The Proton Transfer Reactions of Muscle Pyruvate Kinase*

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SUMMARY

Rabbit muscle pyruvate kinase detritiates phosphoenolpyruvate 3-tritium under conditions of the net forward reaction prior to the release of pyruvate. This exchange requires that release of pyruvate is not rapid with respect to reversal of the proton transfer steps involved in the catalysis of its enolization. This conclusion is consistent with the low tritium isotope effect that is observed for the hydrogen exchange on pyruvate that is activated by ATP. An intrinsic isotope effect for the proton abstraction step as high as 26 can be expected since such high values were observed in the enzymatic enolization of pyruvate activated by other phosphate compounds. Considerable exchange of tritium from tritiated water into remaining phosphoenolpyruvate during the course of the forward pyruvate kinase reaction is observed, indicating that the enzyme-bound pyruvate-ATP complex that is generated in the forward reaction can return to substrate form at a significant rate relative to product release. A kinetic analysis indicates that the release of phosphoenolpyruvate and ADP may be a rate-determining factor in this exchange rate. Thus, the conclusions of earlier workers that muscle pyruvate kinase follows rapid equilibrium kinetics require re-examination. The effects of varying pH, mono- and divalent cations, temperature, and alternative activators on the rates of proton exchange are reported.

The over-all reaction catalyzed by pyruvate kinase consists in the transfer of the phosphoryl group of phosphoenolpyruvate to ATP and the transfer of a proton from water to form the methyl group of pyruvate. This dual functionality is illustrated by the following proposed reaction sequence in which enolpyruvate is considered to be an enzyme-bound intermediate.

Previous work has established that these two functions can be made independent of each other. Pyruvate kinase transfers the phosphoryl from ATP to fluoride (1) or hydroxylamine (2), and pyruvate kinase enolizes pyruvate in the presence of compounds other than ATP, such as P_i, without phosphoryl transfer (3).

The present paper concerns the path that hydrogen follows in going from the medium to the —CH_3 of pyruvate. It has been shown that this hydrogen is introduced stereospecifically from the 2-s; face of C-3 (4-6), but the fact that this proton becomes equivalent to the other two hydrogens of the methyl group by rotation at the C_2-C_3 bond, Sequence 2, makes possible the monitoring of paths and rates of proton transfer by determining the subsequent loss of hydrogen derived from PEP to the medium.

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1 The abbreviation used is: PEP, phosphoenolpyruvate.
Experimental Procedure

Reagents—Pyruvate kinase (rabbit muscle) and lactate dehydrogenase (rabbit muscle) were purchased from Boehringer Mannheim Corp. or Calbiochem. The pyruvate kinase preparations had a specific activity of 210 units per mg of protein and 1 unit of enzyme-catalyzed formation of 1 mole of product per min under the following standard conditions: 100 mM N-tris-(hydroxymethyl)methyl aminoethanol sulfate (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 2 mM ADP, 1 mM PEP, 0.1 mM DPNH, and lactate dehydrogenase. [3-T]Pyruvate was the gift of Dr. P. Melochu. [3-T]PEP was prepared from [3-T]pyruvate with PEP synthase as follows. A reaction mixture contained the following in a volume of 6 ml: 300 μmoles of triethanolamine, pH 7.5; 120 μmoles of MgCl₂; 60 μmoles of sodium [3-T]pyruvate; 3 × 10⁶ cpm per μmole; 90 μmoles of ATP; and 5 units of PEP synthase (7). After 1 hour at 26°, the reaction mixture was diluted to 120 ml, placed on a Dowex 1 (Cl⁻) column (20 × 1.7 cm), which was then washed with water and 0.02 M HCl. The [3-T]PEP was eluted with 0.04 M HCl (7), pooled, neutralized with 2 M NaOH, and stored frozen. The best preparations of [3-T]PEP contained less than 1% pyruvate and less than 0.2% volatile counts; greater than 98% of the counts could be converted by pyruvate kinase to pyruvate and water. Treatment of the [3-T]PEP with acid-washed charcoal removed ADP which eluted from the column with the PEP. To desalt the PEP, it was placed on a G-10 column (2 × 31 cm) equilibrated with 5 mM Tris (pH 7.5) and the PEP was eluted with the same buffer. Methylphosphonate, prepared according to the method of Nichol et al. (8), was supplied by Dr. A. S. Hampton, and fluorophosphate, purified according to the method of Mildvan et al. (9), was provided by Dr. A. S. Mildvan. Z-Phosphoenol-ketobutyrate (6) was the generous gift of Dr. D. B. Sprenger. All other chemicals were obtained commercially and used without further purification, except for TOH and D₂O, which are re-distilled before use. Values for pD are those of the measured "pH" + 0.4 (10).

Primary Hydrogen Isotope Effect in Pyruvate Enolization.—A comparison was made of the rates of exchange of proton and tritium from [3-T]pyruvate with deuterium in D₂O in a single reaction mixture. This approach is free of complications from secondary isotope effects on the rate of bond cleavage since tritium is present only in tracer quantity and D₂O is a common medium. Proton exchange was followed on the Varian HA-100-15 nuclear magnetic resonance spectrometer, as the decrease in relative areas of the pyruvate methyl group signal (2.4 ppm) to the methane peak (3.6 ppm) of Tris. Tritium exchange was followed as TOH production by transferring 0.05 ml aliquots at various times to 0.95 ml of 0.1 M HCl, removing 0.1 ml of this mixture to a distilling tube containing 0.4 ml of 0.025 M NaOH citrate, and upon distillation under reduced pressure, counting the entire distillate. Both measurements were made at a minimum of five time points with each reaction mixture and both gave linear first order plots. Calculation of exchange rates from the three equivalent positions of the pyruvate methyl group was by the equation: 

\[ r_\text{ex} = -\frac{d[\text{pyruvate}]}{dt} \times \text{mg enzyme}^{-1} \times \text{ln}(1 - \text{fraction exchanged}). \]

Partition of Tritium from [3-T]PEP between Pyruvate and Water.—In these experiments, [3-T]PEP and an excess of ADP (or other phosphoryl acceptor) were reacted with pyruvate kinase and a lactate dehydrogenase trap for the pyruvate. The reaction was followed to completion spectrophotometrically by the change in A₃₄₀ due to DPNH oxidation. All experiments were conducted at 26° unless otherwise stated. Tritium released into the solvent was determined by counting the distillate from an 0.1 ml aliquot added to 0.4 ml of 0.1 M sodium citrate, pH 8. The citrate was added to minimize the volatility of lactate acid. No back reaction of free pyruvate, which would release tritium into the solvent, would be observed under these conditions because of the lactate dehydrogenase trap and the high Kₘ for pyruvate in the back reaction (3). No effect on the partition was observed upon increasing the concentration of lactate dehydrogenase or upon simultaneous removal of ATP by reaction with glucose and hexokinase.

Partition of Tritium from Water between PEP or Pyruvate.—In these experiments, PEP and ADP (at half the PEP concentration) were reacted in TOH with pyruvate kinase and a lactate dehydrogenase trap. When the reaction was complete with respect to ADP, the reaction mixtures were frozen, the TOH removed by sublimation, the residue dissolved in 50 ml of H₂O and placed on a Dowex 1 (Cl⁻) column (5 × 1.7 cm), and after a water wash, the [3-T]lactate was eluted with 0.005 M HCl and the [3-T]PEP with 0.5 M NH₄Cl, according to the method of Bartlett (11). Concentrations of lactate and PEP in appropriate fractions were determined on aliquots by enzymatic procedures (12) and identical aliquots were counted for radioactivity to get specific activities. Similar column chromatography and analysis of lactate derived from [3-T]PEP as a function of percentage reaction was used to access any secondary tritium isotope effect in the pyruvate kinase reaction.

Results

In a previous study, enolization of pyruvate was found to require the presence of all the components of the net reaction, K⁺, Mg²⁺, and ATP, with the notable exception that ATP could be replaced by a number of simple diatomic ions such as P₃ and monooester phosphates (3). It was considered of interest to ask whether [3-T]PEP was detritiated by the enzyme under conditions that were either incomplete for carrying out the net reaction or, if complete, the formed pyruvate was trapped with lactate dehydrogenase to prevent its further reaction. This question is of interest for several reasons. The possibility that protonation might be able to precede phosphoryl transfer (Reaction 4) is suggested in analogy to the mechanism for Hg²⁺-catalyzed hydrolysis of PEP suggested by Benkovic and Schray (13), in which a proton replaces Hg²⁺.

\[
\text{Hg}^{2+} + \text{PEP} \rightarrow \text{Hg}^{2+} + \text{P} + \text{H}^+.
\]

By reversal of the first step, in a reaction with randomized hydrogens of the methyl group, it would be possible to obtain exchange of tritium from PEP. A second route for detritiation of [3-T]PEP would be provided if the enzyme itself were capable of accepting the phosphoryl group of PEP, then subsequent ketonization and its reversal would result in tritium exchange, Reaction 5.
Previous experiments by Harrison et al. (14), in which the possibility of a phosphoryl-enzyme intermediate was eliminated using the test of pyruvate to PEP exchange is more demanding of the mechanism than the tritium exchange test since the latter does not require that free pyruvate be formed from PEP in the absence of ADP.

Conditions necessary for the detritiation of [3-T]PEP are shown in Table I. A trapping system for pyruvate (lactate dehydrogenase and DPNH) was included to prevent net reversal of pyruvate formed in the complete system. It is observed that under specific conditions of pyruvate formation a significant portion of the tritium of the substrate was exchanged for protons of the medium. With an amount of enzyme capable of completing the net reaction in only one-tenth the incubation time used, little or no detritiation occurred if any one of the components required for net reaction was omitted. In addition, replacement of K+ by tetramethylammonium, and replacement of ADP by AMP, AMP + Pi, or AMP + NO3 did not lead to hydrogen exchange. Since the formation of pyruvate from PEP is known to be stereospecific (4), any route for exchange requires protonation, randomization of hydrogen by rotation around the C5-C6 bond and proton abstraction. Hence, one can conclude that one or more of these steps depends on phosphoryl transfer to ADP. As evidence that loss of tritium from PEP occurs only during the course of the net reaction, Fig. 1 shows a correlation of detritiation with the formation of pyruvate. Reaction Scheme 3 provides an explanation for the detritiation of PEP during the course of the net reaction. The exchange requires only that Step 4 is not rapid compared with one or more of the paths connecting E with medium protons.

The loss of tritium from [3-T]PEP may greatly underestimate the amount of proton exchange that is occurring if Step 3 of Scheme 3 is rate-determining for the exchange process. To evaluate the contribution that an isotope effect would make, the kinetic isotope effect for hydrogen exchange from pyruvate was measured. In order to do this, a mixture of [3-1H]pyruvate and [3-T]pyruvate (trace) was subjected to pyruvate kinase under exchange conditions in a medium of D2O. The rates of loss of the proton signal of pyruvate and of the appearance of tritium in water were compared with either ATP or an ATP analog to activate the exchange. Three analogs were chosen for their isoelcetronic character, but wide range of basicity. Thus, the pK values of Pi are 2.1, 6.7, and 11.8 (15), those of methylphosphonate, 2.4 and 7.7 (16), and those of fluorophosphate, 0.6 and 4.8 (17).

Table I documents the isotope effects observed in the enolization and the rates of tritium exchange. The isotopic effect seen with ATP as the activator is quite small, δ2P:δ2T = 2.5 to 4, compared with 15 to 25 observed with the analogs. Apparently the C—H bond-breaking step determines the rate of hydrogen exchange with the analog, but may not do so with ATP. That the same step might not be rate determining with ATP and the analogs was suggested by earlier data (3) in which the pH dependence of the enolization process with Pi; and the other analogs followed the pK of the activator, whereas with ATP the rate increased sharply in the region above pH 7.5 where ATP s fully ionized.

### Table I

**Detritiation of [3-T]PEP by pyruvate kinase**

The complete reaction mixture contained 100 mm in tetramethylammonium salt of the buffer, N-tris(hydroxymethyl)methyl-2-aminoisopropyl sulfonate (pH 6.9), 100 mm KCl, 2 mm ADP, 5 mm MgCl2, 1 mm EDTA, 0.1 mm [3-T]PEP (10 cpm), 0.15 mm DPNH, 0.1 unit of lactate dehydrogenase, and 0.1 unit of pyruvate kinase (total volume, 1 ml). Additions made where noted were 5 mm AMP, 25 mm Na2HPO4, 50 mm NaNO3, and 100 mm tetramethylammonium Cl (TMA Cl). Formation of pyruvate was followed at 340 nm. After 10 min, 0.2 ml of each reaction mixture was added to 0.3 ml of 0.1 M sodium citrate (pH 6) and following distillation, volatile counts determined. The complete reaction had terminated in 2 min and no detectable reaction was observed in the other cases. Extensive dialysis of the enzymes was necessary to show no reaction in the absence of KCl.

| Conditions             | Percentage of volatile counts |
|------------------------|-------------------------------|
| Nonenzymatic control   | 2.5                           |
| Complete               | 18.2                          |
| -Mg2+                  | 2.4                           |
| -KCl                   | 3.1                           |
| -KCl | TMA Cl                  | 2.7                           |
| -ADP                   | 2.8                           |
| -ADP + AMP             | 2.5                           |
| -ADP + Pi              | 2.4                           |
| -ADP + AMP + NO3       | 2.4                           |
| -ADP + AMP + Pi        | 2.6                           |

![Fig. 1. Extent of detritiation of [3-T]PEP as a function of extent of reaction](http://www.jbc.org/)
Isotope effect in pyruvate kinase-catalyzed enolization of pyruvate

All activators were present as potassium salts and, to maintain similar ionic strength, 300 mm KCl was added to the ATP reaction mixtures. Besides the above, each reaction mixture contained: 50 mm Tris-Cl; 5 mm MgCl₂; 100 mm potassium [3-T]pyruvate (2 x 10⁶ cpm per pmole); and 0.1 mg of pyruvate kinase in a volume of 1.1 ml of D₂O. Reaction temperature was 32°C.

| Activator          | pH | v₉H | v₉T | v₉H/v₉T |
|--------------------|----|-----|-----|---------|
| ATP, 2 mm          | 9.1| 96.0|38.4 | 2.5     |
| Pₐ, 100 mm         | 9.0| 25.6|1.57 | 15.0    |
| Methylphosphonate, 100 mm | 9.1| 25.1|0.57 | 25.9    |
| Fluorophosphate, 100 mm | 8.8| 3.6 |0.18 | 20.2    |
| ATP, 2 mm          | 7.5| 8.6 |2.14 | 4.0     |
| Pₐ, 100 mm         | 7.7| 24.7|1.47 | 16.8    |
| Fluorophosphate, 100 mm | 6.5| 7.1 |0.5 | 14.1    |
| Methylphosphonate, 100 mm | 7.8| 11.9|0.71 | 16.8    |
| Fluorophosphate, 100 mm | 7.7| 3.3 |0.14 | 23.7    |

In the enolization experiments, the partition of the enzyme among the various intermediate forms is probably close to the equilibrium distribution during most of the period that measurements were made. The equation that describes the exchange rate for hydrogen is

\[ v_{H} = \frac{k_{H} \cdot E_{eq}}{k_{H} + k_{T}} \]

where \( E_{eq} \) is the equilibrium concentration of the enzyme-pyruvate-ATP complex that partitions between proton exchange (\( k_{H} \)) and product dissociation (\( k_{T} \)). The rate constant for proton exchange is made up of rate constants for all the exchange paths. This equation and the full expression for \( E_{eq} \) can readily be derived using the approach of Yagil and Hoberman (18). In the corresponding expression for \( v_{T} \), \( k_{H} \) would replace \( k_{T} \) in the derivation of \( k_{H} \).

In Scheme 3, Step 3 is itself complex in that it includes rotation at C₂-C₃ of bound pyruvate as well as proton abstraction, which may or may not involve a basic group on the enzyme. Likewise, \( k_{T} \) may be a complex constant in which stepwise dissociations of ATP and pyruvate are included. These extensions alter neither the form of Equation 1, nor the central importance of \( E_{eq} \) in the partitioning between proton exchange and product dissociation. A small isotope effect in pyruvate enolization may result from either a slow product dissociation relative to proton exchange (i.e. \( k_{H} < k_{H} \)) or from a non-rate-limiting dissociation of proton from the base on the enzyme (10). Attempting to find conditions where, with ATP, the C—H bond is broken in the relatively slow step, the isotope effect was determined with either Co³⁺ or Mn²⁺ replacing Mg²⁺. Isotope effects (\( v_{H}/v_{T} \)) of 1.5 and 2.2 were observed for Co³⁺ and Mn²⁺ at pH 7.7, respectively, where rates of tritium exchange were 17 and 90.3 pmol per mg of protein. The C—H bond-breaking step does not appear rate limiting under these conditions.

The alternative route for generating the ternary products complex (\( E_{3} \)) from tritiated PEP allows one to measure directly the ratio \( k_{H} \):\( k_{T} \) from a determination of the ratio, called \( R_{H} \), of tritium in the water to that in the pyruvate in the over-all forward reactions, as in Table I. Determinations of \( R_{H} \) were made at several pH values and with Mg²⁺, Mn²⁺, or Co³⁺, as the required divalent cation. The forward velocity (\( V_{for} \)) and the extrapolated maximum rate of tritium exchange from pyruvate with ATP as activator were determined under comparable conditions. The necessary monovalent cation was K⁺, present at its optimal concentration. Table III lists values for \( R_{H} \), \( V_{for} \), and \( v_{H} \), interpolated to a common pH value of 8. The interpolation was necessary because each of these values was found to be sensitive to pH and the variations were dissimilar for each divalent ion (see Figs. 2 and 4).

It will be noted that \( R_{H} \), the partition of tritium of PEP between water and pyruvate, varies considerably with divalent metal ion. At this pH (and at all others examined, see Fig. 3), \( R_{H} \) with Co³⁺ is greater than with Mn²⁺, which is greater than with Mg²⁺. High values of proton exchange prior to product release in the forward reaction should lead to a low isotope effect in ATP-dependent enolization of pyruvate according to Equation 6. Accordingly, the high \( R_{H} \) values observed with Co³⁺ and Mn²⁺ are consistent with the small isotope effects observed in the corresponding enolization reactions. The low \( R_{H} \) value with Mg²⁺ is coupled, however, with an isotope effect in the ATP-dependent enolization that is small relative to the tritium isotope effect of 20 to 25 in the enolization activated by compounds other than ATP. These observations suggest that another step, such as proton release from the enzyme, may be rate limiting in the ATP-dependent enolization and may be responsible for the low \( R_{H} \).

A further deduction can be made from the data in Table III. From Equation 6 for tritium, \( V_{for} = (k_{H} \cdot k_{H}/k_{H} + k_{T}) \cdot E_{eq} \), and from the definition of \( R_{H} = (k_{H} \cdot k_{H}/k_{T}) \), one can determine
that $k_{\text{f}}E_{\text{eqq}}$ equals $V_{\text{at}}(1 + R_T)/R_T$. This value can be compared with $k_{\text{f}}E_{\text{eqq}}$, which is the net maximal forward steady state velocity under initial rate conditions, $V_{\text{for}}$. Accordingly, the ratio of $E_{\text{eqq}}:E_{\text{eqa}}$ equals $V_{\text{at}}(1 + R_T)/R_T - V_{\text{for}}$, all terms of which are determined under comparable conditions at pH 8 (Table III). When both rates are determined at saturation, $E_{\text{eqa}}:E_{\text{eqa}}$ must be equal to or greater than unity unless the differing conditions for measuring $V_{\text{at}}$ and $V_{\text{for}}$ introduce inhibitory factors. To the extent that product release is rate determining for the forward reaction, $E_{\text{eqa}}$ should equal $E_{\text{eqq}}$ since the preliminary equilibrium of all enzyme complexes should partition the enzyme in the same way as under the equilibrium conditions used to measure enolization at saturating concentration of ATP and pyruvate. As indicated in Table III, the ratio $E_{\text{eqa}}:E_{\text{eqq}}$ equals 1.08 for Mg$^{2+}$, 3.07 for Mn$^{2+}$, and 1.09 for Co$^{2+}$. These values are close to the limiting value of 1; this implies, barring some inhibitory factors, that Step 4 must be rate limiting in the forward direction, especially with Mg$^{2+}$ and Co$^{2+}$.

A further test that product dissociation is rate determining, not only in pyruvate enolization (one of the explanations for the low isotope effect with ATP), but also in the net forward reaction (as indicated by the similarity in $E_{\text{eqa}}$ and $E_{\text{eqq}}$), consists in measuring the total reversal of $E_{\text{eqa}}$ to free PEP. This measurement of the appearance of tritium from water into free PEP during the forward reaction was made in the presence of lactate dehydrogenase and DPNH to make free pyruvate unavailable for reversal. The forward reaction was followed in the presence of tritiated water until 50% of the PEP was consumed; when the reaction was terminated, PEP and lactate were isolated, and their specific activities determined. The partition of tritium from water between PEP and pyruvate is designated $PT$ and is a relative measure of $E_{\text{eqq}}$ reversing to free PEP during the forward reaction. In parallel incubations, $R_T$, the partition ratio of tritium from PEP between water and pyruvate, was measured and is a relative measure of $E_{\text{eqa}}$ reversing to $E_{\text{eqa}}$ and whatever other steps are involved in tritium dissociation from the enzyme.

The specific activities of PEP and lactate, the derived value of $R_T$, and the independently measured value of $R_T$ under several conditions are included in Table IV.

It is evident that these values are strongly influenced by pH and divalent cation. The discrimination against tritium incorporation into pyruvate has previously been studied at pH 7 by Simon et al. (20) with a discrimination factor of about 6, as found here also at pH 7.3 with Mg$^{2+}$. However, this is clearly dependent on the choice of conditions since rate limitation in product release is clearly shown with Co$^{2+}$ at pH 8.8 by both the high value of $R_T$ and the specific activity of the lactate which exceeds that of the water. It is also clear that tritium introduced in the ketonization step and retained in the enolization has found its way back to free PEP in rather significant amounts. Thus, with Mg$^{2+}$ at pH 8.5 the reisolated PEP has 26% of the specific activity of the lactate. If complete isotopic equilibration between PEP and water had occurred prior to release of products, this value could have been 66.7% barring an enrichment in tritium. The question of which steps might be rate-limiting for the return of intermediates to PEP will be considered under “Discussion.”

At pH 7.3 with Mg$^{2+}$, the return of $E_{\text{eqa}}$ to free PEP (Py) exceeds the detritiation of $[3\text{-T}]$PEP ($R_T$) by 3-fold. Two processes consistent with Scheme 3 could contribute to this imbalance: (a) an isotope effect would discriminate against tritium in the enolization of pyruvate in Complex $E_3$, and (b) capture of $E-T$ derived from $[3\text{-T}]$PEP by a 2nd molecule of PEP. The isotope effect measured from the discrimination against tritium from the medium in the formation of product is about 6.5 in this experiment, comparing the specific activity of the lactate and water hydrogen. A sensitive test of the second possibility was made possible by the recent reports of Bondinell and Sprinson (5) and Stabbe and Kenyon (6) showing that Z-phosphoenol α-ketobutyrate is an alternate substrate for muscle pyruvate kinase. The second authors (6) reported that phosphoenol-α-ketobutyrate had about the same $K_m$ as PEP, $2.5 \times 10^{-8}$ M, but reacted at only about 0.1% the maximum rate. The following experiment was set up to test for the occurrence of intermolecular tritium transfer, $[3\text{-T}]$Pyruvate (1.8 mM, $1.15 \times 10^{10}$ cpm per mole), Z-phosphoenol-α-ketobutyrate (8 mM), ATP (1.8 mM), ADP (0.15 mM), MgCl$_2$ (4 mM), KC1 (80 mM), and sodium N-tris(hydroxymethyl)methyl-2-aminopropane sulfonate (40 mM) were incubated in 4.4 ml with 1160 units of pyruvate kinase. After 5 hours at 25°, 200 μmoles of HCl were added to convert the α-keto acids to acetate and propionate, and the incubation was diluted 100-fold and passed through a column of Dowex 1 (Cl$^-$(1 x 10 cm). About 16% of the tritium ($1.5 \times 10^{4}$ cpm) were found in the column effluent. These were volatile at alkaline pH and hence represent the exchange with water. Elution with 2 ml HCl brought off 7.2 $\times 10^{4}$ cpm which would represent the acetate and propionate, and elution with 40 ml HCl brought off 255,000 cpm in the region where PEP was expected. Since a comparable sample of the incubation mixture taken at zero time failed to show radioactivity in the PEP region, this latter value represents the tritiated PEP formed in exchange for phosphoenolbutyrate. This is a minimal value since, in spite of the low concentration of formed PEP.

### Table IV

| Conditions* | A | B | C | D |
|-------------|---|---|---|---|
| Specific activities | Mg$^{2+}$, pH | Co$^{2+}$, pH | Mg$^{2+}$, pH | Co$^{2+}$, pH |
| −PEP | $4.21 \times 10^9$ | $4.81 \times 10^9$ | $3.7 \times 10^9$ | $3.45 \times 10^9$ |
| −Lactate | $4.12 \times 10^9$ | $1.82 \times 10^9$ | $1.44 \times 10^9$ | $1.47 \times 10^9$ |
| $P_{\text{T}}$ | 0.154 | 0.039 | 0.394 | 0.149 |
| $R_T$ | 0.063 | 0.117 | 0.464 | 2.21 |

*Each reaction contained 100 mM sodium tris(hydroxymethyl)-methyl aminopropane sulfonate, 100 mM KCl, 5 mM MgCl$_2$ or CoCl$_2$, 1.77 mM ADP, 3.54 mM PEP, 5 mM DPNH, 2 units of lactate dehydrogenase, and 0.1 unit of pyruvate kinase in TOH (2.71 $\times 10^2$ cpm per pmole) in a total volume of 0.5 ml. When the reactions were complete, they were treated as described under “Experimental Procedures.”

$R_T = \frac{TP}{TPEP} - \frac{TP(TOH)}{TP(TOH)}$ (3/2) specific activity of PEP-specific activity of lactate, where (3/2) is the correction factor for T incorporated into PEP, which was subsequently converted into pyruvate.

$R_T = \frac{TP(3\text{-T}PEP)}{TP(3\text{-T}PEP)}$ was determined in identical reaction mixtures except that $3\text{-T}$-PEP (10$^6$ cpm per pmole) was substituted for unlabeled PEP and H$_2$O was substituted for TOH.
relative to phosphoenolpyruvate, the PEP will tend to be reutilized preferentially in view of its 1000-fold higher \( V_{\text{max}} \) (6). A portion of the acetate-propionate mixture was resolved by partition chromatography on silicic acid (21). The acetate had a specific activity of \( 9.1 \times 10^4 \text{ cpm per pmole} \). The propionate, corresponding to 2.4 pmol of the whole incubation, had a specific activity of \( 21.2 \text{ cpm per pmole} \). These counts exceed, by far, the number that could be introduced by way of the tritiated water which would have an average specific activity of 1.6 cpm per pmole of hydrogen. The zero time sample was treated in the same way with 10 pmol of propionate added as carrier. Approximately 550 cpm were recovered in the propionic acid peak of the silicic acid column. This corresponds closely with the experimental result and indicates that no intermolecular tritium transfer occurred.

Although Step 4 may be rate-determining in the forward reaction, the detrition of PEP is far from complete under most conditions. From the fact that only a small tritium isotope effect can be observed in the ATP-dependent enolization of pyruvate, this suggests that the tritium exchange cannot simply occur by Step 3 since this would necessarily impose an isotope effect, but may in fact be limited by dissociation of the pyruvate-derived proton from the enzyme, Steps \( h_1 \) or \( h_2 \). In an attempt to understand the factors that control proton release from the enzyme, a number of studies were undertaken of the sensitivity of \( R_T \) to incubation conditions, and these will be described next.

Using increasing concentrations of urea, no increase was seen in \( R_T \) (Table V). Cottam et al. (22) have previously shown that 2 mM urea dissociates pyruvate kinase from tetramers to dimers, with 68% activity and that the monomers formed in 4 M urea are devoid of activity. The loosening of structure accompanying urea denaturation does not increase proton exchange preferentially. On the contrary, the ratio, \( R_T \), decreases. Furthermore, glycerol, which might favor the interaction of polar groups on the enzyme, raised the relative extent of enolization of bound pyruvate substantially. These two results suggest that it is release of products rather than of proton that is primarily affected by urea and glycerol. If product dissociation is facilitated in urea and attenuated in glycerol, the values of \( R_T \) would show the observed changes.

**Table V**

**Effect of urea and glycerol on partitioning of tritium from PEP between water and pyruvate**

Besides the indicated urea, glycerol, and divalent cation, each reaction mixture contained 100 mM Tris-Cl (pH 7.5), 100 mM KCl, 5 mM MgCl\(_2\) or CoCl\(_2\), 1 mM ADP, 0.01 to 0.1 unit of pyruvate kinase (total volume: 1 ml). Methods as described under “Experimental Procedure.”

| Condition          | \( R_T \) |
|--------------------|-----------|
| 0 mM Urea, 5 mM MgCl\(_2\) | 0.057     |
| 1.0 mM Urea, 5 mM MgCl\(_2\) | 0.043     |
| 1.5 mM Urea, 5 mM MgCl\(_2\) | 0.038     |
| 2.5 mM Urea, 5 mM MgCl\(_2\) | 0.028     |
| 50% Glycerol, 5 mM MgCl\(_2\) | 0.036     |
| 50% Glycerol, 5 mM CoCl\(_2\) | 0.087     |
| 0% Glycerol, 5 mM CoCl\(_2\) | 0.225     |
| 50% Glycerol, 5 mM CoCl\(_2\) | 0.410     |

Phosphoglucone isomerase and ketosteroid isomerase, two proton-transferring enzymes that have protons which slowly exchange with solvent, show an increase in exchange relative to net reaction with an increase in temperature (23, 24). Table VI shows that \( R_T \) for pyruvate kinase with either \( \text{Mg}^{2+} \) or \( \text{Co}^{2+} \) as the activating cation decreases as the temperature is raised. Clearly, the proton exchange with solvent catalyzed by pyruvate kinase diverges from that observed with these other enzymes. An Arrhenius plot yields a \( \Delta H^\circ \) value of -2.8 kcal for \( R_T \) with \( \text{Mg}^{2+} \) and -25 kcal for \( R_T \) with \( \text{Co}^{2+} \).

To further characterize the determinants of \( R_T \), the effects of activating divalent and monovalent cations were examined. Fig. 2 shows the effect on \( R_T \) of three divalent cations most closely related to phosphoenolpyruvate, the PEP tend to be reutilized preferentially in view of its 1000-fold higher \( V_{\text{max}} \) (6). A portion of the acetate-propionate mixture was resolved by partition chromatography on silicic acid (21). The acetate had a specific activity of \( 9.1 \times 10^4 \text{ cpm per pmole} \). The propionate, corresponding to 2.4 pmol of the whole incubation, had a specific activity of \( 21.2 \text{ cpm per pmole} \). These counts exceed, by far, the number that could be introduced by way of the tritiated water which would have an average specific activity of 1.6 cpm per pmole of hydrogen. The zero time sample was treated in the same way with 10 pmol of propionate added as carrier. Approximately 550 cpm were recovered in the propionic acid peak of the silicic acid column. This corresponds closely with the experimental result and indicates that no intermolecular tritium transfer occurred.

Although Step 4 may be rate-determining in the forward reaction, the detrition of PEP is far from complete under most conditions. From the fact that only a small tritium isotope effect can be observed in the ATP-dependent enolization of pyruvate, this suggests that the tritium exchange cannot simply occur by Step 3 since this would necessarily impose an isotope effect, but may in fact be limited by dissociation of the pyruvate-derived proton from the enzyme, Steps \( h_1 \) or \( h_2 \). In an attempt to understand the factors that control proton release from the enzyme, a number of studies were undertaken of the sensitivity of \( R_T \) to incubation conditions, and these will be described next.

Using increasing concentrations of urea, no increase was seen in \( R_T \) (Table V). Cottam et al. (22) have previously shown that 2 mM urea dissociates pyruvate kinase from tetramers to dimers, with 68% activity and that the monomers formed in 4 M urea are devoid of activity. The loosening of structure accompanying urea denaturation does not increase proton exchange preferentially. On the contrary, the ratio, \( R_T \), decreases. Furthermore, glycerol, which might favor the interaction of polar groups on the enzyme, raised the relative extent of enolization of bound pyruvate substantially. These two results suggest that it is release of products rather than of proton that is primarily affected by urea and glycerol. If product dissociation is facilitated in urea and attenuated in glycerol, the values of \( R_T \) would show the observed changes.

**Table VI**

**Temperature dependence of partitioning of tritium from PEP between water and pyruvate**

Each reaction contained 100 mM \( N\)-tris(hydroxymethyl)methyl aminoethane sulfonate (pH 7.3), 100 mM KCl, 5 mM MgCl\(_2\) or CoCl\(_2\), 1 mM ADP, 0.1 mM [3-\( ^3 \text{H} \)-PEP (10\(^6 \) cpm per pmole), 0.15 mM DPNH, 2 units of lactate dehydrogenase, and 0.1 unit of pyruvate kinase (total volume: 1 ml). Methods as described under “Experimental Procedure.”

| Temperature | \( R_T \) with Mg\(^{2+}\) | \( R_T \) with Co\(^{2+}\) |
|-------------|-----------------|-----------------|
| 10°C        | 0.059           | 0.460           |
| 12°C        | 0.053           | 0.304           |
| 21°C        | 0.050           | 0.268           |
| 32°C        | 0.023           | 0.230           |
| 43°C        | 0.020           | 0.199           |

**Fig. 2.** Divalent cation and pH dependence of partitioning of tritium from PEP between water and pyruvate during the pyruvate kinase reaction. Besides the cations present at 5 mM as their chloride salts, the reaction mixtures consisted of 100 mM KCl, 5 mM ADP, 0.1 mM [3-\( ^3 \text{H} \)-PEP (10\(^6 \) cpm per pmole), 0.15 mM DPNH, 2 units of lactate dehydrogenase, and 0.1 unit of pyruvate kinase (total volume: 1 ml). Methods as described under “Experimental Procedure.”

**V**\(_{\text{max}}\) with Mg\(^{2+}\) is from the data of Plowman and Krall (27).
effective in activating the over-all reaction as a function of pH. Qualitatively, all display a pH profile characterized by a plateau at neutral pH and a marked increase at alkaline pH. It is difficult to perform experiments at more elevated pH because the cations are readily oxidized or precipitated. With Mg$^{2+}$, experiments could be performed up to pH 9.8 and $R_T$ was found to increase continually with pH. This suggests that hydroxide ion may become involved in the catalysis of the enolization step. Quantitatively, the metals show differences in the values of $R_T$ at all pH values. It follows from this that if a major pathway of hydrogen exchange involves Step 3, then the divalent cation must play a role subsequent to the phosphoryl transfer step, where these ions have been previously reported to act (25, 26).

On the other hand, if the metal ion has its effect at the phosphoryl transfer step only, then phosphoryl transfer from ATP to enolpyruvate, Step -2, must be a necessary preliminary to the exchange that is, Step 3, and not 3, is on the major route for the exchange.

At neutral pH, the rate of the pyruvate kinase reaction has been observed to decrease in the following order: Co$^{2+} >$ Mg$^{2+}$ > Mn$^{2+} >$ Ni$^{2+}$ (27). At neutral pH, $R_T$ is found to decrease in the order, Co$^{2+} >$ Ni$^{2+} >$ Mn$^{2+} >$ Mg$^{2+}$. This order corresponds neither to the dissociation rate of the ATP-metal complex (28), nor to the affinity of the metal-enzyme complex (25), nor to the metal octahedral radius (29), nor to the Irving-Williams series (30). Rather, as shown in Fig. 3, $R_T$ is a linear function of the electronegativity of the metal ion, as measured by the pKo of water in its coordination sphere (31). $R_T$ is found to equal $10^{-4.54}$ pKo. Such an effect could result from the metal affecting the electronegativity of the base which, on the enzyme, is involved in the proton transfer step. The dissociated form of the base is found to facilitate proton exchange, and the divalent cations appear to affect the concentration of the dissociated form.

The divalent metal and pH effects on the ATP-activated enolization of pyruvate by pyruvate kinase are illustrated in Fig. 4. With Mg$^{2+}$, the enolization rate increases with increase in pH, previously shown by Rose (3), and similarly to the effect of pH on $R_T$. The Co$^{2+}$- and Mn$^{2+}$-dependent rates exhibit pH optima at 7.3 and 8.1, respectively, in contrast to the observations with $R_T$ (Fig. 2). These differences emphasize the fact that $R_T$, being a ratio of rate constants, is independent of factors which determine the absolute rate of $v_T$. The differential effect of the divalent cations on the pH profile of $v_T$ may result from the effects on the “affinities” for ATP and pyruvate or from formation of inhibitory complexes. While the $K_a$ for ATP at pH 7.6 has been found to be 1.33, 0.38, and 8 mM for Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$, respectively, the effect of pH on this $K_m$ and on other kinetic constants awaits further study.

Pyruvate kinase requires a monovalent cation for activity. Table VI shows the effect of various monovalent cations on the partition ratio, $R_T$. It is of interest that those cations that promote the net reaction most effectively, K$^+$, Rb$^+$, and NH$_4^+$.
of tritium from PEP between water and pyruvate

**Table VII**

| Cation       | $r_T$ |
|--------------|-------|
| $NH_4^+$     | 0.038 |
| $K^+$        | 0.030 |
| $Rb^+$       | 0.020 |
| $Cs^+$       | 0.006 |
| $Na^+$       | 0.005 |

**Table VIII**

| Conditions          | $r_T$ |
|---------------------|-------|
| 10 mM KCl           | 0.012 |
| 100 mM KCl          | 0.030 |
| 200 mM KCl          | 0.029 |
| 400 mM KCl          | 0.105 |
| 100 mM KCl          | 0.447 |
| 200 mM KCl          | 0.529 |
| 400 mM KCl          | 0.802 |
| 10 mM KCl, 100 mM TMA-Cl* | 0.013 |
| 10 mM KCl, 200 mM TMA-Cl | 0.014 |
| 10 mM KCl, 400 mM TMA-Cl | 0.017 |

* Besides the listed components, the reaction mixtures contained 100 mM Tris-Cl, 5 mM MgCl₂, 2 mM ADP, 0.1 mM [3-T]PEP (10⁶ cpm per pmole), 0.15 mM DPNH, 1 unit of lactate dehydrogenase, and 0.1 to 2 units of pyruvate kinase (total volume: 1 ml). Procedure as described under "Experimental Procedure."

The reaction mixtures were treated as described under "Experimental Procedure." To determine the contribution of tritium from PEP between water and pyruvate, the reaction mixtures were treated as described under "Experimental Procedure."

The effect of various phosphoryl acceptors on $r_T$ is shown in Table IX. It will be noted that the ATP analog of ADP, $\alpha,\beta$-methylene-adenosine diphosphate, while a poorer substrate by three orders of magnitude, gave the same $r_T$ as ADP. Also, the ribonucleotides (ADP, GDP) gave higher ratios than their corresponding deoxyribonucleotides (dADP, dGDP), suggesting that the 2'-substituent on the sugar could be involved in binding and that substituting a proton for a hydroxyl at C-2 increases the affinity for ATP. This is rate determining in the forward reaction path. This conclusion is derived from two sources. First, the concentration of the ternary products complex in the forward steady state at substrate saturation, $E_{sat}$, agrees well with the concentration of $E_{con}$ established from the side of pyruvate plus ATP at saturation, Table III. Similar experiments, not reported, have been performed at pH 7.3 and 8.6 with similar results. The second evidence is more restricted and comes from the extent of detritiation of [3-T]PEP at pH 7.2 and Co⁺, Table IV. Here, even ignoring a discrimination against tritium in the reversal of Step 3, a factor of 1.8-fold or more, the reversal of that step as measured by $r_T$ is very rapid.

Since the complete system is required for the detritiation of [3-T]pyruvate (3), the irreversible dissociation of either product of the forward reaction would terminate the release into water of tritium derived from [3-T]PEP. It is possible that the rate-determining step is isomerization of the ternary products complex, $E_{con}$, rather than the immediate release of products. Under conditions in which pyruvate is trapped with lactate dehydrogenase plus DPNH, the presence of 2 mM ATP had no effect on the extent of detritiation of [3-T]PEP at pH 7.2 with MgCl₂. Thus, if ATP is the first product released, the subsequent release of pyruvate would have to be too rapid to permit the reconstitution of the ternary products complex to take place at this level of ATP. On the other hand, it was observed in Table VIII that concentrations of $K^+$ well above its activation constant for the reaction (11 mM) (32) had a marked effect that could not be attributed to changes in ionic strength. These results show that the $K^+$ at high concentration is involved in steps subsequent to the phosphoryl transfer step and that $k_{eT}$ and $k_4$ are differentially affected. This might be explained by a low affinity binding of $K^+$ to some kind of effector site; however, an alternative explanation will be considered under "Discussion."

The effect of various phosphoryl acceptors on $r_T$ is shown in Table IX. It will be noted that the ATP analog of ADP, $\alpha,\beta$-methylene-adenosine diphosphate, while a poorer substrate...
effect of high levels of monovalent cation on the forward reaction rate. Further studies will be required to determine whether this explanation pertains to both phenomena. Likewise such an investigation will be required to determine to what extent the large differences in the extent of deuteriation of PEP seen in Table VII with different monovalent cations is to be attributed to such a mechanism.

Although the liberation of products is rate-limiting for the maximum forward velocity, as shown in Table IV, this does not bring with it extensive isotopic equilibration of the substrates with the complex of enzyme and bound products. Thus the back labeling of PEP from tritiated water, \( P_{F} \), is no greater than the net forward rate, and appreciably less under certain conditions. This fact can be brought into harmony with rate limitation at Step 4 in the forward reaction only if it is assumed that the release of PEP, or of ADP if that is required to precede it, is rate-limiting for the back labeling of PEP. This assumption is not inconsistent with a slow Step 4 since Step 1 is not involved in the kinetic expressions for the maximum forward velocity. Initial rate studies by Reynolds et al. (33) have been interpreted in support of random and random interactions of PEP and ADP with pyruvate kinase. This is based on the observation of competitive inhibition by ATP with either substrate and the occurrence of linear double reciprocal plots for both substrates with \( K_m \) values of each found to be independent of the concentration of the invariant substrate. The argument for random interaction is supported by the observation of direct binding studies with both PEP and ADP by ultracentrifugation studies (31) and by kinetic protection and proton relaxation rate studies (26). Furthermore, in the latter work, good correlations between the calculated binary dissociation constants and the kinetic \( K_m \) values were observed with Mn\(^{2+}\) as the activator.

Although these observations can be considered definitive in support of a random order of substrate interaction, the restriction of “equilibrium kinetics” may not be considered to be established. Thus, nonlinear double reciprocal plots were observed by Melchior (34) when (MgADP) was the varied substrate. In addition, the occurrence of linear replots for a random bisubstrate reaction is now considered to be of limited value in deciding whether a quasiequilibrium state persists (34, 35).

The conclusion drawn concerning the rate-limiting role of product release for the maximal forward velocity is unrelated to the particular sequence of phosphoryl and proton transfer that is chosen for the mechanism. The arguments in support of the stepwise mechanism, Reaction 1 are based on the independent occurrence of the two processes and may not be universally accepted. However, for the purposes of placing the data in a context for discussion, this mechanism, expanded in Scheme 3 is assumed to be correct. Considering the steps between 1 and 4, it is observed from Tables IV to IX that the degree to which \( E_3 \) undergoes enolization prior to dissociation of products, as indicated by the value of \( R_T \), varies greatly with the conditions of the incubation. It is evident that the exchange of the –CHO proton with medium occurs prior to release of PEP or pyruvate since an RT of 2.24 (Table IV) is much greater than the observed return of PEP, \( P_F = 0.149 \), would allow and also much greater than could be explained if the methyl tritium became equilibrated with a protonic base, such as lyzine, and in which exchange with the medium followed dissociation of products. Under these particular conditions, pH 8.8 with Co\(^{2+}\), the steps that are involved in enolization and exchange with the medium clearly do not contribute to rate determination of the forward velocity. On the other hand, there must be a small contribution at pH 7.3 with Mg\(^{2+}\) since, as noted in Table IV, the value of \( R_T \) is quite small and a significant, although not maximal, discrimination against the utilization of tritium from the medium is evident in the formation of pyruvate.

It is only indirectly established, from the experiments reported, that the enzyme functions as a base in the enolization process, although this is to be expected. The failure to observe intermolecular trinitium transfer from [3-T]pyruvate to phosphoenolpyruvate tends to rule out the presence of EH\(^+\) per se in the \( h_0 \) step, Scheme 3. In this experiment the phosphoenolpyruvate was present at 300-fold its \( K_m \) for the formation of \( \alpha \)-ketobutyrate and ATP and therefore should have provided an excellent trap for any tritium derived from pyruvate and retained by the enzyme. On the other hand, the very low value of the isotope effect of ATP-dependent enolization of pyruvate, together with the low value of \( R_T \) under certain conditions, strongly suggests that the enolization cannot only involve direct loss of hydrogen to the medium, Step 3', since this would always necessarily show a full isotope effect under such conditions. Among the other steps involved in the exchange loss of tritium from [3-TjPEP, the rotation around \( C_T-C_2 \) of pyruvate is probably much faster than competing steps since such unhindered rotations are known to have rate constants of the order of \( 10^{12} \text{ sec}^{-1} \) (36), and it was determined that the \( R_T \) was the same for both E- and Z-[3-TjPEP.

Concerning the nature of a base catalyst on the enzyme, it is not possible to be definitive at this time. Hydroxide ion in the coordination sphere of the divalent metal could be acting as a base. This would provide a ready explanation for the effect of electronegativity of metal ion on the partition ratio, \( R_T \) (Fig. 3). Alternatively, an amino acid residue coordinate to the metal could serve as a base and show a response to the electronegativity of the metal. The low slope of the linear free energy relationship of Fig. 3 suggests an indirect effect.

Of possible importance for future studies is that the enolization of pyruvate that is activated by analogs of ATP such as \( P_1 \) occurs with a high isotope effect, indicating that the C—H bond cleavage is rate-determining. Thus kinetic studies of the exchange may be expected to reflect the character of the basic group as well as other factors involved in this step. In this respect it is of interest that the pH dependence of enolization of pyruvate with various diatomic activators corresponded to the \( pK_a \) of the activator (3).

As was noted in Fig. 1, the loss of tritium from PEP to the medium closely followed the course of the over-all reaction. In a parallel study to this one, also at pH 7.3, the lactates formed early and at completion of the reaction were compared with respect to their specific activities and found to be within 3% of each other. Thus no secondary isotope effect could be demonstrated. Rather large inverse isotope effects would be expected if either Step 3 or 4 were rate determining (compare with studies of enolase in which were obtained values of \( k_T / k_D = 1.26 \) as a kinetic effect and \( k_T / k_D = 1.4 \) as an equilibrium effect (37)). However, to show a discrimination between trinitiated and normal species of PEP in the course of many cycles of reaction it is required that intermediates prior to the rate-determining step be able to mix with the free PEP pool in order to restore the specific activity of the intermediates to normal. Likewise, discrimination due to a preliminary equilibrium depends on
free communication between the intermediate prior to the rate-limiting step and the free PEP. Thus the failure to see the discrimination in favor of tritiated PEP that would result if the steps prior to Step 4 were at equilibrium is the consequence of the slow reversal of the PEP on step and is consistent with the relatively low rate of back labeling of PEP with tritiated water.

All studies reported in this paper were performed with pyruvate kinase from rabbit muscle. Pyruvate kinase from yeast and human erythrocytes have thus far been examined only in a preliminary way. Both show \( R_T \) values similar to those reported in this paper. The yeast enzyme shows the same \( R_T \) in the presence and absence of fructose 1,6-diphosphate, its allosteric activator. In their study of yeast fermentation of [6-\( ^3\)H]glucose, Saur et al. (38) did a mass spectrometric analysis of the ethanol and found the ratio CH\(_2\)D-CH\(_2\)OH:CHD-CH\(_2\)OH to be 0.21. By the exclusion, as unlikely, of other known routes for the loss of these deuteriums during glycolysis, these authors considered the exchange by enolization of pyruvate by pyruvate kinase to be the most likely explanation of their data (39). From direct experiments with yeast pyruvate kinase acting on [3-\( ^3\)T]PEP, a value of \( R_T = 0.21 \) is not unlikely. In an analogous study with [6-\( ^3\)T]glucose, Simon and Medina (40) concluded that exchange of tritium of pyruvate by pyruvate kinase was the most likely explanation for the low content of tritium in the ethanol produced by yeast.

The loss of tritium from [3-\( ^3\)T]PEP during the course of the pyruvate kinase reaction poses a problem for the preparation of chiral pyruvate with this enzyme (4). Clearly the kinase step must be carried out under conditions that minimize the enolization of pyruvate either during or after its formation. Conditions favoring low values of detrination should be used: low pH, Mg\(_{2+}\) rather than Mn\(_{2+}\), and limiting amounts of K\(^+\) or NH\(_4^+\).

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