ENZYMES OF THE HUMAN ERYTHROCYTE

IV. PHOSPHOGLUCOSE ISOMERASE, PURIFICATION AND PROPERTIES*

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The enzyme, phosphoglucose isomerase, catalyzes the reversible equilibrium reaction:

\[ \text{Glucose-6-PO}_4 \rightleftharpoons \text{fructose-6-PO}_4 \]

The enzymatic interconversion involves a net transfer of hydrogen which migrates as a proton (1). In spite of an early reference (2) to the role and existence of this glycolytic enzyme within a variety of animal tissues, it apparently has not been prepared previously in a significantly purified state from any source. Fractions rich in this enzyme have been separated from rabbit muscle (3), and a purification of a phosphomannose isomerase has also been achieved from this tissue (4). The possibility of obtaining phosphoglucose isomerase in purified form from human erythrocytes was considered after a report (5) which described appreciable activity as associated with these cells.

Previous papers of this series (6-8) have been concerned with the isolation and characterization of various enzymes obtained from the human erythrocyte. The present report will summarize information pertinent to obtaining phosphoglucose isomerase in a highly purified form and will also describe certain characteristic properties of the enzyme.

EXPERIMENTAL

Materials and Methods

Enzyme Source—Washed erythrocytes separated from outdated human blood obtained from the hospital blood bank served as the source of enzyme. The erythrocytes were washed with 1 per cent sodium chloride solution by repeated centrifugation, with care taken to remove adhering

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leucocytes. The washed cells were hemolyzed by the addition of 1 volume of water and used directly for the preparation of enzyme.

**Enzyme Substrates**—Commercial preparations (Schwarz) of glucose 6-phosphate (G-6-P) and fructose 6-phosphate (F-6-P) were purified by several precipitations of the barium salts from dilute ethanol solution. Barium was removed by treatment with Dowex 50 (H), and after neutralization the resulting solutions were standardized by phosphate analyses (9).

**Enzyme Assay**—Routine phosphohexose isomerase assay was based essentially on a method described by Bodansky (5). The method was, however, extended to allow a more generalized application. The assay procedure as utilized follows:

Reaction mixtures were at 1.0 ml. volume and contained 2 μmoles of either G-6-P or F-6-P in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.5 and appropriately diluted enzyme. After incubation (arbitrary period, usually 10 or 20 minutes) at 37°, the reaction was stopped by the addition of an equal volume of acid (usually 5 per cent trichloroacetic acid). Fructose (as F-6-P) was determined directly on these samples by using the Roe reagent (10) as applied by Bodansky (5). Depending upon the substrate used, enzyme activity was followed either by the rate of F-6-P formation or disappearance. Only with the initial crude material (i.e. hemolysate) was it necessary to deproteinize the solution before fructose determination.

**Enzyme Activity**—Enzymatic interconversion of G-6-P and F-6-P was found to proceed to equilibrium from either direction at an equivalent rate, according to conventional first order kinetics. Final equilibrium mixtures contained 60 per cent G-6-P and 40 per cent F-6-P at 37°. At equivalent substrate concentration, therefore, F-6-P is converted at 1½ times (i.e. 60 to 40) greater rate than G-6-P (see below). Equivalent activity values can therefore be obtained by using either as substrate by computing the fraction of theoretical maximal conversion occurring within the selected reaction period.

Enzyme activity could be conveniently expressed in terms of the first order velocity constant and was computed as follows: Activity = 1/t \log_{10} 100/100-X; where t is expressed in minutes and X represents the per cent of maximal conversion of substrate after time t (maximal conversion of G-6-P and F-6-P taken as 60 and 40 per cent of initial concentrations respectively).

A plot of an experiment demonstrating the relationship between activity, enzyme concentration (A), and reaction time (B) as computed according to the above equation, by using G-6-P and F-6-P as substrates, is presented in Fig. 1. It is evident that activity can be expressed as a linear function of enzyme concentration and reaction time with identical values resulting with either substrate.
Enzyme Unit—A unit of enzyme will be defined as that amount yielding 1 activity unit with the previously specified assay conditions. It is obvious from the activity equation that 1 unit of enzyme will correspond to that amount which yields 90 per cent of the theoretical maximal conversion of substrate within a 1 minute reaction period.

Protein Estimation—Protein concentrations were estimated by micro-Kjeldahl nitrogen analysis (11) as well as by tyrosine measurements by using the phenol reagent (12).

![Graph showing the interconversion of G-6-P and F-6-P as a function of enzyme concentration and reaction time.](image)

**Fig. 1.** Interconversion of G-6-P and F-6-P as a function of enzyme concentration (A) and reaction time (B). Reaction mixtures contained designated volumes of appropriately diluted purified enzyme, 0.1 M Tris at pH 7.5, and 2 μmoles of G-6-P or F-6-P, all in a final 1.0 ml. reaction volume.

Results

Enzyme Purification Procedure

Enzyme Separation from Hemoglobin—Selective adsorption of phosphoglucone isomerase from the hemolysate, as successfully applied with other erythrocyte enzymes (6), was found to be inapplicable. A satisfactory procedure for hemoglobin removal was, however, found in the chloroform-alcohol denaturation technique initially described by Tsuchihashi (13) and subsequently successfully applied by others (e.g., (14–16)). Preliminary experiments indicated a rapid rate of hemoglobin denaturation after short periods of vigorous stirring of crude hemolysate with an equal volume of a 1:1 chloroform-methanol mixture. A rapid and near maximal denaturation of both enzyme and total protein resulted after several minutes of vigorous stirring, with little further loss of either occurring after this time (see Fig. 2). Enzyme denaturation during this period was approximately 60 per cent, with total protein at 99 per cent. In subsequent experiments it was found that by carrying out the chloroform denaturation at a lower temperature (−10°) proportionately less enzyme and protein denaturation
occurred (probably superior procedure, although not used, yielding better enzyme recoveries and approximately equivalent purification).

The chloroform denaturation procedure as applied in the purification studies follows (for a summary, see Table I, Step A): A total of 2 liters of washed erythrocytes was prepared and hemolyzed by the addition of an equal volume of ion-free water. The resulting hemolysate was divided into four equal batches. Each 1000 ml. batch was immersed in an ice bath and cooled to near 0° and vigorously stirred (with the aid of a motor-driven stirrer) for a 10 minute period in the presence of an equal volume of cold (0°) 1:1 chloroform-methanol mixture. After the stirring period, 400 ml. of cold water were added and the denatured protein was separated by filtration overnight under reduced pressure in a cold box at near 0°, yielding a clear, light pink to colorless filtrate.

Concentration of Filtrates and Dialysis—The clear filtrates which result from the chloroform treatment required the removal of organic solvents for maximal enzyme stability as well as considerable concentration before further enzyme fractionation (protein concentration usually around 0.1 per cent in filtrates). Attempts at concentration by salting out proteins with ammonium sulfate proved unsuccessful due to considerable enzyme inactivation. Lyophilization of filtrates could be successfully applied with little enzyme inactivation resulting; however, the procedure proved tedious and was ultimately discontinued. An especially satisfactory method was eventually used incorporating a "flash evaporator" (Renco Instruments), leading to a rapid, convenient means of concentration. Details concerning the concentration procedure follow (for a summary, see Table I, Step B).
The filtrate from the chloroform denaturation step was transferred to 2 liter round bottomed boiling flasks and a few drops of antifoam agent were added (octyl alcohol). The flask was connected to a "flash evaporator" and rotated at room temperature. A dry ice trap was incorporated between the vacuum pump and rotator. The organic solvents were rapidly distilled, and the flask was subsequently immersed in a 37° water bath to complete the concentration. The final concentration step was carried out in a 50 ml boiling flask with care taken to avoid evaporation to dryness (enzyme loss was noted when solutions were taken to dryness).

**Table I**

*Purification Protocol of Erythrocyte Phosphoglucone Isomerase as Carried Out on 2000 Ml. of Washed Red Cells*

<table>
<thead>
<tr>
<th>Purification steps*</th>
<th>Protein†</th>
<th>Enzyme‡</th>
<th>Specific activity</th>
<th>Degree of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hemolysate</td>
<td>620,000</td>
<td>5500</td>
<td>0.0089</td>
<td></td>
</tr>
<tr>
<td>Combined chloroform filtrates</td>
<td>3,900</td>
<td>1670</td>
<td>0.43</td>
<td>48</td>
</tr>
<tr>
<td>Dialyzed concentrate</td>
<td>3,300</td>
<td>1440</td>
<td>0.44</td>
<td>49</td>
</tr>
<tr>
<td>Ethanol fraction 1 (0-60%)</td>
<td>431</td>
<td>1250</td>
<td>2.9</td>
<td>330</td>
</tr>
<tr>
<td>Ethanol fraction 2 (50-62%)</td>
<td>81</td>
<td>880</td>
<td>10.8</td>
<td>1200</td>
</tr>
</tbody>
</table>

* For details concerning purification steps, consult the text.
† Protein estimated by nitrogen analyses on original hemolysate and final purified product only. All intermediate fractions analyzed for protein content by tyrosine measurements.
‡ A unit of enzyme defined as that amount capable of converting either G-6-P or F-6-P to 90 per cent of theoretical maximum within a 1 minute reaction period at 37° (consult the text for details concerning assay conditions as well as computation of activity).

The concentrated solution was transferred to a washed cellophane tube (washed and thoroughly soaked in versenate to reduce heavy metal contamination) and dialyzed overnight against 4 liters of ion-free water in the refrigerator. The resulting dark red solution was centrifuged to remove denatured protein. Very little loss of enzyme usually occurred throughout the concentration and dialysis procedures.

**First Ethanol Fractionation**—Subsequent purification of the enzyme leading to the final product was achieved by cold ethanol fractionation. Other purification methods which were tried without success included salt fractionation, the use of various gels, and further selective denaturation techniques.
The relative solubility of enzyme as compared with the other proteins present in the preparation was tested over a range of ethanol concentrations (see Fig. 3). From these results, a good fractionation of the enzyme with the use of ethanol was anticipated. Maximal enzyme stability in ethanol solutions required that the pH be around 7.5 and the temperature at 0° and below. The first ethanol fractionation procedure as applied follows (for a summary, see Table I, Step C).

The dialyzed enzyme concentrate was adjusted to approximately 2 per cent protein concentration and buffered at pH 7.5 in 0.1 M Tris. The resulting solution was cooled to an icy slush, and cold ethanol (at 0°) was rapidly added to a final 60 per cent concentration (v/v). The sediment containing nearly all of the enzyme was removed by centrifugation at 0° and dissolved in a minimal volume of cold water. Abundant denatured protein present was removed by centrifugation and the resulting clear supernatant fluid treated in a flash evaporator to remove excess ethanol. The enzyme solution was practically colorless after the alcohol fractionation procedure and could be stored for prolonged periods at refrigerator temperatures with little or no loss in activity.

Second Ethanol Fractionation—Final enzyme purification was accomplished by refractionation with ethanol. Preliminary studies with small aliquots of the preparation indicated an increased instability of the enzyme in ethanol, requiring a temperature of less than 0° to be maintained during the refractionation procedure. By working at a temperature of −10°, little or no inactivation of enzyme occurred.
The refractionation procedure as applied follows (for a summary, see Table I, Step D).

The enzyme solution was prepared for refractionation by adjusting to pH 7.5 in 0.1 M Tris buffer and diluting to approximately 1 per cent protein concentration. The solution was cooled to an icy slush, and ethanol, previously cooled to -20°C, was rapidly added to a final concentration of 50 per cent (v/v). The resulting sediment was discarded after centrifugation at -10°C, and contained somewhat less than one-half and one-fourth of the original protein and enzyme, respectively. To the supernatant liquor was added more cold ethanol (-20°C) in an amount sufficient to bring the concentration to a final 62 per cent (v/v). The resulting small amount of sediment contained practically all of the remaining enzyme and was removed by centrifugation at -10°C. The sediment was dissolved in a minimal volume of water and treated in a flash evaporator to remove excess ethanol. Insoluble protein present was removed by centrifugation and discarded. The resulting clear, practically colorless solution (slightly yellow) represented the final product. The purified product was found to be stable for months at refrigerator temperatures.

Relative Enzyme Purity

Further purification of the enzyme was not feasible in view of the limited amount of starting material selected. Enzyme preparation on a larger scale was not considered within the scope of the present investigations.

From rate data, the approximate turnover number (i.e., moles of substrate converted per minute per 100,000 gm. of protein) of the preparation was estimated to be in excess of 30,000 (based on an initial rate by using 0.014 M G-6-P as substrate, 37°C reaction temperature, and pH 7.5). From the relatively large turnover number, a high degree of purification was initially assumed. Subsequent examination of the preparation by paper electrophoresis disclosed the presence of at least four components. Further examination suggested that the enzymatically active component constituted approximately 20 to 25 per cent of the total protein and traveled as a cation at pH 8.5 (other protein contaminants traveled as anions or did not move at this pH). The apparent strongly basic character of the enzyme relative to the other proteins present suggested that a final isolation in pure form might be achieved by ion exchange chromatography. Circumstances did not allow a further examination of this possibility.

Properties of Purified Enzyme

Kinetics—The characteristics of G-6-P and F-6-P interconversion by the purified erythrocyte isomerase are summarized graphically in Fig. 4. The reaction proceeds to equilibrium and approaches this position from
either direction at equivalent rate. Equilibrium mixtures were estimated to contain 60 per cent G-6-P and 40 per cent F-6-P, as mentioned previously. The equilibrium position found is identical with that reported for isomerase from human serum (17) and differs somewhat from published figures (2, 18) obtained with enzyme from other sources. The relationship

![Graph](attachment:image.png)

**Fig. 4.** Interconversion of G-6-P and F-6-P by erythrocyte isomerase. Reaction mixtures contained 2 μmoles of G-6-P or F-6-P, 0.1 M Tris buffer at pH 7.5, and appropriately diluted purified enzyme, all in a final 1.0 ml. volume. The reaction time was maintained constant (10 minutes) in one series of experiments, and enzyme concentration varied. In the other series, reaction time was varied by using a single level of enzyme (lowest level).

Fig. 5. Reaction velocity as a function of substrate concentration. Reaction mixtures contained substrates at designated levels, 0.1 M Tris buffer at pH 7.5, and appropriately diluted purified enzyme. S refers to substrate in each case and V is the reaction velocity expressed as micromole of F-6-P formed or transformed.

between the interconversion rate and the reaction time as well as enzyme concentration is demonstrated with the two substrates.

The relative reaction rate was determined over a wide range of substrate concentrations (Fig. 5). The results are summarized graphically by using the familiar double reciprocal plot (19) for convenience of evaluation. In the presence of dilute substrate levels, the response is linear in accord with predicted theory. At substrate concentrations in excess of 0.005 M, deviations occur which lead to rates in excess of that expected. The Michaelis constants ($K_M$) did not differ significantly for the two substrates as estimated from the linear portions of the curves.
The purified enzyme showed a sharp pH optimum at around 8 with F-6-P and a broad peak centering at approximately the same position with G-6-P as substrate (see Fig. 6). These results are essentially similar to those reported for the isomerase present in human serum (17).

Temperature effects on the reaction rate were determined with the purified enzyme in the presence of both G-6-P and F-6-P (see Fig. 7). A temperature range of 15–45° was investigated at increments of 5°. A plot of the log of the reaction rate against the reciprocal of the absolute temperature is shown. A common straight line results from this plot for both substrates over the entire temperature range investigated. From the slope of the single straight line, the apparent activation energy was approximated for the catalyzed reactions to be 11,000 calories.

Enzyme Stability—The thermal stability characteristics of the purified enzyme were determined. Maximal stability to heat was at alkaline pH and centered around 8.5, in support of a basic isoelectric point, as was previously indicated. Demonstrable inactivation did not occur at this pH until a temperature of 47° was approached. Further elevation of the temperature to 50°, however, resulted in a rapid destruction of the enzyme. Enzyme inactivation rates observed at 47° as a function of pH are reproduced in Fig. 8. The denaturation proceeds at constant rate in accord
with first order reaction kinetics. The denaturation rate was independent of the enzyme concentration over a wide investigated range, as would be expected.

Because the addition of non-ionic, surface-active agents and various metal-binding reagents was found to show no measurable effect on the inactivation rate, thermal inactivation of the enzyme was considered to represent a true denaturation process (viz., destruction of hydrogen bonding) rather than inactivation induced by surface forces or by heavy metal contaminants (7). The stability of the enzyme to surface forces could perhaps have been predicted by its proved ability to withstand destruction by the chloroform procedure employed in isolation. Insensitivity of the enzyme toward the usual heavy metal contaminants, as indicated by a lack of ef-

![Diagram](image_url)

**Fig. 8.** Thermal denaturation characteristics as a function of pH. The denaturation was carried out in the presence of 0.1 M Tris-acetate buffers adjusted to appropriate pH. Residual activity was determined in the usual manner, following appropriate dilution.

fect by added metal-binding agents on the stability, suggested the non-sulfhydryl nature of the enzyme. Subsequent reaction of the enzyme in the presence of the more specific sulfhydryl reagents showed little or no inactivation to result when tested with p-chloromercuribenzoate (0.002 M), iodosobenzoate (0.002 M), and iodoacetamide (0.02 M). The suspected resistance to metal inactivation was confirmed by the finding of little effect after incubation of the enzyme in the presence of Co++, Ni++, Pb++, Cu++, Fe++, and Fe+++ (all at 0.001 M). Measurable inactivation did occur, however, in the presence of Zn++, Cd++ (approximately 50 per cent reduction in activity at 0.001 M), and Hg++ (complete inactivation at 0.001 M).

**SUMMARY**

Detailed procedures have been presented for obtaining phosphohexose isomerase in high purity from the human erythrocyte. The isolation tech-
niques involved a preliminary selective denaturation of hemoglobin with the use of chloroform-methanol mixtures as the active agent, followed by cold ethanol fractionation for obtaining the enzyme. The isolated product had a calculated turnover number of approximately 30,000 but was not yet pure.

The purified product was further examined with respect to its kinetic properties and stability characteristics, the results of which were presented.

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