A Study of the Mechanism of O-Phosphorylhydroxylamine Synthesis Catalyzed by Pyruvate Kinase*

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SUMMARY

The non-nucleotide product formed in the hydroxylamine kinase reaction, previously shown to be catalyzed by pyruvate kinase, has been identified as O-phosphorylhydroxylamine. The reaction exhibits an absolute requirement for bicarbonate, a nucleoside triphosphate (which cannot be replaced by phosphoenolpyruvate), and a divalent metal (Mn++ or Zn++, but not Mg++ ions). N-Methylhydroxylamine and, to a lesser extent, N,N-dimethylhydroxylamine also undergo phosphorylation, whereas the O-methyl derivative does not. The Michaelis constants of adenosine triphosphate, bicarbonate, and hydroxylamine for pyruvate kinase from rabbit skeletal muscle are 1.7 × 10^-3 M, 1.9 × 10^-2 M, and 2.4 × 10^-2 M, respectively.

A study of the effects of bicarbonate and hydroxylamine on the kinetics of the pyruvate kinase reaction showed that these compounds are apparently bound at the site which binds pyruvate or phosphoenolpyruvate but do not interact with the site which binds adenosine diphosphate or adenosine triphosphate. Bicarbonate, which is significantly inhibitory at physiological concentrations, is a competitive inhibitor with respect to phosphoenolpyruvate in the presence of either Mg++ or Mn++ ions. With the latter metal the K_i of bicarbonate is 0.02 M. Hydroxylamine exhibited mixed inhibition with respect to phosphoenolpyruvate with Mg++ ions present and uncompetitive inhibition with Mn++ ions, whereas an equimolar mixture of hydroxylamine and bicarbonate gave mixed inhibition kinetics with either of these metals. On the other hand, hydroxylamine, bicarbonate, or both compounds together showed no significant inhibitory kinetic effects with respect to adenosine diphosphate, with either metal present.

No evidence was obtained that N-hydroxy carbamate, formed by the reaction of bicarbonate with hydroxylamine, serves as the substrate in the hydroxylamine kinase reaction. It is proposed that bicarbonate and hydroxylamine occupy the pyruvate-binding site of the enzyme and that Zn++ or Mn++ ions form a complex with the nitrogen atom of hydroxylamine, thereby polarizing the N—O bond and increasing the likelihood of a nucleophilic attack on the terminal phosphorus atom of adenosine triphosphate by the oxygen atom of hydroxylamine with the formation of O-phosphorylhydroxylamine and adenosine diphosphate.

In the course of earlier studies on propionyl coenzyme A carboxylation in Ochoa's laboratory (2) and p-methylcrotonyl-CoA carboxylation in this laboratory (3), two unusual bicarbonate-dependent reactions were found inadvertently. The ATP-dependent phosphorylation of fluoride to yield fluorophosphate (Reaction 1) was attributed to the action of a "fluorokinase" (4, 5) and the crystalline enzyme from rabbit skeletal muscle having this activity was later shown by Tietz and Ochoa (6) to be identical with pyruvate kinase. Similarly, the ATP-dependent phosphorylation of hydroxylamine (Reaction 2) was attributed to the action of a "hydroxylamine kinase" (7) which was highly purified from pig heart and later shown by Kupiecki and Coon (8) to be identical with pyruvate kinase. In contrast to these two reactions, the glycolytic step catalyzed by pyruvate kinase (Reaction 3) is reversible (9-11) and exhibits no bicarbonate requirement.

\[
\begin{align*}
\text{ATP} + \text{fluoride} & \rightarrow \text{ADP} + \text{fluorophosphate} \\
\text{ATP} + \text{hydroxylamine} & \rightarrow \text{ADP} + \text{phosphorylhydroxylamine}
\end{align*}
\]

These reactions all require K+ ions (6, 8, 12) but differ in their specificity for divalent metals. The fluorokinase and pyruvate kinase reactions require Mg++ or Mn++ (6) but do not occur

1 Systematic name, ATP:pyruvate phosphotransferase, EC 2.7.1.40.
with Zn$^{2+}$ ions (8). In contrast, the hydroxylamine kinase reaction is most rapid with Zn$^{2+}$ or Co$^{2+}$, occurs at a lower rate with Mg$^{2+}$, and is absent with Mg$^{2+}$ ions (7).

The present paper is concerned with the identification of the non-nucleotide product of hydroxylamine phosphorylation as the O-phosphoryl derivative and with kinetic and isotopic studies on the role of bicarbonate in the hydroxylamine kinase reaction. The results obtained also provide some insight into the mechanism of the fluorokinase reaction.

**EXPERIMENTAL PROCEDURE**

**Materials**

Pyruvate kinase was prepared from rabbit skeletal muscle by the procedure of Tietz and Ochoa (6) or obtained from Boehringer and Sons. The enzyme was recrystallized once or twice from 0.02 M imidazole-chloride buffer, pH 7.0, by the slow addition of powdered ammonium sulfate to incipient turbidity and was stored as the ammonium sulfate suspension. These preparations, which had a specific activity at 30° of 270 to 310 munits per mg of enzyme, appeared to be homogeneous as judged by the sedimentation pattern in the ultracentrifuge and by polyacrylamide gel electrophoresis at pH 7.3 in Tris buffer or 8.6 in glycine buffer. The protein concentration of pyruvate kinase solutions was estimated from the absorbance at 280 nm, with an extinction coefficient of 0.54 ml mg$^{-1}$ cm$^{-1}$ (13). Crystalline yeast hexokinase was obtained from Sigma, crystalline lactate dehydrogenase from Boehringer and Sons, and frozen rabbit muscle from Pol-Freca Biologicals (Rogers, Arkansas).

O-Phosphorylhydroxylamine was prepared by the reaction of hydroxylamine with phosphoramidate according to the procedure published by Jencks and Gilchrist (14). The purification of the product or of the enzymatically synthesized phosphorylhydroxylamine was accomplished by chromatography on a column of Dowex 1-X8 buffered with 0.01 N imidazole buffer, pH 7.0, by the slow addition of powdered ammonium sulfate to incipient turbidity and was stored as the ammonium sulfate suspension. These preparations, which had a specific activity at 30° of 270 to 310 munits per mg of enzyme, appeared to be homogeneous as judged by the sedimentation pattern in the ultracentrifuge and by polyacrylamide gel electrophoresis at pH 7.3 in Tris buffer or 8.6 in glycine buffer. The protein concentration of pyruvate kinase solutions was estimated from the absorbance at 280 nm, with an extinction coefficient of 0.54 ml mg$^{-1}$ cm$^{-1}$ (13). Crystalline yeast hexokinase was obtained from Sigma, crystalline lactate dehydrogenase from Boehringer and Sons, and frozen rabbit muscle from Pol-Freca Biologicals (Rogers, Arkansas).

**Methods**

**Assay of Pyruvate Kinase**—Pyruvate kinase was estimated spectrophotometrically by the procedure of Bücher and Pfeiferer (13) as modified by Tietz and Ochoa (6). In this procedure the rate of pyruvate formation is estimated by the coupled reactions:

\[(\text{a}) \quad \text{P-enolpyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}, \quad \text{and (b) pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}^+.\]

The standard reaction mixture contained 100 mmoles of imidazole chloride buffer (pH 7.0), 100 mmoles of KCl, 4 mmoles of MgCl$_2$, 1 mmoles of P-enolpyruvate, 5 mmoles of ADP, 0.15 mmoles of DPNH, 5 mmoles of lactate dehydrogenase, and the sample of pyruvate kinase as the last addition in a final volume of 1.0 ml. The temperature was 30°. The rate of disappearance of DPNH was determined with a spectrophotometer equipped with a Gilford multiple sample absorbance recorder. The specific activity of pyruvate kinase is expressed as micromoles of pyruvate formed per min per mg of protein.

**Assay of Hydroxylamine Kinase Activity of Pyruvate Kinase**—Hydroxylamine kinase was assayed by a slight modification of the method described by Kupiecki and Coon (7). Unless stated otherwise, pyruvate kinase was incubated with 200 mmoles of Tris buffer (pH 7.6), 500 mmoles of potassium bicarbonate, 4 mmoles of zinc acetate, 10 mmoles of ATP, and 200 mmoles of neutralized hydroxylamine in a final volume of 3.0 ml for 30 min at 38°. The final pH was 7.5. The enzyme was omitted in control tubes. At the end of the incubation the reaction mixtures were chilled in ice, 1.0 ml of 40% trichloracetic acid and 200 mg of acid-washed charcoal were added, and the mixture was centrifuged. The supernatant solution was again treated with charcoal, any charcoal remaining after centrifugation was removed by filtration, and the phosphorylated hydroxylamine in the solution was quantitatively hydrolyzed in 0.5 N sulfuric acid (final concentration) for 10 min at 100°. The P$_1$ formed was then estimated by the method of Fiske and SubbaRow (17) with 0.1% Elon in 0.3% NaHSO$_3$ as the reducing agent; unless previously subjected to acid hydrolysis O-phosphorylhydroxylamine does not react as P$_1$ under these conditions. Unless stated otherwise, the values reported have been corrected for P$_1$ found to be present prior to acid hydrolysis; this blank may in part represent a small amount of phosphorylhydroxylamine which hydrolyzes to give P$_1$ in the presence of trichloracetic acid. As shown in Fig. 1, the hydroxylamine kinase reaction has a broad pH optimum from about 7.2 to 8.0 in Tris buffer, and other experiments have shown that over a range of Tris concentrations from 0.03 to 0.16 M the shape and magnitude of this curve do not change. The use of 0.03 M Tris-maleic buffer caused a shift in the pH optimum to about 7.0 and a slight decrease in the amount of product formed, whereas a concentration of 0.1 M resulted in a higher optimum range (7.3 to 7.9) but a further decrease in enzyme activity. Because of these findings the hydroxylamine kinase assay was carried out routinely in 0.067 M Tris buffer, pH 7.5. The formation of phosphorylated hydroxylamine was found to be proportional to the pyruvate kinase concentration over the range used, as shown in Fig. 2.
In some instances, as when attempts were made to use P-enolpyruvate as the phosphorl donor in the hydroxylamine kinase reaction, phosphorylated hydroxylamine was estimated by the method originally devised by Flynn, Jones, and Lipmann (18) for the determination of PPi. In this procedure, cysteine is added to the usual reagents for Pi determination and the increase of pyruvate kinase concentration.

In the usual reaction mixture containing hydroxylamine and Zn++ ions at pH 7.2: formate, acetate, malonate, succinate, malate, sulfate, thiosulfate, sulfamate, and glycolate. Kupieccl and Coon (7) had previously shown that glycine, histidine, leucine, and silicate are inactive when substituted for bicarbonate. Thus the Zn++-activated reaction is highly specific for both hydroxylamine as the phosphate acceptor and for bicarbonate as the other required component. Pyruvate kinase apparently possesses a slight but significant ATPase activity, as noted by the liberation of P_i when hydroxylamine was omitted from the usual reaction mixture. The activity observed under such conditions, or when manganese ions were substituted for zinc ions, led to the liberation of approximately 0.11 pmole of P_i in 30 min in the presence of 1.1 mg of enzyme.

The specificity of the hydroxylamine kinase reaction for metal ions has been described elsewhere (7). The absence of tightly bound divalent metals from pyruvate kinase was shown by dialyzing a crystalline preparation of the enzyme and subjecting a lyophilized sample to neutron activation analysis. Sodium ions were detected, but no other metals. We are indebted to Mr. P. LaFleur, who carried out this analysis with the University of Michigan Ford nuclear reactor.

Although ITP and UTP exhibit activity in the hydroxylamine kinase reaction, they are somewhat less effective than ATP (7). However, when 10^{-3} M P-enolpyruvate was substituted for ATP in the hydroxylamine kinase assay system containing 4 \times 10^{-3} M zinc or magnesium ions, no reaction occurred as judged by measurements of P-enolpyruvate disappearance by a decrease in absorbance at 240 nm (19), pyruvate formation by the dinitrophenylhydrazine method (20) or spectral method (13), or the formation of phosphorylhydroxylamine by a colorimetric method which does not detect P-enolpyruvate (18). A possible explanation of the failure of P-enolpyruvate to phosphorylate hydroxylamine is that a site on the enzyme is capable of binding P-enolpyruvate, or hydroxylamine plus bicarbonate, but not both at the same time. Such an explanation would be in accord with the finding of Reynard et al. (21) that this enzyme possesses a binding site for ATP or ADP and a distinct site for P-enolpyruvate or pyruvate and with the observation in this laboratory that hydroxylamine kinase activity is inhibited by the presence of pyruvate or P-enolpyruvate (8). Kinetic evidence bearing on this question is presented elsewhere in this paper.

Michaelis Constants for Hydroxylamine Kinase Reaction—As shown previously, no phosphorylated hydroxylamine is formed from ATP and hydroxylamine in the absence of bicarbonate, Zn^{++} ions, or pyruvate kinase (7). The apparent Michaelis constants, determined from the Lineweaver-Burk plots shown in Fig. 3, were found to be as follows: hydroxylamine, 2.4 \times 10^{-3} M; bicarbonate, 1.9 \times 10^{-2} M; and ATP, 1.7 \times 10^{-3} M. In each instance the K_m value was determined with the other components at the standard concentrations described under “Methods.” In the usual assay ATP is used at the minimal saturating level, 3.3 \times 10^{-3} M, because higher levels are inhibitory. As a result ATP becomes a rate-limiting component when high levels of enzyme are used or when the incubation is carried out for long periods of time. The data from which the K_m of ATP was determined do not include those from experiments with inhibitory levels. Since Michaelis-Menten kinetics were not obtained with Zn^{++}, the K_m has not been calculated. The minimal saturating concentration of zinc acetate is 1.0 \times 10^{-3} M.

Identification of Phosphorylated Hydroxylamine—Attempts to isolate the product of the hydroxylamine kinase reaction as the...
free compound by lyophilization or as a metal salt were unsuccessful because of decomposition. However, the compound was purified chromatographically and identified as O-phosphorylhydroxylamine by several lines of evidence. It may be noted that Jencks and Gilchrist (14) were similarly unable to isolate chemically prepared O-phosphorylhydroxylamine because of its instability. The product present in a typical enzyme reaction mixture was purified by chromatography on Dowex 1, and aliquots were analyzed to determine the content of Pi; (17) and hydroxylamine (22) after hydrolysis in 0.5 N sulfuric acid for 10 min at 105°. The molar ratio of acid-labile phosphate to acid-labile hydroxylamine was found to be 1.02; in contrast, only traces of these materials were present before acid hydrolysis. The most likely compounds which might give such analytical results are the O- and N-monophosphoryl derivatives of hydroxylamine, and these two possibilities were distinguished by determining the acid lability and reactivity with furfural. The rates of hydrolysis of the enzymatic product and of authentic O-phosphorylhydroxylamine were determined at 38° in 0.5 N sulfuric acid. With each compound the reaction was shown to follow first order kinetics, and the unknown and authentic compounds exhibited similar rate constants (0.0004 and 0.0005 min⁻¹, respectively) with half-times of about 105 min. These results suggest that the linkage is P-O-N rather than P-N-O, since P-N compounds are much more labile to acid; for example, phosphoramidate has a half-life of only about 2 min under the same conditions.

Additional evidence supporting the O- rather than N-phosphoryl linkage was obtained by showing that upon reaction with furfural at pH 4.5 in acetate buffer the enzymatically synthesized compound and the known O-phosphorylhydroxylamine gave the same product, as judged by the ultraviolet spectrum. This procedure (14) detects the unsubstituted amino group by the formation of the Schiff base. Under the conditions used, the products formed exhibited maximum absorption at 273 to 274 nm, and the molar extinction coefficients were not significantly different (1.67 and 1.65 x 10⁴ M⁻¹ cm⁻¹ for the enzymatically produced and the authentic O-phosphorylhydroxylamine, respectively). Finally, the activities of the various methyl derivatives of hydroxylamine suggested that the hydroxyl group, rather than the amino, undergoes phosphorylation. The data in Table I indicate that N-methylhydroxylamine is extensively phosphorylated and that the N,N-dimethyl derivative is attacked much more slowly, but nevertheless at a significant rate. On the other hand, O-methyl- and O,N-dimethylhydroxylamine exhibited no activity. Similar results were obtained when the concentration of each of the compounds was doubled. It should be noted that no significant amount of Pi was detected in any of these experiments prior to acid hydrolysis, thus ruling out the possibility of phospho- 

**Table I**

| Substrate tested | Amount of phosphorylated product formed
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂OH</td>
<td>2.74</td>
</tr>
<tr>
<td>CH₂NH₂OH</td>
<td>1.62</td>
</tr>
<tr>
<td>(CH₃)₂NOH</td>
<td>0.19</td>
</tr>
<tr>
<td>NH₂OCH₃</td>
<td>0</td>
</tr>
<tr>
<td>CH₂NHOCH₃</td>
<td>0</td>
</tr>
</tbody>
</table>

*Total Pi detected after acid hydrolysis.

**Possible Role of Hydroxycarbamate**—By analogy to the reaction of bicarbonate and ammonia to give carbamate in aqueous solution (24), one might expect hydroxylamine and bicarbonate to give N-hydroxycarbamate. A modification of a published method (25) was used to detect the carbonate in the presence of bicarbonate. Reaction mixtures containing 1 M ¹⁴C-labeled ammonium bicarbonate or ¹⁴C-labeled potassium bicarbonate and neutral hydroxylamine in a final volume of 1.0 ml were incubated for 30 min at 38°. The solutions were chilled, excess KOH was then added to stabilize the mixture, and the carbonate was precipitated with BaCl₂ and removed by filtration at 0°. A determination of the radioactivity in the clear filtrate indicated that 10 to 30 μmoles of non-barium-precipitable material were present, whereas no significant radioactivity was found in the filtrate when ammonia or hydroxylamine was omitted from the reaction mixture. Similar experiments indicate that, under the conditions used, 1 to 3% of the bicarbonate may in the presence of ammonia or hydroxylamine be converted to the corresponding carbonate, and suggest that hydroxycarbamate might be the actual substrate phosphorylated in the reaction under study. Attempts were then made to detect the predicted phosphorylation product, which would be O-phosphorylhydroxycarbamate. A typical hydroxylamine kinase reaction mixture containing ¹⁴C-bicarbonate was incubated with pyruvate kinase and then submitted to chromatography on a column of Dowex 1 with the results shown in Fig. 4. The traces of radioactivity detected in the peak containing phosphorylhydroxylamine apparently represent only the tail of the bicarbonate peak. If hydroxycarbamate were the substrate, on the other hand, one would expect to find an equimolar amount of radioactive bicarbonate in the phosphate-containing product. Thus it appears that hydroxylamine, rather than hydroxycarbamate, is the actual substrate phosphorylated. However, this conclusion is based on the assumption that phosphorylhydroxycarbamate would be sufficiently stable to survive the experimental conditions used.

**Kinetic Studies**—Since the evidence so far accumulated pro-

![Fig. 3. Lineweaver-Burk plots for ATP, hydroxylamine, and bicarbonate in the hydroxylamine kinase reaction. The velocity is expressed as micromoles of phosphorylhydroxylamine formed in 30 min in the presence of 51 μg of pyruvate kinase.](http://www.jbc.org/issue/163/16/1633)
Fig. 4. Attempt to detect O-phosphorylhydroxycarbamate as a possible product of the hydroxylamine kinase reaction. A reaction mixture containing 50 μg of pyruvate kinase, 500 μmoles of 14C-bicarbonate (50 PC), and the other usual components was incubated for 3 hours. The solution was then diluted to an ionic strength of 0.01 and the pH was adjusted to 7.5 with 0.01 M Tris buffer before the mixture was submitted to chromatography at 4°C on a column (1 X 10 cm) of Dowex 1.

Fig. 5. Lineweaver-Burk plot showing the effect of bicarbonate on the initial velocity of the Mn++-activated pyruvate kinase reaction at various P-enolpyruvate concentrations. In this and subsequent figures the molarity of the inhibitor in the reaction mixtures is indicated in parentheses.

Fig. 6. Effect of bicarbonate on the initial velocity of the Mg++-activated pyruvate kinase reaction at various P-enolpyruvate concentrations. The potassium ion concentration in these experiments was 0.20 M.

Fig. 7. Effect of NH₄OH on the initial velocity of the Mn++-activated pyruvate kinase reaction at various P-enolpyruvate concentrations.

Fig. 8. Effect of NH₄OH on the initial velocity of the Mg++-activated pyruvate kinase reaction at various P-enolpyruvate concentrations.

Fig. 9. Effect of bicarbonate and NH₄OH on the initial velocity of the Mn++-activated pyruvate kinase reaction at various P-enolpyruvate concentrations. The molarity of each inhibitor in the reaction mixture is noted in parentheses.
separate series of experiments using bicarbonate, Mn\(^{++}\) or Mg\(^{++}\) ions, and varying ADP concentrations, no significant inhibitory effects were seen, and the results are therefore not presented. Thus it appears that bicarbonate is bound at the same site as P-enolpyruvate but apparently does not interact appreciably with the ADP-binding site.

The double reciprocal plot in Fig. 7 indicates that in the presence of Mn\(^{++}\) ions hydroxylamine gave mixed inhibition kinetics with respect to P-enolpyruvate, whereas with Mg\(^{++}\) ions competitive inhibition was observed (Fig. 8). On the other hand, no significant effects were noted with either Mn or Mg when the inhibition by hydroxylamine was studied at varying ADP concentrations. When both bicarbonate and hydroxylamine were added (in equimolar amounts), the results indicated mixed inhibition with respect to P-enolpyruvate when either Mn\(^{++}\) or Mg\(^{++}\) ions were present (Figs. 9 and 10). Again, in similar experiments in which 10\(^{-3}\) M P-enolpyruvate was used and the ADP concentration was varied with either Mn or Mg present, the mixture of bicarbonate and hydroxylamine showed no special inhibitory effects. It is therefore concluded that the P-enolpyruvate-binding site is capable of binding both hydroxylamine and bicarbonate, whereas the ADP-binding site is not. Only a limited number of kinetic experiments were carried out with fluoride as a possible inhibitor. As shown in Fig. 11, this substance, like hydroxylamine, gives mixed inhibition with respect to P-enolpyruvate in the presence of Mn\(^{++}\) ions.

Evidence already presented suggests that the formation of hydroxylaminocarbamate from hydroxylamine and bicarbonate is not an essential feature of the hydroxylamine kinase reaction. Data presented in Table II, the combined addition of bicarbonate and fluoride also gave an extent of inhibition not significantly different from the expected additive effects.

**TABLE II**

Comparison of pyruvate kinase inhibitors

The usual conditions were used with 4 \(\times\) 10\(^{-4}\) M Mn\(^{++}\) as the divalent metal.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor concentration</th>
<th>P-enolpyruvate concentration</th>
<th>Observed inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate</td>
<td>0.03</td>
<td>1 (\times) 10(^{-4})</td>
<td>17</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.03</td>
<td>1 (\times) 10(^{-4})</td>
<td>19</td>
</tr>
<tr>
<td>Both</td>
<td>0.03 each</td>
<td>1 (\times) 10(^{-4})</td>
<td>34</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.03</td>
<td>1 (\times) 10(^{-3})</td>
<td>5</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.03</td>
<td>1 (\times) 10(^{-3})</td>
<td>20</td>
</tr>
<tr>
<td>Both</td>
<td>0.03 each</td>
<td>1 (\times) 10(^{-3})</td>
<td>28</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.10</td>
<td>1 (\times) 10(^{-3})</td>
<td>37(^{+})</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.10</td>
<td>1 (\times) 10(^{-3})</td>
<td>4(^{+})</td>
</tr>
<tr>
<td>Both</td>
<td>0.10 each</td>
<td>1 (\times) 10(^{-3})</td>
<td>43(^{+})</td>
</tr>
</tbody>
</table>

\(^{+}\) The final potassium ion concentration was 0.2 M.

indicating that the inhibitory effects of hydroxylamine and bicarbonate on the pyruvate kinase reaction are additive and are presented in Table II. Thus, at either of the P-enolpyruvate concentrations used, there is no indication that a significantly more effective inhibitor is produced when hydroxylamine and bicarbonate are added together. In other experiments not shown here it was found that the preincubation of a mixture of these two compounds for as long as 1 hour before addition to the enzyme reaction mixture had no effect on the extent of inhibition. As indicated in Table II, the combined addition of bicarbonate and fluoride also gave an extent of inhibition not significantly different from the expected additive effects.

**DISCUSSION**

The identification of the non-nucleotide product of the hydroxylamine kinase reaction is based on the observations that it contains equimolar amounts of acid-labile phosphate and hydroxylamine and that it reacts with furfural and undergoes acid hydrolysis in the same manner as the known O-phosphoryl compound prepared in this laboratory as described by Jencks and Gilchrist (14). The possibility should be considered that N-phosphorylhydroxylamine may be the initial product formed, and that it is somehow protected on the enzyme surface from hydrolysis but rearranges, either spontaneously or enzymatically, to give the O derivative. However, the finding that N-methyl- and N,N-dimethylhydroxylamine are phosphorylated, whereas O-methylhydroxylamine is not, suggests that the phosphorylation occurs directly on the oxygen atom of hydroxylamine.

The kinetic data of Reynard et al. (21) indicate that one site of pyruvate kinase binds pyruvate or P-enolpyruvate and another site ADP or ATP, with an apparent overlap of the transferenceable phosphate groups of P-enolpyruvate and ATP. Our earlier finding that both P-enolpyruvate and pyruvate inhibit the hydroxylamine kinase reaction (8) led us to consider the possibility that bicarbonate and hydroxylamine are bound at the same site as these 3-carbon acids. The data in the present paper on the inhibition of pyruvate kinase by hydroxylamine, bicarbonate, or both in the presence of Mg\(^{++}\) or Mn\(^{++}\) ions are in accord with this proposal. Bicarbonate gave kinetics indicating competitive inhibition with respect to P-enolpyruvate, and bicarbonate plus NH\(_2\)OH gave mixed inhibition kinetics with respect to P-enol-
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pyruvate, whereas no significant inhibitory kinetic effects were found with respect to ADP. The results obtained with bicarbonate suggest that this compound may occupy the same location on the enzyme as the carboxyl group of P-enolpyruvate, as has been proposed by Boyer (27). The normal bicarbonate concentration of rat skeletal muscle (28) and of the plasma of vertebrates (29) is somewhat greater than 0.02 mM. Assuming a similar value for rabbit muscle, it appears likely that bicarbonate may significantly inhibit pyruvate kinase under physiological conditions, thus providing a control mechanism which would help to account for the Pasteur effect.

The kinetic data presented and the observation that P-enolpyruvate does not phosphorylate hydroxylamine when substituted for ATP suggest that this compound may occupy the same location on the enzyme as the carboxyl group of P-enolpyruvate, as has been proposed by Boyer (27). The normal bicarbonate concentration of rat skeletal muscle (28) and of the plasma of vertebrates (29) is somewhat greater than 0.02 mM. Assuming a similar value for rabbit muscle, it appears likely that bicarbonate may significantly inhibit pyruvate kinase under physiological conditions, thus providing a control mechanism which would help to account for the Pasteur effect.

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