THE ENZYMATIC SYNTHESIS OF PHOSPHOPYRUVATE FROM PYRUVATE

BY HENRY A. LARDY* AND JOHN A. ZIEGLER†

(From the Departments of Chemistry and Pathological Chemistry, Banting Institute, University of Toronto, Toronto, Canada)

(Received for publication, March 22, 1945)

In the pathway of glycogen synthesis from pyruvate and lactate, one of the steps in the concatenated reactions known as the Embden-Meyerhof scheme which has remained obscure is the mechanism of phosphopyruvate "resynthesis." This problem has been the subject of much speculation and some investigation. Since Meyerhof, Ohlmeyer, Gentner, and Maier-Leibnitz (1) reported the reaction,

\[
\text{Phosphopyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}
\]

to be irreversible, a number of alternate pathways have been proposed for the conversion of pyruvate, lactate, and other carbohydrate fragments to phosphopyruvate.

In accordance with Kalckar's demonstration of phosphopyruvate (hereafter \(\sim\text{phPy}\)) synthesis during the oxidation of fumarate by kidney extracts (3), it was suggested that this enol phosphate arose by a phosphorylation directly coupled with the oxidation of fumarate (Lipmann (2)). The finding that about one-sixth of the newly deposited glycogen, following lactate, pyruvate, or glucose feeding to rats, originated from tissue bicarbonate led Solomon, Vennesland, Klemperer, Buchanan, and Hastings (4) to postulate that the formation of a 4-carbon compound as a precursor of \(\sim\text{phPy}\) is a logical step in glycogen synthesis from pyruvate. Ferdman and Epstein (5) reported \(\sim\text{phPy}\) synthesis during the oxidation of lactate by cat muscle. In view of the fact that their medium contained bicarbonate, it is possible that oxalacetate may have been formed and that subsequent phosphorylation of the 4-carbon acid could have led to \(\sim\text{phPy}\) formation.

Leloir and Muñoz (6) found liver preparations to produce \(\sim\text{phPy}\) during the oxidation of succinate, fumarate, and citrate but not of lactate and pyruvate. Other data presented by these workers have been previously interpreted (7) as indicating that \(\sim\text{phPy}\) may not have arisen by direct phosphorylation of a 4-carbon compound.

* National Research Council (Washington) Fellow in Chemistry 1944–45.
† Aided by a grant from the National Research Council of Canada.

1 Other abbreviations used in this paper are ATP for adenosine triphosphate, ADP for adenosine diphosphate, \(\sim\text{ph}\) for high energy phosphate (2), \(\sim\text{phCr}\) for phosphocreatine.
In a previous attempt to demonstrate the formation of phosphoenolpyruvate, it was found that addition of oxalacetate, or pyruvate plus bicarbonate, to aged rat muscle extracts resulted in a loss of ATP and an increase in inorganic phosphate but no accumulation of enol phosphate could be detected (8).

The experiment on which the Heidelberg workers (1) based their conclusions regarding the irreversibility of Reaction 1 was designed to detect any exchange of \( \sim pH \) between ATP and \( \sim pH \text{Py} \) and did not depend on an accumulation of \( \sim pH \text{Py} \) produced from pyruvate and ATP. The group potential of the enol phosphate has been calculated by Lipmann ((2) p. 110), from thermal data, to be approximately 11,250 calories, or of the same magnitude as that of the two terminal phosphates of ATP. From a thermodynamic point of view, therefore, it seems that, in the steady state, phosphate exchange should occur between ATP and \( \sim pH \text{Py} \). Meyerhof apparently recognized this, for, in discussing the experiment from which it was concluded that Reaction 1 is irreversible, he stated in 1941 (9), “But we must concede that the experimental basis for this negative result is not too large and therefore accept it with some reservation until it is more firmly established.”

The discovery, by Boyer et al. (10, 11), that \( K^+ \) is necessary for the transfer of \( \sim pH \) from \( \sim pH \text{Py} \) to the adenylic system bears strongly on the validity of that conclusion, for the Heidelberg workers employed an enzyme extract which has been dialyzed for 12 hours and the protocol of their experiment does not indicate the addition of any potassium salt. The reaction has therefore been investigated with radioactive phosphorus, and the relation of various cations to the reversal of Reaction 1 and to the oxidative phosphorylation of \( d \)-glyceraldehyde 3-phosphate has been studied.

**Methods**

All experiments reported in this paper were made with a dialyzed extract of acetone-precipitated rat muscle extract prepared as described elsewhere (11). \( dL \)-Glyceraldehyde 3-phosphate (Fischer-Baer ester) was synthesized according to the new procedure of Baer and Fischer (12); the other substrates were as previously described (10, 11). All acids were neutralized with sodium hydroxide and the final pH of the reaction mixtures was always 7.4. No bicarbonate was added in any of the experiments.

The enzyme reaction mixtures were deproteinized with trichloroacetic acid and immediately placed in an ice-salt bath. Inorganic phosphate

---

2 The cozymase used in these experiments was kindly supplied by Dr. D. F. Green of Merek and Company, Inc., Rahway, New Jersey, the desoxycorticosterone acetate by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey.
was separated from the filtrate as MgNH₄PO₄·6H₂O by treatment with magnesia mixture (13). ~phPy was then hydrolyzed by the alkaline iodine procedure of Lohmann and Meyerhof (14).

Following removal of the excess iodine under acid conditions the inorganic phosphate liberated was either separated for determination of radioactivity by treatment with magnesia mixture or, in other experiments, was determined quantitatively after removal of the iodoform. The labile phosphate groups of adenosine polyphosphates were hydrolyzed by 1 N HCl for 7 minutes at 100° and the inorganic phosphate was separated with magnesia mixture. The inorganic phosphate fractions were analyzed by a micro modification of the Fiske and Subbarow method (15) adapted to a photoelectric colorimeter.

The radioactivity of aliquots of the phosphate fractions was determined with a Lauritsen electroscope. The activity, measured as net rate of deflection, is expressed as divisions of deflection per minute per mg. of phosphorus.

~phCr was determined in the fraction of the filtrate soluble in 2 per cent CaCl₂ at pH 8.2 (16). The alkali lability of triose phosphate (17) interferes with the accurate determination of both ~phCr and ~phPy and therefore the ester is converted to inorganic phosphate before proceeding with the fractionations. This was accomplished by making the filtrates containing triose phosphate 1 N with NaOH and holding for 30 minutes at room temperature.

Results

Preliminary experiments with an undialyzed extract of muscle acetone powder demonstrated that inorganic phosphate containing P³² was incorporated into the labile phosphate of ATP during the oxidation of d-glyceraldehyde 3-phosphate and that this labeled ~ph was in turn introduced into ~phPy. Since K⁺ is necessary to catalyze Reaction 1 (10, 11) and in view of the fact that the Heidelberg workers used a dialyzed enzyme and added no potassium salts to their reaction mixture, it seemed reasonable that the distribution of ~ph between ATP and ~phPy was contingent on the presence of K⁺. The results of experiments testing this possibility are shown in Table I. The ~phPy in these experiments was produced enzymatically (18) by incubating the phosphoglycerate and enzyme for 10 minutes at 25°, followed by 10 minutes at 37° before the addition of fluoride. Experimental incubation time was measured from the addition of ATP to deproteinization.

The traces of radioactivity appearing in the ~phPy fraction at zero

3 We are indebted to Mr. D. Scott of the Banting and Best Department of Medical Research for the radioactivity measurements.
time are the result of incomplete precipitation of inorganic phosphate by the first treatment with magnesia mixture. In five other zero time samples the degree of contamination of the \( \sim \text{phPy} \) fraction never exceeded that obtained in Experiments A and B. The decrease in activity of the inorganic fraction during incubation is the result of P\(^{32} \) transfer to other fractions and of the increase in inorganic phosphate due to spontaneous decomposition of 1,3-diphosphoglycerate (19, 20).

The equilibrium between \( \sim \text{phPy} \) and ATP, which in these experiments was followed by the distribution of P\(^{32} \), can be seen to be hastened by the addition of K\(^+ \). The incorporation of P\(^{32} \) into the various fractions is the result of the following equilibrium reactions.

\[
\begin{align*}
\text{(2)} & \quad d \text{Glyceraldehyde 3-phosphate} + \text{inorganic phosphate} + \text{cozymase} \rightleftharpoons 1,3\text{-diphosphoglycerate} + \text{reduced cozymase} \\
\text{(3)} & \quad \text{Reduced cozymase} + \text{pyruvate} \rightleftharpoons \text{cozymase} + \text{lactate} \\
\text{(4)} & \quad 1,3\text{-Diphosphoglycerate} + \text{ADP} \rightleftharpoons 3\text{-phosphoglycerate} + \text{ATP} \\
\text{(5)} & \quad \text{Pyruvate} + \text{ATP} \rightleftharpoons \text{phosphopyruvate} + \text{ADP}
\end{align*}
\]

Phosphate exchange between \( \sim \text{phPy} \) and ATP occurred to an appreciable extent even without added potassium but, as shown in Table II, the

### Table I

**Exchange of High Energy Phosphate between Phosphopyruvate and Adenosine Triphosphate**

At the time of fluoride addition each tube contained the following (expressed in micromoles): phosphoglycerate 80, phosphopyruvate 20, lactate 100, MgCl\(_2\) 20, MnSO\(_4\) 8, cozymase 0.3, inorganic phosphate (containing P\(^{32} \)) 33, and 1.6 ml. of enzyme solution. After the addition of 200 micromoles of NaF, 3.2 micromoles of adenosine triphosphate were added, giving a final volume of 4 ml. Incubated 15 minutes at 37°.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>KCl added</th>
<th>P(^{32} ) content of phosphate fractions expressed in divisions per min. per mg. F</th>
<th>Inorganic</th>
<th>Adenosine triphosphate</th>
<th>Phosphopyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>200</td>
<td>62</td>
<td>0.0</td>
<td>15</td>
<td>1.3</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>46</td>
<td>15</td>
<td>8.6</td>
<td>5.7</td>
</tr>
<tr>
<td>A</td>
<td>200</td>
<td>42</td>
<td>15</td>
<td>8.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Zero time</td>
<td>None</td>
<td>63</td>
<td>1.0</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>&quot;</td>
<td>53</td>
<td>15</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>B</td>
<td>200</td>
<td>47</td>
<td>14</td>
<td>6.3</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>200*</td>
<td>49</td>
<td>13</td>
<td>8.4</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* 2 mg. of desoxycorticosterone acetate were added.
enzyme was only partially depleted of K+ by the 48 hour dialysis. The enzyme solution must have retained sufficient quantities of K+ to catalyze the transfer of phosphate from ~phPy to ADP but at a rate not greater than three-sevenths that obtained with added potassium.

K+, Mg++, and Mn++ in Relation to Phosphorylations Coupled with Oxidation of d-Glyceraldehyde 3-Phosphate—From the above results it appeared that the incorporation of inorganic phosphate into ATP (through the

<table>
<thead>
<tr>
<th>Other additions</th>
<th>Phosphocreatine synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>KCl</td>
</tr>
<tr>
<td>micromoles</td>
<td>micromoles</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

TABLE II
Extent of Dialysis of Enzyme and Proof of Fluoride Inhibition

Each tube contained the following additions (expressed in micromoles) in a final volume of 1 ml.: phosphoglycerate 10, creatine 50, adenosine triphosphate 0.16, inorganic phosphate 100, and 0.5 ml. of enzyme solution. Incubated 10 minutes at 37°.

<table>
<thead>
<tr>
<th>KCl added</th>
<th>Phosphocreatine synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>micromoles</td>
<td>micromoles</td>
</tr>
<tr>
<td>None</td>
<td>8.3</td>
</tr>
<tr>
<td>200</td>
<td>7.4</td>
</tr>
</tbody>
</table>

oxidation of glyceraldehyde phosphate) was influenced less by potassium than the equilibrium between ~phPy and ATP. The effect of K⁺, Mg⁺⁺, and Mn⁺⁺ on the oxidative phosphorylation was therefore investigated. The results in Table III show that added K⁺ was not required for Reactions 2, 3, and 4. It was shown by Boyer et al. (10) that K⁺ is not required for the phosphorylation of creatine by ATP; since Mg⁺⁺ is required for the latter phosphorylation (21), its requirement for Reactions 2, 3, and 4 could not be tested by the system shown in Table III.
In experiments with adenylic acid as \( \sim \)ph acceptor, it was found (Table IV) that Mg\(^{++}\) or Mn\(^{++}\) additions were also unnecessary for the oxidation of glyceraldehyde phosphate and the transfer of \( \sim \)ph from 1,3-diphosphoglycerate to the adenylic system. The addition of traces of ATP was not required to initiate the phosphorylation of adenylic acid. In the phos-

**Table IV**

*Fixation of Inorganic Phosphate during Oxidation of Fischer-Baer Ester and Transfer of Phosphate to Adenylic Acid*

Each tube contained the following additions (expressed in micromoles) in a final volume of 2 ml.: inorganic phosphate 20, \( dl \)-glyceraldehyde 3-phosphate 20 (of \( d \) component), cozymase 0.15, pyruvate 200, adenylic acid 10 (capable of accepting 20 micromoles of high energy phosphate), NaF 100, and 0.7 ml. of enzyme solution. Incubated 8 minutes at 37°.

<table>
<thead>
<tr>
<th>Other additions</th>
<th>Adenosine triphosphate taken up</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl(_2)</td>
<td>MnSO(_4)</td>
</tr>
<tr>
<td>micromoles</td>
<td>micromoles</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table V**

*Synthesis of Phosphopyruvate during Oxidation of Fischer-Baer Ester*

Each tube contained the following additions (expressed in micromoles) in a final volume of 2.25 ml.: inorganic phosphate 150, pyruvate 300, \( dl \)-glyceraldehyde 3-phosphate 20 (of \( d \) component), cozymase 0.15, hexose diphosphate 20, NaF 120, adenosine triphosphate 0.4, MgCl\(_2\) 20, MnSO\(_4\) 10, and 0.63 ml. of enzyme solution. Incubated at 37°.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time of incubation</th>
<th>KCl added</th>
<th>Phosphopyruvate ( \sim )ph synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>None</td>
<td>0.00</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>200</td>
<td>0.58</td>
</tr>
</tbody>
</table>

phorylation of creatine by \( \sim \)phPy, adenylic acid is not nearly as effective a \( \sim \)ph carrier as the higher phosphorylated adenylic compounds (21, 11) but where there is an accumulation of \( \sim \)ph in the adenylic system, as in Table IV or in the phosphorylation of adenylic acid by \( \sim \)phPy (other experiments), additions of ADP or ATP do not accelerate the process.
Synthesis of Phosphopyruvate—Since Reaction 5 was shown in the above experiments with P$^{32}$ to be reversible, the synthesis of $\sim$-phPy from pyruvate should occur when the supply of $\sim$-ph is continually being renewed by another reaction. As shown in Table V, $\sim$-phPy was synthesized from pyruvate when the $\sim$-ph was produced by oxidation of glyceraldehyde 3-phosphate, but only when K$^+$ was added. The $\sim$-phPy values for each sample are corrected for traces of inorganic phosphate remaining after the treatment with magnesia mixture. Although the amounts of $\sim$-phPy synthesized are small, they are far greater than the limit of accuracy of the analytical methods (0.05 micromole) and it must be remembered that the 3-phosphoglycerate produced also competes with pyruvate for the $\sim$-ph. The 1,3-diphosphoglycerate produced in the competitive reaction spontaneously decomposes (19, 20) to give inorganic phosphate and 3-phosphoglycerate which may again accept $\sim$-ph. Hexose diphosphate was added in these experiments to prevent removal of d-glyceraldehyde 3-phosphate$^4$ by the isomerase-aldolase equilibrium.

The concentration of NaF in these experiments is far greater than that required completely to inhibit enolase (23) (see also Table II). Further evidence that the $\sim$-phPy produced did not come from phosphoglycerate is the fact that $\sim$-phPy appeared only when K$^+$ was added.

**DISCUSSION**

It has been experimentally demonstrated by the distribution of P$^{32}$ and by direct synthesis that pyruvate may be enzymatically phosphorylated by ATP in the presence of K$^+$ and Mg$^{++}$ to produce $\sim$-phPy. The implications of this finding for the mechanism of glycogen synthesis are evident. It is no longer necessary to postulate the occurrence of a 4-carbon intermediate in the synthesis of $\sim$-phPy from pyruvate. It is a thermodynamic fact that a pathway of $\sim$-phPy synthesis from pyruvate through a 4-carbon intermediate, regardless of its mechanism, can be energetically no more economical than the direct synthesis by Reaction 5. In the intact organism the continually applied "potential" of $\sim$-ph produced by the oxidation of carbohydrate (see (2) and (24)) or fat (25) can supply the energy for the synthesis of hexoses from pyruvate. The finding of Buchanan, Hastings, and Nesbett (26) that high concentrations of K$^+$ were necessary to obtain glycogen formation from pyruvate in liver slices can be explained, at least in part, by the necessity of K$^+$ for Reaction 5.

$^4$ Only the d component of the Fischer-Baer ester condenses in the presence of rat muscle isomerase and aldolase at low temperature to produce hexose diphosphate (H. A. L., unpublished data). l-Glyceraldehyde 3-phosphate differs from the free l-glyceraldehyde in that the latter can condense with dihydroxyacetone phosphate to form l-sorbose 1-phosphate (22).
The present findings do not rule out the possibility of \(~\text{phPy}\) formation, by direct oxidative phosphorylation of a 4-carbon dicarboxylic acid, but they do eliminate such a 4-carbon compound as an obligatory intermediate in the conversion of pyruvate to \(~\text{phPy}\).

The mechanism by which bicarbonate is incorporated into glycogen will probably be disclosed by further studies on the reversibility of pyruvate decarboxylation (27, 28) and on the extent to which the enol group of oxalacetate can shift between carbon atoms 2 and 3 (29, 30).

**SUMMARY**

It has been demonstrated by two separate enzymatic techniques that the reaction

\[
\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{phosphopyruvate} + \text{ADP}
\]

is reversible.

\(P^{32}\) incorporated into ATP during the oxidation of glyceraldehyde 3-phosphate was in equilibrium with that in phosphopyruvate.

Pyruvate was enzymatically phosphorylated when high energy phosphate was continually supplied by the oxidation of glyceraldehyde 3-phosphate. The importance of \(K^+\) for the synthesis of phosphopyruvate from pyruvate was demonstrated.

\(K^+\) and \(Mg^{++}\) or \(Mn^{++}\) are needed in much lower concentrations (if at all) for the oxidation of glyceraldehyde 3-phosphate and the transfer of high energy phosphate to the adenylic system than for the transfer from phosphoglycerate through phosphopyruvate to adenylylate.

I am indebted to Professors Hermann O. L. Fischer and Andrew Hunter, in whose laboratories these experiments were conducted, for kindly furnishing supplies and equipment, and to Professor Erich Baer who introduced to me the synthesis of \(dl\)-glyceraldehyde 3-phosphate. (H. A. L.)

**BIBLIOGRAPHY**

THE ENZYMATIC SYNTHESIS OF PHOSPHOPYRUVATE FROM PYRUVATE
Henry A. Lardy and John A. Ziegler

J. Biol. Chem. 1945, 159:343-351.

Access the most updated version of this article at http://www.jbc.org/content/159/2/343.citation

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/159/2/343.citation.full.html#ref-list-1