Studies on the Mechanism and Stereochemical Properties of the Oxalacetate Decarboxylase Activity of Pyruvate Kinase*

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When cod fish muscle oxalacetate decarboxylase catalyzes the decarboxylation of oxalacetate in the presence of NaBH₄, L-lactate results from the reduction of enzyme-bound pyruvate. However, d-lactate results when borohydride reduces the binary enzyme-pyruvate complex formed by adding pyruvate from solution, as reported by others. This observation suggests that there are alternate mechanisms for reduction that are either kinetically or sterically determined for the E-pyruvate forms produced in the two directions. In the process of investigating the mechanism of reduction, the cod fish muscle decarboxylase was discovered to be identical with pyruvate kinase. Decarboxylase activity appears to take place at a site which overlaps the phosphoenolpyruvate binding site on this enzyme, as discussed in the following paper. Crystalline rabbit muscle pyruvate kinase also contains significant decarboxylase activity indicating that the two reactions may be structurally related functions.

In the presence of K⁺, orthophosphate, or ATP the rabbit muscle enzyme catalyzes the detritiation of enzyme-bound pyruvate formed during decarboxylation before release of pyruvate from the enzyme, in analogy with the detritiation of pyruvate formed from P-[3-³H]enolpyruvate in the kinase reaction. This observation is consistent with the formation of an enolpyruvate intermediate common to the kinetic pathways of both reactions. Since the decarboxylase reaction is completely stereospecific, within the limits of detection, going with retention of configuration, the protonation of the enolpyruvate intermediate is completely determined by the enzyme as is the case with the enolpyruvate intermediate generated from P-enolpyruvate in the kinase reaction.

The present study grew out of an attempt to determine if the cod muscle decarboxylase reaction occurred with retention of configuration as found for other decarboxylases. The subsequent discovery that the decarboxylase was, in fact, identical with pyruvate kinase and that both the decarboxylase and kinase reactions take place at overlapping sites on the enzyme, as described in the following paper (4), prompted the investigation of the mechanistic and stereochemical relationship between these two activities.

MATERIALS AND METHODS

Commercial preparations of the following materials were used: oxalacetic acid (Sigma), sodium borohydride, 90.8% (Matheson), tricyclohexylammonium P-enolpyruvate (Calbiochem), disodium adenosine triphosphate (Sigma), and monosodium 2-ketoglutarate (Sigma). Sodium tetrafluoroborate (ROC/RIC) was crystallized once from water. Tritiated ethanol solvent and stored under anhydrous conditions. Tritiated pyruvate was generously provided by Dr. H. P. Meloche. The calcium phosphate-cellulose column used for purifying the enzyme was prepared according to Kosicki (5). The NAD⁺-agarose used in the affinity column was a product of P-L Biochemicals. Fresh frozen cod fish muscle was generously provided by Mr. John D. Kaylor of the National Marine Fisheries, Gloucester, Mass. P-enolpyruvate carboxylase from spinach was generously provided by Drs. H. Miziorko and A. S. Mildvan. All commercial enzyme preparations were from Boehringer.

Purification of Oxalacetate Decarboxylase Activity—The enzyme used in these experiments was prepared by the method of Kosicki through the calcium-phosphate step (5) after which filtration through

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an NAD+‐agarose column was used to remove lactate dehydrogenase, the major protein contaminant in the decarboxylase after the calcium phosphate column. The peak tubes from the 0.1 M phosphate elution of the calcium phosphate column were concentrated immediately to a minimum volume by ultrafiltration. An aliquot of the concentrate containing approximately 4 mg of protein was layered onto an NAD+‐agarose column (4 cm × 0.5 cm) equilibrated in 0.06 M phosphate buffer, pH 6.8, and eluted with the same buffer. Essentially 100% of the decarboxylase activity, representing 32% of the protein, was eluted in the void volume while greater than 98% of the lactate dehydrogenase activity remained adsorbed to the column. The specific activity of the decarboxylase was 0.91 unit/mg based on an extinction coefficient of 0.78 for protein absorbance at 280 nm and was homogeneous by all of the physical criteria described in the following paper (4). The enzyme was stored at liquid nitrogen temperature.

**Assay of Oxalacetate Decarboxylase**—Activity was determined in a coupled assay system with lactate dehydrogenase from the decrease in absorbance at 340 nm of NADH. Solid oxalacetic acid, which exists as the enol tautomer, must first be equilibrated in buffer with the keto form, the true substrate for the decarboxylase (6). The substrate was first dissolved in water (4 mg/ml) and incubated at room temperature for 10 min. Then the solution was diluted with an equal volume of 0.1 M triethanolamine chloride buffer, pH 7.6, and allowed to stand for an additional 15 min. In the basic assay, 0.1 ml of this stock solution was diluted to 1 ml in triethanolamine chloride buffer, 50 mM, pH 7.6, containing 0.1 mM of NADH, 1.5 mM of MnSO4, and 3 units of dialyzed lactate dehydrogenase maintained at 37° in a 1-cm cuvette. The optical decrease of absorbance at 340 nm reflects the decarboxylation of oxalacetate. The more rapid rate of decrease after addition of enzyme represents enzymic decarboxylation. The 0.00 to 0.50 expanded scale of a Cary 14 spectrophotometer was used to follow absorbance change.

**Preparation of [3-3H]Aspartate**—This compound was prepared by converting [3-2H]pyruvate to [3-3H]oxalacetate with pyruvate carboxylase which, in turn, was coupled to glutamate-oxalacetate transaminase to convert [3-3H]oxalacetate to [3-3H]aspartate. The reaction mixture contained the following compounds in a volume of 1 ml: 200 μmol of Tris, pH 7.6, 5 μmol of ATP, 7 μmol of MgCl2, 150 μmol of NaHCO3, 60 μmol of KCl, 0.05 μmol of [3-3H]pyruvate, 10 units of glutamate dehydrogenase, 2.5 units of glutamate-oxalacetate transaminase, 0.2 unit of pyruvate carboxylase. After the optical density at 340 nm goes to a minimum, 0.1 ml of 2 N HClO4 was added and the mixture precipitated by centrifugation. The reaction mixture was neutralized to pH 7.2 with KOH, diluted to 25 ml with distilled water, and placed onto a Dowex 1 column (1 cm × 0.8 cm). The column was eluted with a linear 0 to 2.5 mM gradient collecting 4.4 ml fractions. The [3-3H]aspartic acid product is usually found between Fractions 28 and 32. The product was lyophilized as a dry powder.

**Preparation of Chirally Labeled [3-3H, 14H]Malates**—The L-malates containing deuterium and tritium stereospecifically located at C-3 were prepared by the method of Rose (7).

**Method for Borohydride Reductions**—In studies of the borohydride reduction of pyruvate generated by enzymic dehydrogenation, the oxalacetate substrate was generated in situ by the P-enolpyruvate carboxylase reaction in order to avoid having oxalacetate present at high concentration during the reduction. A solution of 1.07 units of cod muscle oxalacetate decarboxylase was prepared in 0.57 ml of 0.52 M potassium phosphate buffer, pH 7.0, containing 1.5 μmol of reduced glutathione, 2.5 μmol of NaHCO3, 0.5 μmol of MnSO4, and 1.05 μmol of P- [3-3H]enolpyruvate (4.52 × 104 cpm/μmol). A peristaltic pump was used to deliver a steady flow of borohydride solution (0.85 ml/hour of a 0.045 M stock solution in 1 × 10−3 M NaOH) to this mixture. Immediately after starting the peristaltic pump, 0.035 unit of P-enolpyruvate carboxylase was added. After 2 hours, the reaction was terminated by the addition of 0.7 ml of 0.1 M HClO4. The protein precipitate was removed by centrifugation and the supernatant acidified to pH <1.0 with 10 N H2SO4. The isolation of lactic acid from this mixture was carried out by the procedure described below.

**Procedure for the borohydride reduction of the mixture of pyruvate and lactate dehydrogenase** was like that used by Kosicki and Westheimer (2). Cod muscle oxalacetate decarboxylase, 0.97 unit, was added to a solution containing 200 μmol of KH2PO4, pH 7.0, and 0.5 μmol of MnSO4. The total volume was 0.45 ml. Stock sodium borohydride (in 1 × 10−3 M NaOH) and sodium pyruvate (in 0.5 M KPO4, pH 7.0) were simultaneously infused separately into the mixture containing the enzyme using a peristaltic pump at rates of 0.41 μmol/min and 1.5 μmol/min, respectively. After 4 min the reaction was terminated by the addition of 0.2 ml of 2 N HClO4. The protein precipitate was removed by centrifugation and the supernatant acidified to pH <1.0 with 10 N H2SO4. This mixture was diluted to sodium bicarbonate and extracted with ether by the procedure described below. A control experiment was run using the condition described above only in the absence of decarboxylase.

**Isolation of Borohydride Reduction Products**—The acified reaction mixtures were added to enough dry, water-washed silica acid (Mallinckrodt) to form a free flowing powder. The silica acid was stirred with 100 to 150 ml of anhydrous diethylether in a glass-stoppered flask overnight. Care was taken to minimize exposure of the solution to air during this time to prevent peroxides of ether from forming. Ether peroxides will readily decarboxylate any traces of unreduced pyruvate in the reaction mixture to acetic acid which migrates near to lactic acid on Dowex 1–Cl− columns. After overnight stirring, 2 ml of 1 × 10−3 M NaOH were added to the ether supernatant. The ether was removed from this mixture on a rotary evaporator.

**Lactate and Malate Assays**—Total lactic acid was determined by the colorimetric procedure of Barker (8). d- and L-lactate were determined by the method of Holzer and Soling (9) from the increase in optical density at 366 nm (ε = 9.1 × 103) at pH 9.5 due to production of acetylpyridine NAD+ (0.88 mM) with either d- or L-specific lactate dehydrogenase (Boehringer) at pH 9.5. The oxidation of lactate under these conditions goes to about 90% completion. The correction factor due to incomplete reaction was obtained by running standards of lactate with the unknown. A modification of the lactate assay was used for the determination of l-malate as described by Holzer and Soling (10).

**Hydrolysis of Borate Ester of Lactate**—The borate esters of lactate, generated from the reduction of pyruvate by borohydride under the conditions described above, were converted to unesterified lactic acid by treatment with acidic methanol (0.25% HCl) and 50% methanol at 25° for 16 hours. The methanol was removed on a rotary evaporator to a volume of about 0.5 ml, taking care not to allow the solution to go dry. The remaining aqueous solution was mixed with glycine buffer, pH 9.5, for enzymic lactate analysis.

**RESULTS AND DISCUSSION**

**Proton Exchange Reactions of Oxalacetate Decarboxylase**—To determine whether the enzymic decarboxylation of oxalacetate proceeds with retention or inversion of configuration, stereospecifically labeled [3-3H, 14H]oxalacetate was to be treated with cod muscle decarboxylase in H2O medium and the chirality of the derived pyruvate established. The feasibility of this approach, however, critically depends on there being no extensive exchange of the methyl protons of pyruvate with solvent water during the course of the reaction.

As shown in Table I, this was indeed the case with the cod muscle decarboxylase since greater than 60% of the tritium of [3-3H]oxalacetate was lost to water on being converted to pyruvate. In this experiment [3-3H]oxalacetate was generated from P-[3-3H]enolpyruvate, using spinach P-enolpyruvate carboxylase, in the presence of excess decarboxylase. Lactate dehydrogenase was present to trap the pyruvate. The presence of a trace of malate dehydrogenase in the reaction mixture assured that a small portion of the oxalacetate pool would be trapped as malate. Since the specific activity of the isolated malate was close to that of the starting P-[3-3H]enolpyruvate, tritium was not lost either during the carboxylation of P-enolpyruvate or from elimination of the oxalacetate. The low specific activity of the isolated lactate was probably due to catalyzed enolization of pyruvate before its dissociation from the surface of the decarboxylase. Pyruvate kinase is known to catalyze a similar exchange in the conversion of P-[3-3H]enolpyruvate to pyruvate in the presence of ADP (11). In the likely
event that the protons of the methyl group of pyruvate are made equivalent by torsion about the C 2-C 3 bond, rapid equilibration of the methyl group protons with solvent could result before dissociation from the enzyme if $k_d$ were greater than $k_s$ in Equation 2. To prevent enolization of enzyme-bound pyruvate generated during the course of the decarboxylation, an attempt was made to reduce it with NaBH₄, thus introducing a new step, $k_4(BH_4^-)$, in competition with $k_4$ (Equation 2).

Borohydride Trapping of Enzyme-Pyruvate Intermediate Formed from Decarboxylation—Kosicki and Westheimer (2) have previously demonstrated that when pyruvate is reduced with NaBH₄ in the presence of the cod muscle decarboxylase the resulting lactic acid is optically active, containing an excess of the D isomer. They concluded that some of the pyruvate must be reduced on the surface of the enzyme at a catalyzed rate comparable to that of the decarboxylation reaction. An experiment was set up using conditions similar to those of the first experiment, Table I, except that DPNH and lactate dehydrogenase were replaced with NaBH₄, which was constantly infused into the enzyme mixture in the presence of P-[3-¹H] enolpyruvate. The exact conditions of this experiment are described under “Materials and Methods.”

Fig. 1 shows the elution profile from Dowex 1-Cl⁻ (top plate) of this reaction mixture. The first peak had a specific activity of approximately 5.0 ± 1.0 x 10⁴ cpm/μmol based on the colorimetric p hydroxydiphenyl assay for total lactate. Surprisingly, this peak had almost the same specific activity as the escaping P-[3-¹H] enolpyruvate (4.52 ± 10⁴ cpm/μmol) based on an enzymic assay using lactate dehydrogenase specific for the L enantiomer of lactate. (4.55 x 10⁴ cpm/μmol); i.e., the first peak was essentially optically pure L-lactate. Starting with 1.0 μmol of P-[3-¹H] enolpyruvate, 12.4% of the radioactivity was found in the first peak. The second peak shown in Fig. 1 (top plate) containing about 7.4% of the radioactivity was not a substrate for L-lactate dehydrogenase immediately after elution from the Dowex 1-Cl⁻ column. However, after acidification and treatment with methanol, a procedure which hydrolyzes borate esters as described under “Materials and Methods,” the peak assayed as racemic lactic acid. Apparently, the first peak resulted from the stereospecific reduction of pyruvate on the surface of the enzyme while the second peak was produced from the nonstereospecific reduction by BH₄⁻ of pyruvate not bound to an enzyme surface. This peak is most likely a borate ester on the basis of its ready hydrolysis in the presence of acidic methanol. It contained no organic phosphate by the method of Fiske and SubbaRow (12). Apparently, some feature of the mechanism of BH₄⁻ reduction of pyruvate free in solution results in borate esters formation while reduction on the enzyme gives an unesterified lactic acid. The remaining counts on the column were eluted with 40 mM HCl as two poorly resolved peaks containing malate and unreacted P-enolpyruvate, not shown in Fig. 1. As an additional check that the

### Table I

<table>
<thead>
<tr>
<th>Enzyme-Pyruvate</th>
<th>Oxaloacetate pool (as lactate)</th>
<th>Pyruvate (as lactate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>89,200 cpm/μmol</td>
<td>89,900 cpm/μmol</td>
<td>35,400 cpm/μmol</td>
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**Detritiation during decarboxylation of [3-H]oxalacetate**

The reaction mixture contained the following compounds in a volume of 1 ml: 35 μmol of Tris, pH 7.2, 2.6 μmol of reduced glutathione, 1.3 μmol of DPNH, 4.4 μmol of NaHCO₃, 0.90 μmol of MnSO₄, 0.71 μmol of P-[3-¹H] enolpyruvate, 20 units of lactate dehydrogenase, 0.016 unit of malate dehydrogenase, 1.3 pmol of DPNH, 4.4 pmol of NaHCO₃, 0.90 pmol of KPO₄, 0.042 unit of phosphoenolpyruvate carboxylase (spinach), and 0.016 unit of malate dehydrogenase. Lactate and malate were separated with 2 mM and 40 mM HCl, respectively, on a Dowex 1-Cl⁻ column.

**Fig. 1.** Two kinds of tritiated lactate formed with NaBH₄ in decarboxylation of [3-H]oxalacetate. Top plate, 2 mM HCl elution of counts from the Dowex 1-Cl⁻ column used to resolve the lactate acid from the reaction mixture where BH₄⁻ was present during the enzymic decarboxylation of [3-H] oxalacetate generated from P-[3-¹H] enolpyruvate (4.52 ± 10⁴ cpm/μmol) with phosphoenolpyruvate carboxylase and HCO₃⁻. Refer to “Materials and Methods” for exact conditions. Bottom plate, elution of counts from Dowex 1-Cl⁻ column used to resolve a reaction mixture where a portion of the initial peak (top plate) was oxidized with L-specific lactate dehydrogenase (LDH) in the presence of acetylpyridine NAD⁺.
first peak was all \(\text{L}-\text{lactate}\), a portion of it was oxidized to pyruvate in the presence of the acetylpyridine analog of DPN\(^+\) and \(\text{L}-\text{specific}\) lactate dehydrogenase and chromatographed on Dowex 1-Cl\(^-\). As shown in Fig. 1 (bottom plate) essentially all of the counts were eluted with 40 \text{mM HCl} where pyruvate is found. Any oxidized \(\text{d}-\text{lactate}\) should have appeared in the 2 \text{mM HCl} region. No contaminating \(\text{d}-\text{specific}\) lactate dehydrogenase activity was found in the \(\text{L}-\text{specific}\) lactate dehydrogenase used in this experiment.

In an important control experiment, [2-\(\text{\textsuperscript{14}C}\)]pyruvate was reduced by NaBH\(_4\) in the absence of enzyme. As shown in Fig. 2 (top plate) a single peak was eluted with 2 \text{mM HCl}. This was not a substrate for \(\text{L}-\text{lactate} \text{dehydrogenase}\) (Fig. 2). When a portion of this peak was acidified, treated with methanol, and rechromatographed it migrated as free lactate acid and assayed as racemic lactate (Fig. 2, bottom plate).

To be certain that \(\text{L}-\text{lactate} \text{production}\) reflected the action of the decarboxylase and not reduction of pyruvate on P-enolpyruvate carboxylase, this experiment was repeated by infusing NaBH\(_4\) (0.60 \text{\mu mol/min}) and oxalacetate 0.0376 \text{\mu mol/min}) in separate streams into a solution containing 0.94 unit of the decarboxylase from cod muscle. When a total of 1.11 \text{\mu mol} of oxalacetate had been infused, the reaction mixture was acidified and processed in the same way as described above. The first peak eluted from the Dowex 1-Cl\(^-\) column was pure \(\text{L}-\text{lactate}\) (\(\sim 0.10\) \text{\mu mol}) and the second was the borate ester of racemic lactate (\(\sim 0.28\) \text{\mu mol}). The results of this experiment are in accord with those found previously where oxalacetate had been infused, the reaction mixture was acidified, and the resulting solution was chromatographed on Dowex 1-CI\(^-\). When a total of 1.11 \text{\mu mol} of oxalacetate had been infused, the reaction mixture was acidified and processed in the same way as described above. The first peak eluted from the Dowex 1-Cl\(^-\) column was pure \(\text{L}-\text{lactate}\) (\(\sim 0.10\) \text{\mu mol}) and the second was the borate ester of racemic lactate (\(\sim 0.28\) \text{\mu mol}). The results of this experiment are in accord with those found previously where oxalacetate was enzymically generated in the reaction mixture instead of being added directly and indicate that optically pure \(\text{L}-\text{lactate}\) is produced by the decarboxylase.

**Borohydride Trapping of Enzyme-Pyruvate Complex Formed by Addition of Pyruvate from Solution**—Based on optical rotation Kosicki and Westheimer (2) concluded that an excess of \(\text{d}-\text{lactate}\) was formed when free pyruvate was added to the presence of the cod decarboxylase. Their experiment was repeated using the methods described here for isolating lactic acid. As shown in Fig. 3, two radioactive peaks were eluted from the Dowex 1-Cl\(^-\) column, one of which migrates as unesterified lactic acid (A) and the other as the borate ester of lactic acid (B). Using \(\text{L}-\text{specific}\) and \(\text{d}-\text{specific} \text{lactate dehydrogenase}\), peak A was found to be greater than 90% \(\text{d}-\text{lactate}\) while peak B analyzed as the hydrolyzable borate ester of racemic lactate. The yield of \(\text{d}-\text{lactate}\) from both peaks was 72 \(\pm 4\)% which compares well with the 25% excess \(\text{d}-\text{lactate}\) originally reported by Kosicki and Westheimer (2). In retrospect it is evident that in their isolation of lactic acid these authors used conditions that would have led to the hydrolysis of any borate esters present and hence could not have discriminated between lactate resulting from the reduction of pyruvate on the enzyme surface and that occurring free in solution.

The singular result requiring further exploration is that BH\(_4^-\) stereospecifically reduces the \(\text{re}\) face of enzyme-bound pyruvate formed from the decarboxylation of oxalacetate while the opposite (or si) face is stereospecifically reduced in the enzyme-pyruvate complex formed by direct addition of pyruvate to the enzyme from solution. Assuming that the two stereospecific reduction reactions are catalyzed by the same enzyme species in the apparently homogeneous enzyme preparation, three general hypotheses may potentially explain the above results. First, pyruvate generated on the enzyme from decarboxylation may be at a site distinct from the site to which pyruvate, added from the product side, binds. Differences between these topographically distinct complexes result in the formation of different enantiomers of lactate after BH\(_4^-\) reduction. Second, alternate enzyme-pyruvate forms at the same site may be reduced by BH\(_4^-\) depending on which kinetic sequence pyruvate is generated at the active site (Equation 3). Differences in the conformation of these complexes would expose different faces of the carbonyl bond of pyruvate to reduction by BH\(_4^-\). A third hypothesis assumes that BH\(_4^-\) binds to a site on the enzyme which is identical with or overlaps with the presumed \(\beta\)-carboxyl binding site for oxalacetate. Borohydride binds to this site.
Mechanism of Oxalacetate Decarboxylase

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\[
+ \text{Oxalacetate} \rightarrow \text{Enolpyruvate} \rightarrow \text{Pyruvate} \rightarrow \text{Lactate}
\]

\[
\text{Oxalacetate} + \text{H}^+ \rightarrow \text{Pyruvate} + \text{CO}_2
\]

\[
\text{Pyruvate} + \text{BH}_4^- \rightarrow \text{L-lactate}
\]

\[
\text{Pyruvate} + \text{BH}_4^- \rightarrow \text{D-lactate}
\]

\[
\text{Oxalacetate} + \text{BH}_4^- \rightarrow \text{L-lactate}
\]

Before reducing the enzyme-pyruvate complex, formed by adding pyruvate from solution, to given D-lactate (Equation 4). On the other hand, if the CO_2 formed from decarboxylation of oxalacetate leaves the enzyme after pyruvate, the BH_4^- could act only at the α face of the carbonyl giving L-lactate (Equation 5). A similar hypothesis involving specific BH_4^- binding to the β-carboxyl site was proposed by Kosicki and Westheimer to explain their failure to observe stereoselective reduction of oxalacetate by the decarboxylase (2). Apparently only racemic malate was recovered.

**Effect of BF_4^- on Decarboxylase Activity**—As a test of the presence of a common binding site for BH_4^- and oxalacetate on the decarboxylase, the effect of NaBF_4, a presumed analog of NaBH_4, on the K_m of oxalacetate in the decarboxylase assay was examined and found to be a linear competitive inhibitor with a K_i of 18 mM (Fig. 4). As a comparison, 100 mM F^- or NO_3^- gave less than 20% inhibition when oxalacetate was at its K_m concentration (0.25 mM). This corresponds to a K_i of >200 mM.

**Effect of BF_4^- on Enzymic Enolization of Pyruvate**—Importantly, NaBF_4 does not inhibit the enzyme-catalyzed enolization of pyruvate first observed by Kosicki (3), indicating that a ternary E-BF_4^-pyruvate complex can form (Fig. 5).

**Evidence That Decarboxylase and Pyruvate Kinase Activity Are due to Single Active Site**—The failure of NaBF_4 to inhibit the enolization of pyruvate would also result if the enolization were due to another unrelated enzyme present in the decarboxylase preparation. Since rabbit muscle pyruvate kinase is known to catalyze this reaction (13), P-enolpyruvate-ADP phosphorvtransfer activity in the cod muscle enzyme was assayed for by coupling to lactate dehydrogenase. Unexpectedly, the kinase activity exceeded by 230-fold the oxalacetate decarboxylase rate in this apparently homogeneous preparation of decarboxylase. The dilemma of the inconsistent reduction stereochemistries seemed, briefly, to be explained by the presence of two different enzymes since Phillips et al. recently reported that pyruvate kinase (of rabbit muscle) catalyzes the reduction of pyruvate by BH_4^- to give D-lactate as the predominant form (14). However, this was not the case since subsequent studies demonstrated convincingly that both the decarboxylase and kinase activities are properties of a single enzyme. Evidence supporting this conclusion based on protein fraction procedures, rates of inactivation of the two activities, and modifier effects is presented in the following paper (4). In addition, Tsai and Mildvan have established that the Mn^2+ activator constants for both the decarboxylase and pyruvate kinase activities are similar for the cod muscle enzyme preparation. Therefore, it became of particular interest, because of the opposite actions of NaBH_4 to ask whether the kinase and decarboxylase activities occur at the same site on the enzyme.

The conclusion that both activities occur at the same or overlapping sites is supported by the findings of the following paper that (a) P-enolpyruvate is a strong linear competitive inhibitor with respect to oxalacetate, K_i = 3.75 μM, in the cod muscle decarboxylase reaction, (b) 4-ethoxalacetate, previously shown to be a strong inhibitor of cod muscle decarboxylase, 1 has approximately the same K_i value, ~11 μM, as a linear
Mechanism of Oxalacetate Decarboxylase

competitive inhibitor against oxalacetate and P-enolpyruvate in the decarboxylase and pyruvate kinase reactions, respectively, and (c) both P-enolpyruvate and oxalacetate exhibit cooperative binding kinetics in their respective reactions and are almost equally responsive to fructose 1,6-bisphosphate, an allosteric activator which shifts the Hill constant toward one (4). Finally, as evidence that decarboxylase and kinase may be structurally linked activities, crystalline rabbit muscle pyruvate kinase was observed to have significant decarboxylase activity (0.5 unit/mg) that was completely inhibited by P-enolpyruvate.

Effect of K+, P, and ATP on Enolization—The enolization of pyruvate by pyruvate kinase is known to require K+ and ATP (or P) as well as Mg2+ (or Mn2+) (13). Thus the enolization of pyruvate formed during oxalacetate decarboxylation (Table I) may be expected if the same catalytic site is involved since KPO4 was present at 10 mM. Using rabbit muscle pyruvate kinase it is observed that neither K+ nor ATP (or P) is required for decarboxylase activity, nor do they significantly influence the oxalacetate to pyruvate rate. On the other hand it could be shown (Table II) that both K+ and ATP are required for the loss of tritium from [3-3H]oxalacetate to occur. Exchange was previously seen (Table I) with K+ and P, and the cod enzyme. It follows that the same factors required for dehydrogenase conversion of pyruvate cause dehydrogenation of the pyruvate generated on the enzyme from oxalacetate. However, since neither K+ nor P (or ATP) is required for the formation of pyruvate from oxalacetate, it would seem that their role is not a mechanistic one but a kinetic one. Robinson and Rose have suggested that the effect of high K+ in causing excessive enolization of P-[3-3H]enolpyruvate to form the P-enolpyruvate-ADP phosphotransferase reaction is to slow the synthesis of the enzyme-bound pyruvate (11). A similar explanation would satisfy the observations with [3-3H]oxalacetate. Here ATP (or P) is also needed whereas with P-[3-3H]enolpyruvate the ATP is generated on the enzyme. The absence of effects of K+ and ATP on decarboxylase reaction rates suggests that neither the ketonization nor product release step is rate-determining for decarboxylation.

Although the similar effect of K+ in causing the excessive enolizations from pyruvate derived from either oxalacetate or P-enolpyruvate suggests that the same site is involved for both reactions, it should be noted that under the same conditions the pyruvate derived from oxalacetate is much more sensitive to excessive enolization. Thus at 50 mM KCl and 10 mM ATP about 80% detritiation was observed with [3-3H]oxalacetate, whereas only 10% would be seen with P-[3-3H]enolpyruvate (11). Likewise in Table I more than 60% of the tritium of [3-3H]oxalacetate was lost in decarboxylation by the cod enzyme. Since detritiation at the prochiral carbon of oxalacetate, if it occurred, would probably be stereospecific, it is unlikely that the difference would be due to enolization of oxalacetate. The finding that in the presence of NaBH4 no tritium is lost (Fig. 1) is further evidence that the detritiation occurs at the pyruvate level only.

Stereochemistry of Decarboxylation—Oxalacetate, stereospecifically labeled with deuterium and tritium at C-3, was decarboxylated with the rabbit muscle enzyme after rigorously excluding K+, NH4+, and P, from the reaction mixture. Under these conditions it was not necessary to trap the pyruvate produced. Fig. 6 shows the scheme for establishing whether decarboxylation involves retention or inversion of configuration using (3R) [3-3H]oxalacetate, generated from (3R) [3-3H]malate, as a substrate for the decarboxylase. The pyruvate product was isolated and converted to malate in a coupled system using pyruvate carboxylase and malate dehydrogenase. The carboxylation reaction is known to be mediated by an intramolecular isotope effect where k, / k, = 3.3 (7). The L-malate product was then treated with fumarase which labels only the pro-R hydrogen isotope during the formation of fumarate. If the decarboxylation reaction involves retention of configuration and (3R) [3-3H]oxalacetate is the substrate, the fumarase step will result in a majority of the tritium being lost to water. On the other hand, if inversion is involved, most of the tritium will be retained in fumarate.

The results of Table III indicate retention of configuration. Using (3R) [3-3H]malate as a source of the substrate, 84% of the counts of the recovered malate were lost to solvent in the fumarase step, close to the expected discrimination on the basis of the reported intramolecular isotope effect in the pyruvate carboxylase step (7). Retention of configuration is confirmed using (3S) [3-3H]malate as the starting material since most of the label is found in the pro-S position of malate in the fumarase step. The poorer discrimination observed with this enantiomer likely reflects the greater decrease in specific activity going from the starting malate to the malate species used in the fumarase reaction. Contaminating NH4+ and P, in the incubation mixture during the decarboxylation reaction could account for this decrease.

It has been proposed that the enzymes of pyruvate metabolism are related in evolution as suggested by similar stereochemistries in all reactions involving P-enolpyruvate (2-s face addition) and in proton replacement on pyruvate (retention). Retention has also been observed with malic enzyme (malate dehydrogenase decarboxylating) and with pyruvate carboxylase.

Chiral Pyruvate—The establishment of retention of configuration for the decarboxylation reaction of rabbit muscle pyruvate kinase provides a direct method for producing chirally labeled [3H2,3H]pyruvate, that is more convenient, using only commercially available enzymes, than that previously described (7). For example, deuterium can be introduced into the pro-R position of malate using the fumarase equilibrium in
catalyzed process (Table III). Thus, each step in the reaction, which includes C–C bond cleavage and subsequent protonation of the enolpyruvate intermediate, is completely determined by the enzyme. From earlier studies on the stereochemistry of the pyruvate kinase reaction (7), proton addition to enolpyruvate derived from P-enolpyruvate was shown to come from the 2-si face. If it were certain that the enolpyruvate formed from oxalacetate had the same orientation on the enzyme as that from P-enolpyruvate, it could be concluded that both CO₂ departure and proton addition occur from the 2-si face as well. However, this may be an unsafe assumption. First, K⁺ and ATP or P, are not required for, nor do they significantly affect, the decarboxylase reaction while these compounds are required in the catalyzed enolization of pyruvate (13). Second, the loss of tritium to solvent from [3-3H]oxalacetate during decarboxylation is much greater than the loss to solvent from p-[3-3H]enolpyruvate during the kinase reaction. The ratio of rate constants for enolization and pyruvate-off steps (k₄ and k₅ of Equation 2) should be the same if the enzyme-pyruvate complex formed from oxalacetate and P-enolpyruvate were the same. Finally, the alternate borohydride reduction stereochemistry may be a consequence of an additional E-pyruvate intermediate in the decarboxylase reaction that normally produced in the kinase reaction (Equation 3).

Attempts to catch enzyme-bound pyruvate generated from P-enolpyruvate with NaBH₄ has not yet been successful. Earlier results of Robinson and Rose (11) (Table III) showed that in the P-enolpyruvate–pyruvate reaction most of the enzyme was present in the enzyme-pyruvate form. The greater accessibility to BH₄⁻ of the oxalacetate-derived enzyme pyruvate complex may be related to its greater exchange rate with water, referred to already.

All of the differences cited might result from an interaction between the active site and oxalacetate not like that interaction found in the enzyme-bound intermediates of the pyruvate kinase fraction. This interaction may be reflected in the low Kₘ (0.2 mM) of oxalacetate and K₅ of 4-ethylmalolactate (~11 µM) for the cod muscle enzyme compared to the K₄₅ of ~3 mM for pyruvate inhibition of decarboxylase activity reported by Schmitt et al. (1). It might be that a strong interaction with the C-4 carboxyl group puts the enzyme into a conformation that allows different interactions with the medium and active site to occur throughout the catalytic process.

**CONCLUDING REMARKS**

The oxalacetate decarboxylase activity of pyruvate kinase exhibits stereospecificity as would be expected of an enzyme-
REFERENCES

Studies on the mechanism and stereochemical properties of the oxalacetate decarboxylase activity of pyruvate kinase.

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