase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>ribonuclease</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>desoxyribonuclease</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>aspartate carbamyltransferase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>dihydroorotase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>dihydroorotic dehydrogenase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>orotidylic pyrophosphorylase</td>
<td>Activity Ratio</td>
</tr>
<tr>
<td>orotidylic decarboxylase</td>
<td>Activity Ratio</td>
</tr>
</tbody>
</table>

*Numbers in superscript refer to references of published reports from which calculations were made.
†Denotes experiments in man, all others were in rabbits.
‡Denotes reference to present work.

were inhibited from 50–75 per cent with 5-FOA. With 6-Azur, inhibition occurred regularly only in the reticulocytes. Repeated experiments in normocytes showed very slight or no inhibition by 6-Azur, possibly reflecting inability of the normocyte to effect the phosphorylation of 6-azauridine (6-Azur) to 6-azauridylic acid, the metabolically active compound.16

Experiments with both cell types using ring labeled OA-C14 repeatedly showed no C14O2 release. Similarly, it was not possible to demonstrate utilization of CAA-1-C14 by either the reticulocyte or the normocyte. This may be related to poor penetration of this dicarboxylic acid into the cells.

To investigate the possibility that the activities observed in intact red cells might be due to the low levels of contaminating white blood cells, experiments of the above nature were carried out using intact white cells alone in amounts
Reticulocytosis and Pyrimidine Biosynthesis

Table 3.—Orotic Acid Metabolism in Intact Rabbit Reticulocytes and Normocytes
(Direct Enzyme Assays are Included for Comparative Purposes)*

<table>
<thead>
<tr>
<th></th>
<th>Intact Cells</th>
<th>Hemolysates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OA-C¹⁴</td>
<td>OA-C¹⁴ + 5F-OA</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>12.9*</td>
<td>6.4</td>
</tr>
<tr>
<td>(95% retics)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normocytes</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>(5% retics)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results expressed as µ mole C¹⁴O₂ released /10⁶ cells/hour.

Abbreviations include: OA-C¹⁴, carboxyl-labeled orotic acid-C¹⁴; 5F-OA, 5-fluoroorotic acid; 6-Azur, 6-azauridine; OA → OSP, orotidylic pyrophosphorylase; OSP → UMP, orotidylic decarboxylase.

5–7 times greater than that ordinarily found as contaminants in the red cell preparations. Such experiments indicated that the contaminating white cells could account for no more than 1–2 per cent of the activities observed in the intact red cell preparations.

Discussion

Observations on the effect of erythrocyte age on enzyme activity have usually been confined to the changes occurring during reticulocytosis (maturation) or less frequently to changes occurring during aging of the normocyte per se (senescence). The studies reported here relate only to the gross alterations in enzyme activity found in the reticulocyte and the time required for the average erythrocyte enzyme activity to return to its baseline level. The data do not allow accurate conclusions to be drawn concerning enzyme changes during the 60 day survival time of the mature rabbit erythrocyte. Such information could be obtained only by the use of a method for separating young from old normocytes, based on some property such as differential osmotic fragility. There are also sources of variation in the use of induced reticulocytes as a cell type. Such cells show a marked heterogeneity on a morphologic scale of maturity. In addition, recent evidence indicates that in the rat the reticulocytes induced by phenylhydrazine hemolysis may be abnormal cells in that they are larger, mature more slowly, and are cleared from the blood stream more rapidly than are their normal counterparts. Despite these limitations of the technic employed, it is felt that the data obtained reflect with acceptable validity the changes in activities of pyrimidine biosynthetic enzymes which occur during normal reticulocytosis and maturation.

The mature non-nucleated erythrocyte contains neither DNA nor RNA and—as would be anticipated—has not been shown to be capable of protein synthesis. The major nucleotides found in the erythrocyte are those of adenine. Guanine nucleotides are present at approximately one-tenth those of adenine, and pyrimidine nucleotides are present in trace amounts only, not as yet accurately measured. Recent studies have demonstrated a block in the de novo synthesis of purine nucleotides which develops during cell maturation. The exact location of the missing enzyme or enzymes has not been identified, but probably occurs prior to the formation of 5-aminomidazole-4-carboxamide...
Despite this block in de novo synthesis, the purines of erythrocyte adenosine triphosphate and guanosine triphosphate exhibit renewal rates similar to the purines of mixed organ ribonucleic acid. Evidence has been presented that this may occur by a salvage synthesis utilizing preformed purines, although the functional significance of this demonstrated pathway has yet to be clarified. The mature erythrocyte also exhibits a complete block in the de novo synthesis of pyrimidine nucleotides due to the absence of the particular enzyme dihydroorotic dehydrogenase. The data presented here show that dihydroorotic dehydrogenase activity is present in the reticulocyte, disappearing at a rate approximating the fall in residual reticulocyte count. The other enzymes in the de novo synthesis of uridine-5'-phosphate are correspondingly increased in activity in the immature erythrocyte. In close analogy to previous studies on purine nucleotides, the reticulocyte contains the enzymes necessary for the de novo synthesis of pyrimidine nucleotides; the normocyte has a block in de novo synthesis. Although the individual enzymes could be assayed in reproducible activities in hemolysed erythrocytes, the utilization of CAA-1-C14 as a pyrimidine precursor in intact cells could not be demonstrated. This may be related to poor penetration of this dicarboxylic acid into the cell.

Although chromatographic studies of the acid soluble fraction from erythrocyte hemolysates have revealed only trace amounts of nucleotides which might contain pyrimidines, enzymatic studies indicate the presence of some pyrimidine-containing cofactors in the adult erythrocyte. This extremely low concentration would make difficult any determination of the renewal rate of erythrocyte pyrimidine nucleotides, comparable to that determined for purine nucleotides. If a similar renewal rate did pertain, synthesis of pyrimidine nucleotides might occur from exogenous orotic acid (distal to the block at dihydroorotic dehydrogenase) or by salvage synthesis utilizing preformed pyrimidine bases. Orotic acid has not been detected in plasma. In urine it has been found only in the syndrome of orotic aciduria or following the use of certain antimetabolites, notably 6-azauridine. A low rate of utilization of orotic acid for uridine-5'-phosphate synthesis by the mature erythrocyte has been shown in the present study. The possibility of pyrimidine nucleotide renewal from free pyrimidines by nucleotide pyrophosphorylase activity in the presence of 5-phosphoribosylpyrophosphate has not been excluded. Free pyrimidine bases may be made available by the degradation of erythrocyte nucleotides. The presence of small amounts of uracil and cytosine in urine suggests that these pyrimidine bases are also available in low levels in plasma.

It must be noted that carbamylphosphate synthetase has not been detected in hemic cells. This enzyme is found in very low activity in mammalian tissues except the liver, where its activity is greatly enhanced by a function in the urea cycle. It is possible that the indirect assay employed was too insensitive to detect the minute amounts of this labile product presumably formed. It is also possible that the carbamyl group of citrulline may serve to distribute a carbamylphosphate precursor to non-hepatic tissues.

This report is but one of a series concerning changes in activities of enzymes coincident with maturation of the reticulocyte. (For purposes of comparison, these previous reports, together with the studies reported in this paper, have
been summarized in table 2. In this maturation over the short period of several hours, the reticulocyte changes from a metabolically active cell capable of synthesizing protein, heme, purine nucleotides, pyrimidine nucleotides, and containing an active tricarboxylic-acid cycle to an anomalous glycolytic-dependent cell incapable of carrying out any of these synthetic functions. Loss of an enzyme sequence does not necessarily occur in toto during erythrocyte maturation, for some enzymes remain which can only be presumed to be vestigial. As a general rule, particulate enzymes are lost; soluble enzymes remain, even if at greatly reduced activities. Although they contribute to an understanding of the events occurring during erythrocyte maturation, the studies reported here do not add information concerning the metabolic events which determine the life span of the mature erythrocyte.

SUMMARY

Five sequential enzymes leading to the formation of uridine-5'-phosphate were studied in acetophenylhydrazine-induced reticulocytes in the rabbit. All of these enzymes—aspartate carbamyltransferase, dihydroorotase, dihydroorotic dehydrogenase, orotidylic pyrophosphorylase, and orotidylic decarboxylase—decreased markedly in activity during in vivo maturation and aging of the reticulocytes. In analogy to previous studies on purine nucleotide biosynthesis, it is concluded that the reticulocyte, but not the mature erythrocyte, is capable of de novo pyrimidine nucleotide biosynthesis.

SUMMARIO IN INTERLINGUA

Cinque enzymas sequential ducente al formation de uridina-5'-phosphato esseva studiate in reticulocytos induce in conilios per medio de acetophenylhydrazina. Omne iste enzymas—carbamyltransferase de aspartato, dihydroorotase, dihydroorotic dehydrogenase, pyrophosphorylase orotidylic, a decarboxylase orotidylic—decresceva marcatemente in lor activitate durante le curso de maturation in vivo e de senescentia del reticulocytos. Il es concludite—per analogia con previe studios del biosynthese de nucleotidos de purina—que le reticulocyto, sed non le matur erythrocyto, es capace de biosynthese ab novo de nucleotidos de pyrimidina.

REFERENCES


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