ON THE MECHANISM OF ACTION OF MUSCLE AND POTATO PHOSPHORYLASE

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It has recently been reported by Doudoroff, Barker, and Hassid (1) that an exchange occurs between the phosphate group of glucose-1-phosphate and inorganic phosphate in the presence of sucrose phosphorylase and in the absence of acceptor. On the basis of this exchange, they postulate a mechanism involving the formation of an enzyme-glucose complex, thus endowing the enzyme with the ability to transfer glucose to fructose and other acceptors; viz.,

\[
\text{Glucose-1-phosphate} + \text{enzyme} \rightleftharpoons \text{enzyme-glucose} + \text{phosphate}
\]

\[
\text{Enzyme-glucose} + \text{fructose} \rightleftharpoons \text{enzyme} + \text{sucrose}
\]

This mechanism has been confirmed by these authors by studying the reaction with arsenate (2). When glucose-1-phosphate, enzyme, and arsenate were mixed, again in the absence of acceptor, free glucose was formed. This could only have been due to the spontaneous decomposition of glucose-1-arsenate. Thus, the arsenate must have exchanged with the phosphate through the intermediate formation of an enzyme-glucose complex.

The proposed mechanism which might be described as an exchange of a bond between substrate and enzyme defines the nature of the substrate-enzyme interaction in contrast to the usual formulation which merely postulates the formation of an enzyme-substrate complex. This type of mechanism expressed in a generalized form, namely,

\[
A-B + \text{enzyme} \rightleftharpoons \text{enzyme-A} + B
\]

might well be applicable to other enzymatic reactions.

It seemed of interest to test the validity of this mechanism for the mode of action of muscle and potato phosphorylase and consider the possibility that \( A \) may be either glucose or phosphate. No exchange was found to occur between inorganic phosphate (labeled with P\(^{32}\)) and glucose-1-phosphate in the presence of muscle or potato phosphorylase when the addition of polysaccharide as acceptor was omitted. The absence of exchange was confirmed in the case of potato phosphorylase by the failure of arsenate to yield free glucose in a reaction mixture containing glucose-1-
phosphate but no acceptor. There was also no exchange of glucose (labeled with C\textsuperscript{14}) with glucose-1-phosphate during enzymatic synthesis of polysaccharide by muscle phosphorylase. The labeled glucose did not exchange with glycogen either in this system, which precluded a mechanism involving the transfer of a glucosidic bond through an interaction of enzyme and polysaccharide primer.

One further possibility was explored; namely, that an exchange might occur between adenylic acid which activates muscle phosphorylase b and inorganic phosphate. No exchange was observed. Thus the mechanism of enzyme action involving the type of bond transfer exhibited by sucrose phosphorylase is not apparent for the phosphorylases investigated in the present study and the extent of its applicability to other enzyme reactions awaits further investigation.

**EXPERIMENTAL**

*Separation of Phosphates*—The inorganic phosphate and glucose-1-phosphate in the reaction mixtures were separated as barium salts at pH 8.5 to 9. Inorganic phosphate was contained in the precipitate, glucose-1-phosphate in the supernatant fluid.

The precipitate was redissolved in dilute HCl and the precipitation at pH 8.5 to 9 was repeated. The resulting precipitate was dissolved, barium was removed with Na\textsubscript{2}SO\textsubscript{4}, and the inorganic phosphate was then precipitated as the ammonium magnesium salt. After being washed with ammonium magnesium mixture, this fraction contained no organic phosphate.

To the soluble barium salts inorganic phosphate was added (in order to dilute contaminating inorganic P\textsuperscript{32}) and again removed by precipitation at pH 8.5 to 9. This was repeated several times. After removal of the barium the solution was found to contain besides glucose-1-phosphate a small amount of orthophosphate (1 to 8 per cent of the glucose-1-phosphate) as determined by the Fiske-Subbarow method (3). All values of radioactivity of glucose-1-phosphate listed in Tables I and II are corrected for the slight contamination which never exceeded 0.6 per cent of the orthophosphate radioactivity.

In experiments in which the phosphorylase reaction had gone to equilibrium, the recovery of glucose-1-phosphate could be improved by removal of the synthetic polysaccharide before fractionation with barium. Very little phosphate was removed with the polysaccharide when the latter was precipitated at pH 5 in 50 per cent ethanol. The supernatant fluid was then brought to pH 8.5 to 9 with Ba(OH)\textsubscript{2} and more ethanol was added to raise its concentration to 66 per cent. The resulting precipitate which contained inorganic phosphate and glucose-1-phosphate was separated into the two fractions as described above.
Exchange of Glucose-1-Phosphate and Inorganic Phosphate—It will be seen in Table I that in the aliquot to which starch had been added as a primer in the presence of potato phosphorylase the reaction attained equilibrium and that inorganic phosphate and glucose-1-phosphate had the same radioactivity (635 and 630 counts per minute per mM, respectively). Without addition of primer, however, no reaction took place and the glucose-1-phosphate showed no radioactivity. It will be noted from Table I that this was also the case in the experiment in which muscle phosphorylase served as catalyst. When a suboptimal amount of primer was added (12 mg. per cent of glycogen) the reaction was still far from equilibrium, but nevertheless the isolated glucose-1-phosphate had some radioactivity (47 counts per minute per mM), showing the extent to which the reverse

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Poly-saccharide</th>
<th>Reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per cent</td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>min.</td>
</tr>
<tr>
<td>Potato phosphorylase</td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>125*</td>
<td>30</td>
</tr>
<tr>
<td>Crystalline muscle-phosphorylase a</td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>12†</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>250†</td>
<td>20</td>
</tr>
</tbody>
</table>

* Soluble starch.
† Glycogen.

reaction (glycogen + inorganic phosphate → glucose-1-phosphate) had taken place. With high concentration of primer (250 mg. per cent of glycogen) equilibrium was reached in the presence of muscle phosphorylase. In this experiment the polysaccharide was not removed before fractionation and the recovery of glucose-1-phosphate was so low that its radioactivity could not be measured.

Exchange of Glucose-1-Phosphate with Glucose—In the experiment summarized in Table II the phosphorylase reaction was allowed to go to equilibrium in the presence of glucose containing C\(^14\). Glycogen was added to prime the reaction. After precipitation of the polysaccharide with 50 per cent ethanol, the phosphates were precipitated from the alcoholic fluid as barium salts and separated as already described. The isolated glucose-1-phosphate was found to contain no radioactivity. Glucose
remained in the 66 per cent alcoholic supernatant solution. Air was bubbled through the solution to remove the ethanol and the barium was precipitated with sulfuric acid. The amount of glucose in the solution was determined by the Nelson method (4). The glucose was found to have the same radioactivity in the experimental and control samples. No exchange with either glucose-1-phosphate or with glycogen could have taken place.

Exchange between Adenylic Acid and Inorganic or Glucose-1-Phosphate—Muscle phosphorylase b, which is active only when adenylic acid is added, was the enzyme used in this experiment as indicated in Table III. The reaction mixture contained initially 16 micromoles of glucose-1-phosphate and 5.5 micromoles of adenylic acid per ml. The reaction was allowed to go to equilibrium in the presence of P³² (orthophosphate) and hence there was incorporation of P³² in glucose-1-phosphate. Then the reaction mixture was made 1.0 N with HCl and heated to 100° for 7 minutes. This led to hydrolysis of glucose-1-phosphate and left ribose phosphate (derived from adenylic acid) as the sole water-soluble barium phosphate. After isolation of the ribose phosphate by the procedure described for the isolation of glucose-1-phosphate, its ribose content (5) and phosphate content (3) were found to be in good agreement. There was no radioactivity found in the ribose phosphate. The radioactivity values of the inorganic phosphate given in Table III were measured after the hydrolysis of glucose-1-phosphate.

### TABLE II

*Exchange of Glucose (3755 Counts per Minute per Mg.) with Glucose-1-phosphate and Glycogen in Presence of Muscle Phosphorylase a*

<table>
<thead>
<tr>
<th></th>
<th>Recovered glucose</th>
<th>Recovered glucose-1-phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts per min. per mg.</td>
<td>counts per min. per mg.</td>
</tr>
<tr>
<td>Control (no enzyme)</td>
<td>3805</td>
<td>0</td>
</tr>
<tr>
<td>Experimental</td>
<td>3835</td>
<td>0</td>
</tr>
</tbody>
</table>

### TABLE III

*Exchange of Inorganic Phosphate (P³²) with Adenylic Acid in Reaction of Glucose-1-phosphate and Glycogen in Presence of Muscle Phosphorylase b*

<table>
<thead>
<tr>
<th></th>
<th>Inorganic phosphate</th>
<th>Ribose phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts per min. per mg</td>
<td>counts per min. per mg</td>
</tr>
<tr>
<td>Control (no enzyme)</td>
<td>2090</td>
<td>0</td>
</tr>
<tr>
<td>Experimental</td>
<td>1940</td>
<td>0</td>
</tr>
</tbody>
</table>
SUMMARY

No exchange occurred between inorganic phosphate and glucose-1-phosphate in the presence of muscle phosphorylase a or potato phosphorylase in the absence of polysaccharide as acceptor. Furthermore the phosphate of adenylic acid did not interchange in a complete reaction mixture containing inorganic phosphate (P\textsuperscript{32}O), glucose-1-phosphate, glycogen, and muscle phosphorylase b. The possibility of an exchange between glucose (labeled with C\textsuperscript{14}) and glucose-1-phosphate was investigated in the presence of muscle phosphorylase and was found to be negative. The implications of these results are discussed in so far as they affect the mechanism of these enzyme reactions.

The authors wish to thank Dr. Barker and Dr. Hassid of the University of California for the radioactive glucose used in this work.

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