SERUM PHOSPHOHEXOSE ISOMERASE*

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In 1933 Lohmann (1) demonstrated the existence of phosphohexose isomerase, the enzyme which mediates the reversible conversion of glucose-6-phosphate to fructose-6-phosphate, in dialyzed frog muscle extracts and in simple aqueous extracts of yeast, heart, kidney, liver, and brain. Tankó (2) and, more recently, Somers and Cosby (3) have found this enzyme to be present in pea meal. Slein (4) has obtained a partially purified preparation of phosphomannose isomerase from rabbit muscle. Although several studies have referred to the wide-spread distribution of phosphohexose isomerase in tissues (4, 5), this enzyme has received relatively little investigative attention.

The present study shows that a considerable degree of phosphohexose isomerase activity is present in the serum of several species, including man. The properties of this enzyme in human blood serum are characterized, and quantitative data on its activity in various sera from patients with disease are submitted, in order to indicate the possible physiological significance of this enzyme in serum.

EXPERIMENTAL

Fructose-6-phosphate and glucose-6-phosphate were obtained as the barium salts (Schwarz). The values for the hydrolysis of the organic phosphorus of these compounds in N HCl at 100° were determined according to Robison's procedure (6) and were in good agreement with the values reported by Robison (6) and by Lohmann (7). Fructose was determined by the reaction with resorcinol and HCl essentially in accordance with Roe's method (8), except that the heating period was 15 minutes. The

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1 Slein has pointed out that, in order to name Lohmann’s isomerase unambiguously according to substrate, the primary product of mannose-6-phosphate isomerization would have to be known. For the present, therefore, it would seem advisable to employ the term, phosphohexose isomerase, as previous writers have done, to designate the enzyme which mediates the interconversion of the glucose-6- and fructose-6-phosphates.

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value for fructose-6-phosphate was 61.5 per cent of that for free fructose and was in good agreement with the value given by Umbreit and his associates (9).

Most of the experiments were performed with the sodium salts of the hexose phosphates prepared from the barium salts by precipitation with Na₂SO₄. The barium salts were employed in a few experiments as indicated in the text. Twice centrifuged serum without visible evidence of erythrocytes² or hemolysis was used.

The conversion of fructose-6-phosphate (F-6-P) to glucose-6-phosphate (G-6-P), or the reverse action, was followed by adding serum, warmed to 37°, to a mixture of buffer (sodium diethyl barbiturate or Michaelis' (10) sodium acetate-diethyl barbiturate) and of either fructose- or glucose-6-phosphate, which had been kept in a thermostat regulated to 37.00° ± 0.05°. The contents were mixed rapidly, and samples were withdrawn at suitable times and were added to trichloroacetic acid to stop the reaction. The determination of the fructose color value in appropriate aliquots of the trichloroacetic acid filtrate was made the basis for following the changes in the concentration of fructose-6-phosphate, in accordance with the procedure used by Somers and Cosby (3). The cherry-red solutions resulting from the interaction with resorcinol and HCl were read in tubes of 16 mm. diameter at 490 mλ in a Coleman junior spectrophotometer.

Results

Changes in Glucose-6-phosphate and Fructose-6-phosphate in Presence of Serum—A number of experiments were performed in which the time-course of the interconversion of these substrates in the presence of human serum was followed for several hours. For example, in one experiment at pH 7.7 and 0.0024 m sodium fructose-6-phosphate, the optical density of solutions containing the equivalent of 0.4 cc. of reaction mixture decreased from an initial value of 0.600 to 0.502 at 30 minutes, 0.438 at 60 minutes, and 0.288 at 240 minutes, and remained fairly constant for the next 2 hours. As a result of the phosphatase activity of the serum, inorganic phosphate was also slowly liberated. This liberation amounted, per 0.4 cc. of reaction mixture, to 0.4 γ at 30 minutes, 0.8 γ at 60 minutes, 2.5 γ at 240 minutes, and 4.2 γ at 395 minutes.

The question whether inorganic phosphate is liberated from fructose-6-

² We observed that as little as 1 volume of erythrocytes in 100,000 volumes of serum could be visibly detected as a red pellet after a single centrifugation. Therefore, although it was found that erythrocytes also had considerable phosphohexose isomerase activity, calculation as well as direct determination showed that the increment due to possible contamination by erythrocytes was negligible under our experimental conditions.
phosphate or from glucose-6-phosphate has been studied in the case of liver phosphatase (5, 11). If any of the phosphate formed by the action of the serum phosphatase does arise from the cleavage of fructose-6-phosphate, then the calculation of the concentration of this compound at various stages of the enzyme action should take into account the possible presence of free fructose which has 1/0.61 the color value of the combined fructose. The observed readings may be corrected by means of the following formula:

\[
\text{Observed optical density} = (0.61 R \times y) + (1 - 0.61) R \times a \times y
\]

where \( y \) is the amount of fructose-6-phosphate that would be present if there were no hydrolysis, \( R \) is the optical density produced in the Roe procedure by a unit weight of fructose, and \( a \) is the hydrolyzed fraction of fructose-6-phosphate.

Calculation of the concentration of fructose-6-phosphate from the data cited above on the basis of the assumptions that (a) the inorganic phosphate rose solely from the glucose-6-phosphate formed, that (b) it rose solely from fructose-6-phosphate, or that (c) it came in approximately equal parts from both compounds, yielded, within experimental error, the same values at any particular stage during the conversion of the first 20 to 30 per cent of the fructose-6-phosphate. However, the equilibrium value, expressed as the fraction of fructose-6-phosphate initially present, differed in the three instances. It was 47 per cent on the basis of the assumption that the inorganic phosphate came from the glucose-6-phosphate being formed, 38 per cent on the basis that the phosphate rose entirely from the cleavage of fructose-6-phosphate, and 42 per cent on the basis of the assumption that the phosphate came in approximately equal parts from both hexose phosphate compounds.

In general, whether glucose-6-phosphate or fructose-6-phosphate was used as the substrate initially present, corrections for the possible liberation of fructose during the first part of the reaction were negligible, but the composition of the mixture of fructose-6-phosphate and glucose-6-phosphate at equilibrium was 45 to 55 per cent on the basis of the first assumption, 35 to 65 per cent on the basis of the second, and about 40 to 60 per cent on the basis of the third assumption.3

3 The question may be raised whether the point of equilibrium or its rate of attainment is influenced by serum enzymes other than phosphatase. The possibility of glucose-6-phosphate being changed to glucose-1-phosphate would seem to be negligible, first because the ratio of G-6-P to G-1-P at equilibrium in the phosphoglucomutase reaction is 95 to 5 per cent, and, secondly, because with glucose-1-phosphate as substrate no evidence of phosphoglucomutase activity was found in any of the plasmas of a group of four normal persons and eight with disease. The possibility that fructose-6-phosphate might be converted to fructose-1,6-diphosphate under
Fig. 1 shows the rates at which equilibrium is approached from either substrate under the influence of rat serum. The rate of liberation of inorganic phosphate was also determined, and the observed fructose color values were corrected on the basis that about half the liberated phosphate rose from the cleavage of fructose-6-phosphate. The equilibrium point, as may be seen, was at 40 per cent F-6-P and 60 per cent G-6-P.

The equilibrium point on any of the three assumptions concerning the locus of phosphatase actions falls well within the range of values given in the literature for tissue phosphohexose isomerases. Several texts (13, 14) note a value of about 30 to 32 per cent fructose-6-phosphate and 68 to 70 per cent glucose-6-phosphate, but this is apparently based upon Lohmann's results with frog muscle (1). Lohmann submitted the following our experimental conditions is also negligible. Adenosine triphosphate, necessary for the mediation of this reaction, is not present in serum (12). The diphosphate is readily hydrolyzed in N HCl or H₂SO₄ at 100°, namely to the extent of 37 per cent in 10 minutes and 67 per cent in 1 hour (7). Analyses of mixtures of plasma or serum with fructose-6-phosphate, which had been incubated for periods as long as 24 hours, gave no evidence of the formation of the labile diphosphate ester.
values for the fructose-6-phosphate at equilibrium in other tissues: heart 34, yeast 33, kidney 40, and liver 44 per cent.

Hydrolyzable Phosphorus at Equilibrium Point—Analysis of the acid-hydrolyzable properties of the organic phosphorus in the equilibrium mixture supported the view that the serum was mediating the reaction, glucose-6-phosphate ⇌ fructose-6-phosphate. In the present work, the fraction of organic phosphorus hydrolyzed in 1 hour in \( \text{NHCl} \) at 100°, in accordance with Robison's procedure (6), was found to be 3.4 per cent for glucose-6-phosphate and 40.4 per cent for fructose-6-phosphate. Table I shows that the phosphorus hydrolyzed in 1 hour at equilibrium in reaction mixtures, containing initial concentrations of 0.004 M barium-glucose-6-phosphate, 0.012 M sodium diethyl barbiturate, and 0.01 cc. of a potent serum per cc. of reaction mixture, averaged 17.2 per cent in four experiments. This value was in good agreement with the average value, 17.9 per cent, calculated on the assumption that the glucose-6-phosphate was converted to fructose-6-phosphate.

| Table I |
|---|---|
| Theoretical and Found Values of 1 Hour Acid-Hydrolyzed Phosphorus at Equilibrium in Conversion of Glucose-6-phosphate to Fructose-6-phosphate by Human Serum |
| Experiment No. | Fraction of G-6-P converted as determined by fructose color value | 1 hr. acid-hydrolyzed P at equilibrium mixture of G-6-P and F-6-P as fraction of organic P |
| | per cent | per cent | per cent |
| 1 | 38 | 17.5 | 20.6 |
| 2 | 36 | 16.7 | 13.0 |
| 3 | 41 | 18.6 | 19.7 |
| 4 | 41 | 18.6 | 15.5 |
| Average | 39 | 17.9 | 17.2 |

Serum Phosphohexose Isomerase Activity in Various Species—Table II shows that phosphohexose isomerase activity was also present in the serum of the adult rat, dog, cat, and the 4 day-old chick. The extent of the activity is expressed as the initial reaction velocities with which fructose-6-phosphate and glucose-6-phosphate are converted into each other. It may be noted that at a comparable pH range the rate of conversion of glucose-6-phosphate into fructose-6-phosphate was greater than the reverse reaction. The serum alkaline phosphatase activities of the various sera are also noted.

Stability of Serum Phosphohexose Isomerase—It appeared of value to determine some of the conditions governing human serum phosphohexose
isomerase activity, particularly in view of the fact that these aspects do not seem to have been previously studied in any tissue or extract containing this enzyme. As a preliminary step to such studies, it was found that the isomerase was quite stable. Thus sera could be kept for about 8 hours at room temperature after collection and about 1 to 2 weeks at ice box temperature without showing any decrease in activity. For example, aliquots of a batch of pooled serum in a concentration of 0.08 cc. of serum per cc. of reaction mixture were tested at a pH of 6.6 in a solution containing 0.004 M barium glucose-6-phosphate and 0.012 M sodium diethyl barbiturate buffer. The activities, expressed as micrograms of fructose formed as fructose-6-phosphate per minute per cc. of reaction mixture, were 1.38, 1.45, and 1.45 at 1, 3, and 6 days after collection.

Inactivation of Human Serum Phosphohexose Isomerase at Varying Temperatures—Aliquots of serum were incubated in a water bath at various temperatures (±0.5°) for 10 minutes. The tubes containing these aliquots were then transferred to the thermostat at 37° and the isomerase activity was determined. Table III shows that incubation at 40° caused no change in activity, but that there was a definite decrease at 45° and 50°. Incubation at temperatures over 55° resulted in a practically complete loss of enzyme activity.

Effect of pH—Fig. 2 shows that serum phosphohexose isomerase ac-

<table>
<thead>
<tr>
<th>Species</th>
<th>F-6-P</th>
<th>G-6-P</th>
<th>Alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fructose disappeared per cc. per min.</td>
<td>Fructose formed per cc. per min.</td>
<td>Bodansky units</td>
</tr>
<tr>
<td>Rat</td>
<td>3.45</td>
<td>5.25</td>
<td>36.3</td>
</tr>
<tr>
<td>Dog 1</td>
<td>0.46</td>
<td>0.76</td>
<td>1.36</td>
</tr>
<tr>
<td>&quot; 2</td>
<td>0.63*</td>
<td>1.00*</td>
<td>2.27</td>
</tr>
<tr>
<td>Cat 1</td>
<td>3.05</td>
<td>4.00</td>
<td>0.77</td>
</tr>
<tr>
<td>&quot; 2</td>
<td>11.0</td>
<td>15.6</td>
<td>1.49</td>
</tr>
<tr>
<td>Chick†</td>
<td>12.0</td>
<td>17.3</td>
<td>52.8</td>
</tr>
</tbody>
</table>

* These determinations were carried out with a concentration of 0.1 cc. of serum per cc. of the reaction mixture; the resultant values were, therefore, divided by 2 to give values shown in Table II.
† Pool of serum from 4 day-old chicks; slight hemolysis.
tivity was absent at pH's below about 4.0, that it rose at higher pH values to attain a fairly broad optimum at about 7 to 8 when glucose-6-phosphate was the substrate, and thereafter declined again. With sodium fructose-6-phosphate in an equimolar concentration, the pH-activity curve seemed somewhat steeper and the pH optimal range somewhat narrower and in a slightly more alkaline region. In most of the series of experiments, a final concentration of 0.012 M sodium diethyl barbiturate was used at all pH values in order to keep the factor of salt concentration constant.

**Table III**

*Inactivation of Serum Phosphohexose Isomerase by Incubation for 10 Minutes at Varying Temperatures*

Activity of incubated enzyme tested at 37°, 0.012 M sodium diethyl barbiturate buffer (pH 7.5), and (a) 0.00156 M sodium fructose-6-phosphate and 0.067 cc. of serum per cc. of the reaction mixture, or (b), 0.008 M sodium glucose-6-phosphate and 0.1 cc. of serum per cc. The reaction velocity was calculated for the initial portion of the reaction.

<table>
<thead>
<tr>
<th>Temperature of inactivation °C</th>
<th>(a) Fructose-6-phosphate</th>
<th>(b) Glucose-6-phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of disappearance of F-6-P as fructose γ per cc. per min.</td>
<td>Activity as fraction of unactivated enzyme</td>
</tr>
<tr>
<td>Control</td>
<td>2.53</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>2.60</td>
<td>103</td>
</tr>
<tr>
<td>45</td>
<td>1.50</td>
<td>59</td>
</tr>
<tr>
<td>50</td>
<td>1.17</td>
<td>46</td>
</tr>
<tr>
<td>55</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

The use of Michaelis' acetate-diethyl barbiturate buffer yielded essentially the same pH-activity relationship.

*Effect of Enzyme Concentration*—It has been shown that the velocity of an enzyme reaction is directly proportional to the concentration of enzyme, provided that necessary activators are present and that inhibitors are absent from the enzyme preparation (15, 16). Table IV shows that the phosphohexose isomerase activity of human serum is directly proportional, within experimental error, to the concentration of serum throughout a 10-fold variation of the latter.

*Influence of Substrate Concentration*—The initial reaction velocities were determined at concentrations of barium glucose-6-phosphate ranging from $1 \times 10^{-3}$ M to $12 \times 10^{-3}$ M. Plotting of the reciprocals of the reaction velocities against the reciprocals of the substrate concentrations, in accordance with the Lineweaver-Burk transformation (17) of the Michaelis-
Fig. 2. pH-activity curve with human serum phosphohexose isomerase. Concentration of substrate (sodium salt) in the reaction mixture 0.002 M, of sodium diethyl barbiturate buffer 0.012 M, of pooled human serum 0.1 cc. per cc. of the reaction mixture; temperature, 37°. The initial reaction velocities were determined. For the experiments with fructose-6-phosphate (upper part of figure), Michaelis' acetate-diethyl barbiturate buffer of varying pH values (10) was also employed in a concentration of 0.2 cc. per cc. of the reaction mixture. The velocities thus obtained are designated with X.

**Table IV**

Proportionality between Phosphohexose Isomerase Activity and Concentration of Serum

<table>
<thead>
<tr>
<th>Concentration of serum as volume per cc. reaction mixture</th>
<th>Reaction velocity as reciprocal of time required to form 100 γ of fructose as F-6-P per cc. reaction mixture (min⁻¹)</th>
<th>Reaction velocity (K) concentration of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.0180</td>
<td>0.90</td>
</tr>
<tr>
<td>0.04</td>
<td>0.0323</td>
<td>0.81</td>
</tr>
<tr>
<td>0.10</td>
<td>0.083</td>
<td>0.83</td>
</tr>
<tr>
<td>0.20</td>
<td>0.167</td>
<td>0.84</td>
</tr>
</tbody>
</table>
The value of the Michaelis constant, $K_m$, was 0.00122 mole per liter.

**Effect of Metal Ions**—Magnesium in concentrations up to $10^{-3}$ or $10^{-2}$ m exerted no activating effect. This is similar to the finding of Lohmann, who observed that this ion did not activate the phosphohexose isomerase of frog muscle (1). The experiments were performed with 0.0016 m sodium fructose-6-phosphate and 0.012 m sodium diethyl barbiturate buffer.

The pH ranged from 7.5 to 7.8. Cobalt and manganese which, like magnesium, are activators of a number of enzymes involved in glycolysis (19), similarly failed to exert any effect in the concentrations tested; namely, $1 \times 10^{-4}$ and $1 \times 10^{-3}$ m. Beryllium, which is a potent inhibitor of tissue phosphatase (20, 21), had no inhibitory action in concentrations ranging from $10^{-6}$ to $10^{-3}$ m. Zinc did not inhibit at $10^{-4}$ or $10^{-3}$ m, but at $10^{-3}$ m the inhibition was about 50 to 60 per cent with sodium fructose-6-phosphate as substrate and about 40 per cent with sodium glucose-6-phosphate (0.002 m). The pH in these experiments was usually between 7.40 and 7.50. The effect of copper (Cu$^{2+}$) was not evaluated since it was found...
that the presence of this ion in the filtrate subjected to the Roe procedure led to an orange color instead of the typical cherry-red color and caused a decrease in the transmission at 490 mμ.

Correlation between Serum Phosphohexose Isomerase and Serum Alkaline Phosphatase Activities—The phosphohexose isomerase activity of a number of sera from patients with disease was determined in duplicate under the following conditions: concentration of sodium fructose-6-phosphate, 0.0015 M; sodium diethyl barbiturate buffer, 0.012 M; serum, 0.04 cc. per cc. of reaction mixture; temperature, 37°; pH 7.5 to 7.6. Readings were made at 20 and 40 minutes after the start of the reaction, and one or both readings were used for the calculation of initial reaction velocities. A number of the sera were from patients with cancer who had metastases which involved the skeletal system or the liver, and which, therefore, led to high serum alkaline phosphatase activities. In the course of these determinations it was observed that individuals with high serum alkaline phosphatase activities also tended to have high serum phosphohexose isomerase activities.

Fig. 4 shows a plot of the serum phosphohexose isomerase activities against the alkaline serum phosphatase activities for eighteen of a group of nineteen patients. The reason for excluding this one patient from this

![Graph showing the correlation between serum phosphohexose isomerase and serum alkaline phosphatase activities.](http://www.jbc.org/)

Fig. 4. Correlation of phosphohexose isomerase and alkaline phosphatase activities in sera of patients with disease. See the text for the details of methods of determination.
plot will be noted presently. Computation of the correlation coefficient, \( r \), yielded a highly significant value for a positive correlation; namely, +0.91 with a \( t \) value of 9. The regression equations were

\[
Y' = 0.104X + 0.100 \\
X' = 7.96Y + 2.30
\]

where \( X \) and \( Y \) were the observed alkaline phosphatase and phosphohexose isomerase activities, respectively, and \( X' \) and \( Y' \) are the predicted values for these two enzymes, respectively. The value for \( \sigma_{Y \times X} \), the standard error of \( Y \) as estimated from \( X \), was 0.68. The value for \( \sigma_{X \times Y} \), the standard error of \( X \) as estimated from \( Y \), was 6.0.

It may be shown statistically that the patient, M. C., did not belong to the population represented by the plot in Fig. 4, and that it was, therefore, proper to exclude him. This patient repeatedly had, at different times during his hospital stay, a low serum phosphohexose isomerase activity in the presence of an extremely high serum alkaline phosphatase activity. On two occasions the latter was 59 and 68 Bodansky units; on the basis of Equation 1, the phosphohexose isomerase activities should have been, respectively, 6.3 \( \gamma \) and 7.2 \( \gamma \) of fructose, as F-6-P, converted per cc. of reaction mixture per minute. Actually these activities were 0.70 and 0.53 \( \gamma \) of fructose converted per cc. per minute, or less than the values derivable from Equation 1 by more than 7 to 8 times the standard error.

**DISCUSSION**

The present investigation has shown that there is a considerable degree of phosphohexose isomerase activity present in the serum of such divergent species as the chick, rat, dog, cat, and man. Its presence in other species is also indicated. A number of studies have shown or indicated that this enzyme is widely distributed in tissues, and the question arises which of these tissues is the source of the enzyme in serum. Our observation that there is a significant correlation between the serum alkaline phosphatase and the serum phosphohexose isomerase activities in a group of patients with metastatic cancer of the skeleton or liver raises the problem whether the mechanisms governing the levels of these two enzymes in the serum are in general similar, or whether they are different and are merely frequently affected simultaneously in patients such as those considered here. Studies are in progress to obtain further information concerning this problem.

**SUMMARY**

1. A considerable degree of phosphohexose isomerase activity has been shown to be present in the serum of the rat, cat, dog, chick, and man.
2. The following characteristics of serum phosphohexose isomerase activity have been studied: the equilibrium point in the action on the hexose-6-phosphate esters, stability at room and ice box temperature, inactivation by heat, effect of pH, effect of enzyme and substrate concentration, and the effect of metal ions.

3. A significant correlation was found to exist between the phosphohexose isomerase and alkaline phosphatase activities in the sera of patients with cancerous disease. The implications of this relationship are briefly discussed.

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