THE EFFECTS OF NUCLEOSIDES ON PHOSPHATE AND PENTOSE METABOLISM IN ERYTHROCYTE GHOSTS

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Erythrocyte ghosts have been found to be peculiarly useful for the study of the metabolism of nucleosides (1, 2). Preparations made by osmotic hemolysis were found to be unable to metabolize glucose. Adenosine, however, caused a disappearance of inorganic phosphate from ghost plasma suspensions with an equivalent production of organic acid-soluble ester phosphate. There was a simultaneous disappearance of nucleoside pentose. The data reported here extend the previous study to include other nucleosides and to measure some comparative effects of erythrocytes with those of ghosts. Further evidence for the metabolic utilization of inorganic phosphate by the ghosts is provided by measurements of the activation energy of inorganic phosphate disappearance from ghost plasma suspensions.

EXPERIMENTAL

Properties of Ghosts—Ghost suspensions were made and equilibrated essentially as described previously (1). Ghosts so prepared were morphologically intact and relatively free from debris although heterogeneous in size and pigmentation. These were reconstituted with plasma to give cell counts (B phase contrast, 430 X) of about 5 X 10^6 cells per c.mm. The packed cell volume was determined by sedimentation of the suspension in capillary hematocrit tubes for 10 minutes at 12,000 X g. The mean corpuscular volume, determined from many experiments under optimal conditions of cell counting (two chambers, two calibrated pipettes), was 56 ± 1.7 cu.μ. In obtaining this value, the trapped plasma in the packed cell mass was estimated at 5.7 ± 0.7 per cent with I^131-labeled albumin dialyzed with agitation against 0.165 M KI for 4 hours. The capillary hematocrit tubes were scratched with a diamond and broken at the cell-plasma interface. The cell and plasma fractions were ground separately in test tubes containing 2 ml. of water and the radioactivity was counted in a well type scintillation counter. The mean corpuscular hemoglobin was 6.4 ± 1.6 μgm. per cell, or 21.5 per cent of the hemoglobin

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content of the intact cells from which the ghosts were made. In fifteen experiments, glucose utilization by ghosts suspended in plasma was measured as reducing sugar present in barium hydroxide filtrates (3). The quantities of glucose which disappeared from the medium were found to be insignificant (8 μmoles of glucose per hour per 10^{12} cells). The standard error was 7.2 with a "t" of 1.11, and \( P > 0.2 \). For whole blood free from white cells, the glucose utilized was 176 μmoles per hour per 10^{12} cells (with a standard error of 6.0).

Cell-free hemolysates were obtained by osmotic hemolysis with 6 volumes of water, rendered isotonic with 1 volume of hypertonic NaCl solution, and made free from ghosts by centrifugation at 2300 × g for 40 minutes at 4°.

**Equilibration Technique**—Ghost plasma suspensions and whole blood free from white cells were incubated with substrates at 37° in 100 ml. silicone-treated flasks under 5 per cent CO₂-95 per cent O₂ for 4 hours at shaking rates of 50 to 60 oscillations per minute. Phosphate was added as 0.1 M Na₂HPO₄-KH₂PO₄ buffer (pH 7.4). Nucleosides were dissolved in plasma which had been equilibrated to 37°. In experiments with radioactivity, P³² as NaH₂P³²O₄ in Sörenson phosphate buffer was added to give a final concentration of 0.5 μc. per ml. of cell suspension. In all of the experiments the intact cells and plasma were 1 day old and the ghosts were prepared from fresh blood, kept at 4° overnight, and used the following day. Cell counts were made on samples from each flask within 1 hour after the start and again before the end of each experiment. Hematocrits (capillary technique) and hemoglobin measurements on 0.2 ml. aliquots were taken at hourly intervals. In the experiments with radioactivity, plasma was separated by centrifugation at 2°.

**Methods**

The analytical methods were the same as those presented previously (1). Unless otherwise given, all analyses were made on filtrates (1:10) of the cell suspensions made with 10 per cent trichloroacetic acid.

**Results**

**Phosphate Uptake by Erythrocyte Ghosts**—As reported with adenosine (1), inosine (Fig. 1, A) and guanosine (Fig. 1, B) stimulated the disappearance of inorganic phosphate from ghosts and erythrocytes suspended in plasma. On a cell for cell basis, stimulation of phosphate uptake by inosine showed the ghosts to be more effective than the red cells, as had also been observed with adenosine. Whether or not guanosine differed significantly from either inosine or adenosine was not readily determined. The ghost-free hemolysate from 10^{12} erythrocytes was found
to esterify more than twice the amount of phosphate than that of either ghosts or erythrocytes (1.4 μmoles with inosine and 1.2 μmoles with guanosine).

The phosphate exchange between cells and plasma with P$^{32}$-labeled phosphate as influenced by inosine is demonstrated in Fig. 2. Inosine effected a steady state of exchange of phosphate between either red cells or ghosts and plasma, whereas the specific activities of phosphate without inosine declined in the plasma during the same interval. The decreases

![Figure 1](http://www.jbc.org/)

**Fig. 1.** The effect of inosine and guanosine on the disappearance of inorganic phosphate from whole blood and ghost plasma suspensions. **A**, the effect of inosine. Red cells (●) and ghosts (○) were incubated at 37°C with inosine (8.4 mM) and 2.0 mM phosphate. The average pH was 7.4 ± 0.05 for red cells and 7.5 ± 0.05 for ghosts. The red cell (●) and ghost (○) controls contained 2.0 mM phosphate but no inosine. The average pH for the red cell control was 7.4 ± 0.05 and for the ghost control, 7.5 ± 0.05. **B**, the effect of guanosine. The red cells (●) and ghosts (○) were incubated at 37°C with guanosine (10.0 mM) and phosphate (2.0 mM). The average pH during the incubation was 7.4 ± 0.05 for red cells and 7.5 ± 0.05 for ghosts. The red cell (●) and ghost (○) controls contained (2.0 mM) phosphate but no guanosine. The average pH was 7.4 ± 0.05 for red cells and 7.1 ± 0.05 for ghosts.

in specific activities of inorganic phosphate without nucleoside are apparently due to a breakdown of cellular ester phosphate, followed by diffusion out of the cell and subsequent dilution of the radioactive phosphate of the plasma. As was found with adenosine (1), inosine favored the influx of phosphate into both ghosts and erythrocytes and retarded the decomposition of the cellular ester phosphate during the equilibration interval. Appreciable quantities of radioactive ester phosphate were found to diffuse from the ghosts into the plasma when incubated with inosine. Measured as the difference between total acid-soluble ester phosphate and inorganic phosphate, these amounted to 24 per cent in the 1st half-hour and 43 per cent in 4 hours of the total amount of inorganic phosphate.
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(3.43 μmoles per ml. of plasma) which disappeared from the plasma. The specific activities of inorganic phosphate of the plasma, for ghosts and inosine, were computed, therefore, by multiplying the counts per minute per micromole of phosphate by the fraction of inorganic phosphate present in the total acid-soluble ester phosphate of the plasma.

Dependence of Phosphate Uptake upon Temperature—To confirm the metabolic nature of the phosphate exchange in ghosts, kinetic experiments were run at four temperatures between 27–47°. Samples were withdrawn at 15 minute intervals for the 1st hour and at 30 minute intervals for the next 2 hours. Rate constants for the reaction were calculated for the 1st 1.5 hours of the reaction. During this time, the reaction followed first order kinetics, giving velocity constants (k) equal to 2.303 times the slope, when log \( C_0/C_t \) was plotted against time (\( C_0 \) was the concentration at time, zero, and \( C_t \) was the concentration at time, t). The energy of activation (E) was calculated from the Arrhenius equation \( d \ln k/dT = E/RT^2 \) by plotting log k against 1/T from which the slopes of the lines gave

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**Figure 2.** The effect of inosine on the exchange of inorganic phosphate between erythrocytes or ghosts and plasma. R.S.A. is the relative specific activity of phosphate as counts per minute per micromole of inorganic phosphate, normalized to an added P** count of 1 \( \times \) 10⁶ counts per minute per ml. of blood and a cell count of 4 \( \times \) 10¹⁰ per c.mm. Ghosts and inosine (X), ghosts (O), red cells and inosine (●), and red cell control (0) were incubated under the conditions given in Fig. 1. The curve for ghost and inosine has been obtained by use of the expression, total counts per minute per ml. of plasma/micromoles of inorganic phosphate per ml. of plasma \( \times \) fraction of inorganic phosphate in the total acid-soluble phosphate of the plasma filtrate.
The energies of activation for phosphate uptake by red cells, ghosts, and hemolysates with inosine were found to be 28,000, 19,000, and 9000 calories per mole, respectively. At 47° the phosphate exchange was kinetically first order in the 1st hour, permitting reliable calculations of the rate constant.

**Metabolism of Nucleoside Pentose**—The effects of nucleosides on pentose...
metabolism in ghosts are indicated in Fig. 4. Appreciable amounts of nucleoside pentose were observed to metabolize in 4 hours as there were decreases in the chromogen with orcinol, although no significant differences existed among the nucleosides tested. The magnitude of pentose metabolism in ghosts was comparable to that in erythrocytes (1 mmole per 10^12 erythrocytes for inosine in 4 hours). The pentose metabolized by cell-free hemolysates greatly exceeded that in the cellular components (1.3 mmoles for inosine and 2.3 mmoles for guanosine, respectively, in 4 hours by the hemolysate from 10^12 cells). The color reaction for pentose was determined on the entire acid-soluble fraction after 40 minutes of heating at 100°. Spectra in orcinol showed a single peak at 670 nm in all cases. This indicated that there was no contribution to the pentose color by heptoses. The absence of heptose was also confirmed by the colorimetric.

**TABLE I**

*Effect of Concentrations of Inorganic Phosphate on Rate of Nucleoside Pentose Metabolism in Erythrocyte Ghosts*

The values are given in micromoles per 10^12 cells.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Initial pentose</th>
<th>Initial inorganic phosphate</th>
<th>Pentose metabolized per 3 hrs.</th>
<th>Inorganic phosphate esterified per 3 hrs.</th>
<th>Moles inorganic phosphate</th>
<th>Moles pentose</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-29a</td>
<td>1260</td>
<td>120</td>
<td>120</td>
<td>30</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>6-29c</td>
<td>1320</td>
<td>960</td>
<td>660</td>
<td>440</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>6-29d</td>
<td>1460</td>
<td>2750</td>
<td>980</td>
<td>1100</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>6-29e</td>
<td>1280</td>
<td>3730</td>
<td>820</td>
<td>880</td>
<td>1.07</td>
<td></td>
</tr>
</tbody>
</table>

Erythrocyte ghosts were incubated with inosine and inorganic phosphate at 37°.

methods of Dische (4). The measurement of the decrease in nucleoside pentose with orcinol reagent is feasible because relatively overwhelming amounts of nucleosides were present. The amounts of nucleoside pentose added were of the order of 10 μmoles per ml., whereas pentoses normally present were 1.4 μmoles per ml. of red cell suspension, 0.5 μmole per ml. of ghost suspension, and 0.1 μmole per ml. of hemolysates. The value for the metabolism of nucleoside pentose (Fig. 4) is minimal. For example, conversion of nucleoside pentose to ketopentose would cause a decrease in absorbancy, due to a smaller extinction coefficient of the orcinol chromogen of the ketopentose.

Correlations of ribose utilization with inorganic phosphate disappearance are made in Table I. When sufficient phosphate is present, the ratios of the amounts of inorganic phosphate esterified to pentose metabolized show, for ghosts, that nucleosides effect the interaction of 1 mole of phosphate per mole of pentose. The ratio was dependent upon the amounts of ex-
tracellular phosphate and reached a limiting value of approximately 1 at 2.75 mmoles of phosphate per $10^{12}$ cells (Experiment 6-29d). Further increases in phosphate were not more effective. Increase of inosine from approximately 1460 to 3000 μmoles per $10^{12}$ cells increased the pentose metabolized from 980 to 1360 μmoles.

**DISCUSSION**

The study of catabolic utilization of nucleosides by erythrocytes has extended the knowledge of metabolism in such cells, particularly during storage, when decomposition of the phosphorylated carbohydrate esters occurred (5, 6). Adenosine and inosine have been shown to effect an appreciable resynthesis of phosphate esters, although these as yet have not been well characterized. Rubenstein et al. (7) have demonstrated the hydrolysis of inosine by erythrocytes with the production of hypoxanthine. It was postulated that the pentose constituent of nucleosides entered a pentose phosphate pathway. Since the amounts of 2,3-diphosphoglycerate increased significantly in erythrocytes incubated with adenosine (8, 9), it has been further theorized that purine ribosides are potential sources of glyceraldehyde 3-phosphate (10, 11). These data and those with ghosts are consistent with the reaction scheme shown:

\[
\text{Adenosine} \rightarrow \text{inosine} \rightarrow \text{hypoxanthine} + \text{ribose 5-PO}_4
\]

\[
\text{Ribose 5-PO}_4 \rightarrow \text{ribulose 5-P} \rightarrow \text{xylulose 5-P} \rightarrow \text{triose PO}_4 \rightarrow \text{lactic acid}
\]

Hence, nucleosides appear to provide the erythrocyte with an accessory source of glycolytic intermediates. The nucleoside-induced esterification in ghosts and hemolysates is especially significant, as the nucleoside-deficient preparations were unable to utilize glucose or to esterify inorganic phosphate. The deficiency in glucose utilization by ghosts, caused presumably by instability of hexokinase, would thus be partially compensated. The activation energy of 19,000 calories per mole for phosphate uptake by the ghost confirms the metabolic nature of the phosphate transfer from plasma to cells as a much lower value (about 4000 calories per mole) would be expected if diffusion was the major factor governing the entry of inorganic phosphate. The metabolic character of ghosts is further substantiated by the stoichiometry of the conversion of inorganic phosphate to ester phosphate (1) and a mole for mole equivalence of phosphate esterified to nucleoside pentose metabolized. A value of 7300 to 7500 calories per mole was found for the activation energy of phosphorolysis of inosine by a purified preparation of nucleoside phosphorylase from human erythrocytes (12). This agrees moderately well with the value found here for cell-free hemolysates (9000). Either of these values, when added to 19,800 calories per mole (8) or 10,700 calories per mole (13) for erythrocytes...
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without nucleosides, gives a sum which agrees with the value found here for erythrocytes with nucleosides (28,000 calories per mole). These additive magnitudes suggest at least two independent mechanisms for the phosphate transfer in erythrocytes when nucleosides are present. Further, the decreasing order of energy values in erythrocytes, ghosts, and hemolysates suggests that alterations in permeability affect the access of enzymes to nucleosides with appropriate effects on their kinetics.

SUMMARY

Ghosts and erythrocytes were found to metabolize the pentose of guanosine and inosine, with a simultaneous esterification of inorganic phosphate. In the ghost, under conditions of saturation of the cells with nucleosides and inorganic phosphate, a limiting value of 1 mole of phosphate esterified per mole of pentose metabolized was observed. The activation energy for phosphate exchange in the presence of nucleosides (19,000 calories per mole) confirmed the metabolic nature of the ghost reaction. Differences among ghosts, erythrocytes, and hemolysates may be attributed to alterations in permeability caused by hemolysis.

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