ERYTHROCYTE METABOLISM

I. PURINE NUCLEOSIDE PHOSPHORYLASE*

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Previous studies from this laboratory on the preservation of erythrocytes stored in vitro have shown that the storage lesion, as characterized by the decline in level of organic phosphate esters, may be reversed by incubating the aged cells with certain purine nucleosides (1). Pyrimidine nucleosides were ineffective in this respect, as was the addition of the purine bases and ribose, alone or in combination. These observations, together with the fact that reincorporation of inorganic phosphate into the organic phosphate fractions paralleled the restoration of viability (2), suggested that the initial reaction was being carried out by a purine nucleoside phosphorylase,

\[
\text{Purine riboside} + P_i \rightarrow \text{purine} + R-1-P
\]

an enzyme whose existence in the erythrocyte had been suggested previously by the work of Dische (3), and more recently by Prankerd and Altman (4).

Preliminary reports of this investigation have established the role of nucleoside phosphorylase in the preservation of erythrocytes (5, 6). The present communication will describe the preparation, assay, and properties of this enzyme, which, in many respects, resembles the purine nucleoside phosphorylase found in liver (7) and brain (8).

EXPERIMENTAL

Materials—Whole blood, stored in ACD (National Institutes of Health, Formula B), was made available through the cooperation of Dr. Richard Czajkowski of the King County Central Blood Bank. Xanthine oxidase was isolated from cream by the method of Mackler et al. (9), through the first ammonium sulfate fractionation. Crystalline beef liver catalase was obtained from the Worthington Biochemical Corporation.

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (10) and aged for at least 3 months before use.


1 The following abbreviations are used: P_i, inorganic phosphate; R-1-P, ribose-1-phosphate; R-5-P, ribose-5-phosphate; ACD, acid-citrate-dextrose medium; Tris, tris(hydroxymethyl)aminomethane.
Adenosine, inosine, and cytidine were obtained from the Schwarz Laboratories, Inc., uridine and thymidine from the California Foundation for Biochemical Research, xanthosine, xanthine, guanosine, hypoxanthine, and R-5-P from the Nutritional Biochemicals Corporation, uric acid from the Eastman Kodak Company, and ribose from the Mann Research Laboratories.

2,6-Diaminopurine riboside was generously supplied by Dr. G. B. Brown, adenosine mono- and trisulfate by Dr. A. Hock, and R-1-P by Dr. H. L. A. Tarr.

Methods—Since previous studies (7, 8, 11, 12) with nucleoside phosphorylases from various sources had shown that Reaction 1 is actually a reversible equilibrium favoring the synthetic direction, it was desirable in the present study to shift the equilibrium in the direction of breakdown. This has been accomplished either by oxidizing the liberated purine base, hypoxanthine, with xanthine oxidase (7) or by the use of arsenate to "trap" the pentose moiety.

For most purposes, the nucleoside phosphorylase may be assayed satisfactorily by coupling the system with xanthine oxidase and following the reaction manometrically. The manometer cups contained 10 μmoles of inosine, 100 μmoles of phosphate buffer, pH 7.5, 0.01 ml. (100 units) of catalase, 50 μmoles of ethanol, 0.1 ml. of xanthine oxidase (0.9 mg. of protein), and the phosphorylase, all contained in 2.8 ml. Inosine was omitted in the blanks. The center well contained 0.2 ml. of 6 M KOH; the cups were gassed with air and equilibrated in the water bath at 38° for 10 minutes before closing the stop-cocks. Over several hours, the rate of oxygen uptake was linear with time and linear with phosphorylase concentration (cf. Fig. 1), provided that the total uptake did not exceed 125 μl. per hour. 1 unit of phosphorylase is defined as that amount which causes the uptake...
of 1 microatom of oxygen per hour. Specific activity is defined as units of enzyme per mg. of protein. Protein was estimated by the biuret method (13) with crystalline bovine serum albumin as the standard, and hemoglobin was determined spectrophotometrically at 540 m\(\mu\) (14).

For preparations of the enzyme which were freed from hemoglobin, it was possible to follow the coupled reaction of phosphorylase with xanthine oxidase by the spectrophotometric method of Kalckar, i.e. by following the appearance of uric acid at 290 m\(\mu\) (7). The assay system in 1 cm.

![Fig. 2. Spectrophotometric assay as described in the experimental section](image_url)

The above assays require that inosine be used as substrate. A more general method, applicable to all reactive purine ribonucleosides, is obtained by substituting arsenate for phosphate and measuring the ribose liberated.

\[
Purine-\text{ribose} + \text{arsenate} \rightarrow \text{purine} + \text{ribose-1-arsenate} \tag{2}
\]

\[
\text{Ribose-1-arsenate} + \text{H}_2\text{O} \rightarrow \text{ribose} + \text{arsenate} \tag{3}
\]
The assay system contained 10 μmoles of the nucleoside, 20 μmoles of arsenate, 100 μmoles of Tris buffer, pH 7.5, phosphorylase, and water to make 3.0 ml. The tubes were sealed and incubated for 3 hours at 38°. At the end of this time, the tube contents were heat-deproteinized by immersion in a boiling water bath for 5 minutes, the denatured protein was removed by centrifugation, and 1.0 ml. samples of the supernatant fluid were taken for ribose analysis by using the method of Nelson for reducing sugars (15).

RESULTS AND DISCUSSION

Purification of Enzyme—Fresh, human erythrocytes were washed three times by suspension and centrifugation in 3 volumes of cold 0.9 per cent sodium chloride and resuspended in saline solution to approximately the original hematocrit (about 50). The cell suspension was hemolyzed by quick freezing (dry ice and ethylene glycol monomethyl ether) and quick thawing three times, and the stroma fraction was removed by centrifugation.

90 ml. of hemolysate (Fraction I) were diluted with 360 ml. of cold water, the solution was taken to pH 5.4 with 1 N acetic acid, and the precipitate was removed by centrifugation. The supernatant fluid was adjusted to pH 7.5 with 1 N sodium hydroxide and dialyzed overnight against 10 liters of water. In 250 ml. portions, the solution was then readjusted to pH 5.5 and 600 ml. of water and 150 ml. of calcium phosphate gel (about 15 mg. per ml. of dry weight) were added in that order. The mixture was stirred mechanically for 15 minutes, followed by centrifugation. The supernatant fluid was discarded, and the gel was washed three times, each with 130 ml. of water by suspension and centrifugation. The enzyme was eluted from the gel with 60 ml. of 0.5 M phosphate buffer, pH 8.0, by suspension and centrifugation. The gel was treated as before with an additional 60 ml. portion of buffer and the two supernatant fluids were combined and dialyzed with mechanical stirring for 2 hours against water (Fraction II).

In 50 to 60 ml. portions, the dialyzed enzyme was brought as rapidly as possible to 60° and held at this temperature for 5 minutes. The solution was cooled rapidly in an ice bath, and the denatured protein was removed by centrifugation.

The supernatant fluid was brought to approximately 60 per cent of saturation by adding, in small portions, 42.6 gm. of solid ammonium sulfate per 100 ml. of solution. After the final addition, the solution was stirred gently for an additional 15 minutes, and the precipitate was recovered by centrifugation, dissolved in 8 to 10 ml. of water, and dialyzed for 2 hours.

\(^2\) Unless otherwise specified, all operations are carried out at 0-5° and centrifugations are performed at 2300 × g for 30 minutes in the International refrigerated centrifuge, model PR-1.
with stirring against water. After dialysis, any denatured material was removed by centrifugation. The supernatant fluid (Fraction III) may be stored in the frozen state or lyophilized and stored frozen as a dry powder. Under these conditions the enzyme remains active over several months of storage.

A summary of a representative purification procedure is given in Table I, where it is seen that there is about a 150-fold over-all purification from the hemolysate stage with a 50 per cent yield of enzyme recovered. In all preparations the specific activity of Fraction III varied between 300 and 500.

Since hemoglobin comprises more than 90 per cent of the total protein in hemolysates, it is evident that the removal of this chromoprotein constitutes the major problem in the purification of any erythrocyte enzyme. In the present procedure, the hemoglobin remains in solution and is discarded when the enzyme is adsorbed onto calcium phosphate gel. The phosphorylase, however, retains a reddish color when eluted from the gel, and this color is not diminished by repetition of the gel treatment. Even at the higher stage of purity (Fraction III), the enzyme has a reddish color.

The absorption spectrum of the purified enzyme (peaks at 576, 408, 290 (shoulder), and 275 mp) is somewhat similar to that of highly purified methemoglobin reductase (16), although no reductase activity can be demonstrated in the phosphorylase preparations.

Component Study of Reaction—By using the manometric assay system, wherein the phosphorylase reaction is coupled with xanthine oxidase, it was possible to demonstrate an absolute requirement for (1) the substrate, inosine, (2) phosphate (or arsenate), (3) the phosphorylase, and (4) xanthine oxidase (cf. Table II and Figs. 3 and 4). It was possible to show also a partial requirement for the catalase-ethanol system to remove hydrogen peroxide; the lack of an absolute requirement for the latter system indicated that one of the enzymes in the assay still contains traces of endogenous catalase.

### Table I

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Volume ml.</th>
<th>Phosphorylase activity units</th>
<th>Protein mg. per ml.</th>
<th>Specific activity units per mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Hemolysate</td>
<td>90</td>
<td>27,630</td>
<td>140*</td>
<td>2.2</td>
</tr>
<tr>
<td>II. Calcium phosphate gel eluate</td>
<td>125</td>
<td>22,700</td>
<td>2.7</td>
<td>67</td>
</tr>
<tr>
<td>III. 60% ammonium sulfate</td>
<td>12</td>
<td>13,800</td>
<td>3.4</td>
<td>340</td>
</tr>
</tbody>
</table>

* Based upon hemoglobin determination.
In manometric experiments in which limiting amounts of substrates (1 to 2 \( \mu \)moles) were used in order to obtain complete oxidation, it was observed that an additional 1 microatom of oxygen was taken up when inosine was compared with hypoxanthine. It would appear, therefore, that the ribose phosphate, formed in the phosphorylase reaction, is metabolized further along an oxidative pathway. In this connection, it is of interest to recall the observation of Francoeur and Denstedt (17) that ribose-5-phosphate is peroxidized by \( \text{H}_2\text{O}_2 \) in the presence of catalase, although the oxidation product was not characterized. The further metabolism of the pentose moiety, liberated in erythrocyte phosphorylase reaction, is presently under investigation.

**Products of Reaction**—During the initial phases of this investigation it had been determined that the enzyme had an absolute requirement for

### Table II

**Component Study**

Manometric assay as described in experimental section, except that the complete system contained 100 \( \mu \)moles of Tris buffer, pH 7.5, and 20 \( \mu \)moles of phosphate. Reaction time, 210 minutes.

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>( \text{O}_2 ) uptake microatoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34.3</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>0.5</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>0.1</td>
</tr>
<tr>
<td>Phosphate</td>
<td>5.1</td>
</tr>
<tr>
<td>Arsenate, replacing phosphate</td>
<td>25.0</td>
</tr>
<tr>
<td>Ethanol and catalase</td>
<td>27.8</td>
</tr>
</tbody>
</table>

![Graph](http://www.jbc.org)  

**Fig. 3.** Manometric assay as described in the experimental section with amounts of inosine as indicated.
phosphate or arsenate (cf. Fig. 4), and that in the latter case the liberated ribose could be measured colorimetrically. When inosine was used as substrate, the reaction product, hypoxanthine, could be linked to xanthine oxidase. These observations, and the other close similarities between the erythrocyte enzyme and its counterparts in liver (7) or brain (8), left little doubt that the phosphorylase reaction in this case proceeded according to Reactions 1 to 3. Additional evidence for this assumption was obtained from experiments wherein aliquots from phosphorylase reaction mixtures were subjected to paper chromatography. The purine nucleosides may be

separated from the free bases by chromatography in Solvent System A, isobutyric acid-water-concentrated ammonia (66:33:1), and located by inspection under ultraviolet light (Mineralite). Under these conditions, it was shown that hypoxanthine ($R_F = 0.63$) was released from inosine ($R_F = 0.50$) and guanine ($R_F = 0.66$) from guanosine ($R_F = 0.53$). In addition, the nature of the ribose moiety released was ascertained by chromatographing aliquots of reaction mixtures in Solvent System A and in a second system, Solvent System B, methanol-88 per cent formic acid-water (70:25:5), and by making use of the molybdate spray reagent (18) to locate phosphate esters and aniline trichloroacetate reagent (19) to locate free sugars. As expected, in the presence of arsenate, free ribose ($R_F$ in Solvent System A = 0.48; $R_F$ in Solvent System B = 0.67) was the only

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**Fig. 4**

Spectrophotometric assay as described in the experimental section with the indicated amounts of phosphate (△) and arsenate (○).

**Fig. 5**

Spectrophotometric assay as described in the experimental section with the phosphate concentrations expressed in reciprocal units on the abscissa. Individual determinations in the absence (○) and presence (△) of 2 μmoles of pyrophosphate were made. Initial velocity ($v$) ($\Delta \log I_0/I$ at 290 nm over a 10 minute interval) is expressed in reciprocal units on the ordinate.
reaction product. When phosphate was substituted for arsenate, R-1-P ($R_f$ in Solvent System A = 0.24; $R_f$ in Solvent System B = 0.54) was the principal product. In crude hemolysates or preparations of the partially purified enzyme, several phosphate esters in addition to R-1-P were observed on the paper chromatograms. One of these has been identified by comparison with a known standard as R-5-P ($R_f$ in Solvent System A = 0.23; $R_p$ in Solvent System B = 0.67). This substance undoubtedly arises from R-1-P through the action of phosphoribomutase in erythrocytes (20).

Evidence that the erythrocyte phosphorylase can catalyze Reaction 1 in the direction of nucleoside synthesis can be obtained also by paper chromatographic methods. In a typical experiment, 100 μmoles of R-5-P, 100 μmoles of hypoxanthine, and 20 μmoles of Mg$^{++}$ were incubated for 90 minutes at 37° with 20 ml. of stroma-free hemolysate. Neutralized perchloric acid filtrates were prepared from this reaction mixture. Hypoxanthine was determined with xanthine oxidase, and an aliquot of the filtrate was subjected to paper chromatography in Solvent System A. It was found that 77 μmoles of hypoxanthine remained after incubation (23 μmoles of hypoxanthine having been utilized). Quantitative densitometry of the paper chromatograms indicated a ratio of 0.28 for the inosine and hypoxanthine areas. From the above enzymatic estimation of 77 μmoles of hypoxanthine remaining, it follows that 0.28 X 77 μmoles or 22 μmoles of inosine have been formed during the reaction, an amount which is in good agreement with that of the hypoxanthine utilized.

Kinetic Properties of Enzyme—The enzyme displays a broad pH optimum in the range pH 7.0 to 8.5. There is no diminution of activity when phosphate buffer is replaced by Tris or glycylglycine buffer, provided that phosphate or arsenate is present in catalytic amounts.

The effects of inosine, phosphate, and arsenate concentrations upon the reaction are illustrated in Figs. 3 and 4, respectively. The Michaelis constant ($K_m$) values, calculated by the Lineweaver-Burk method (21) from the double reciprocal plots, are $1.0 \times 10^{-3}$ M for inosine, phosphate, or arsenate. The $K_m$ value for inosine with the purified enzyme is of the same order of magnitude as noted previously for the over-all utilization of nucleosides during the rejuvenation of intact stored cells (1). It is also in good agreement with the value reported by Robins et al. (8) for the brain.

It is of interest that R-1-P gives a yellow color with the molybdate spray reagent (18) and a brown color with the aniline-trichloroacetae reagent (19), whereas glucose-1-phosphate, which moves with essentially the same $R_f$ value in these solvent systems, gives a blue color with the first reagent and no reaction with the second. This difference in behavior is due undoubtedly to the greater lability of the phosphate ester linkage in R-1-P.

Photovolt densitometer, model 301-A, phototube B, filter 5265, wave-length, 253 μm.
enzyme, but considerably larger than that for the beef liver enzyme (11, 22). The $K_m$ value for phosphate is likewise in good agreement with that reported for the brain enzyme (8), but again larger than that for the beef liver preparation (11). From Fig. 4 it can be seen that the maximal velocity obtained with arsenate is only 44 per cent of that with phosphate. This finding is in agreement with the value of 53 per cent reported by Rowen and Kornberg for the beef liver phosphorylase acting upon nicotinamide riboside (11).

Substrate Specificity—In a previous communication (6), it was pointed out that the crude hemolysate contains the necessary enzymes to split inosine, adenosine, guanosine, xanthosine, and 2,6-diaminopurine riboside. If the rate with inosine is expressed arbitrarily as 100, the relative rates of splitting of the other nucleosides are 65, 60, 40, and 25, respectively. The purified enzyme cleaves only inosine and guanosine with the relative rates of 100 and 60. Adenosine mono- and trisulfates, and the pyrimidine ribonucleosides, thymidine, uridine, and cytidine, are not cleaved in either the crude or purified systems. In addition, pyrimidine nucleosides do not inhibit the splitting of purine nucleosides. The above results are interpreted to mean that the purified enzyme splits only inosine and guanosine, in agreement with the substrate specificity noted by Kalckar for rat liver phosphorylase (7), and that in the crude hemolysate other enzymes must convert the other nucleosides to one of these two actual substrates. It is known that erythrocytes contain a powerful adenosine deaminase,6 which is in keeping with our finding that equimolar amounts of ammonia are produced when aged erythrocytes are rejuvenated with adenosine (23). It is evident that adenosine is converted directly to inosine before phosphorolysis.

In preliminary experiments, it has been shown that ribose is liberated when xanthosine is split by crude hemolysates in the presence of arsenate. However, no oxygen uptake is observed when xanthosine is split by the phosphorylase-xanthine oxidase system. This would appear to rule out both the direct splitting of xanthosine to xanthine, or a conversion of xanthosine to inosine followed by splitting to hypoxanthine, since both hypoxanthine and xanthine would react in the xanthine oxidase system. Accordingly, the most plausible explanation would appear to be the conversion of xanthosine to guanosine, followed by phosphorolysis; this possibility is now being investigated.

Effect of Activators and Inhibitors—The enzyme is not inhibited by sodium Versenate or by fluoride, and this evidence, along with the lack of any demonstrable activation by a variety of metal ions, suggests that no metal ion is involved in the phosphorylase reaction. The enzyme is in-

6 Bo Norberg, private communication.
NUCLEOSIDE PHOSPHORYLASE

hibited completely in the presence of $10^{-4}$ M $p$-chloromercuribenzoate or $10^{-3}$ M iodoacetate, which suggests that the enzyme contains essential thiol groups. An inhibition of 50 per cent in the presence of $10^{-4}$ M 2,4-dinitrophenol is of interest, since dinitrophenol is known to "uncouple" oxidative phosphorylation (24), a reaction similar to that of Reaction 1 wherein $P_i$ is converted to organic phosphate. However, the inhibition of oxidative phosphorylation occurs at lower levels of dinitrophenol ($10^{-6}$ to $10^{-5}$ M).

The enzyme is inhibited competitively by pyrophosphate, as shown by the plot of Fig. 5. From these data, a $K_i$ value of $3.3 \times 10^{-4}$ M may be calculated for pyrophosphate. The finding that pyrophosphate has a higher "affinity" for the enzyme than the natural cosubstrate, phosphate, is of interest, although the significance of this observation to erythrocyte metabolism is as yet unknown.

The authors are indebted to Miss Marion Hennessey, Mrs. Mary Jane Osborn, and Mrs. Cherie Howry for their expert assistance on this problem.

SUMMARY

1. Purine nucleoside phosphorylase has been purified about 150-fold from erythrocytes by means of adsorption and elution from calcium phosphate gel, selective heat denaturation, and ammonium sulfate fractionation.

2. The enzyme is specific for inosine and guanosine. In the presence of phosphate, the nucleoside is cleaved to the purine base and R-1-P. When phosphate is replaced by arsenate, ribose is the product.

3. Inosine, phosphate, and arsenate all have $K_m$ values of $1.0 \times 10^{-3}$ M with the erythrocyte phosphorylase.

4. The enzyme catalyzes both the breakdown and synthesis of inosine.

5. The phosphorylase is inhibited by $p$-chloromercuribenzoate, iodoacetate, 2,4-dinitrophenol, and pyrophosphate.

BIBLIOGRAPHY


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