Studies on *Escherichia coli* Pyruvate Dehydrogenase Complex

I. EFFECT OF BROMOPYRUVATE ON THE CATALYTIC ACTIVITIES OF THE COMPLEX*

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SUMMARY

Bromopyruvate inactivates the pyruvate dehydrogenase complex of *Escherichia coli* in a thiamine pyrophosphate (TPP)-dependent process. The catalytic activities of the individual enzyme components within the complex are not destroyed by bromopyruvate under similar conditions, but the activities of the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase components are reduced in thiamine pyrophosphate-independent processes. Radioactivity from [2-¹⁴C]bromopyruvate is irreversibly bound in the presence or absence of thiamine pyrophosphate, but the amount of radioactivity bound in the presence of thiamine pyrophosphate is 3.9 times the amount bound in its absence. The data are consistent with the interpretation that TPP-dependent inactivation involves the action of bromopyruvate as an irreversible inhibitor which is directed into the complex by specific, but not necessarily irreversible, interaction with the pyruvate dehydrogenase component and ultimately blocks some site within the complex which is essential for catalytic activity in the over-all reaction but not in partial reactions catalyzed by the component enzymes.

The pyruvate dehydrogenase complex of *Escherichia coli* is a large multienzyme complex which catalyzes the reaction described by Equation 5 (1, 2). It is generally believed that in its physiological state this complex is engaged

\[ \text{H}^+ + \text{pyruvate} + E_1\cdot\text{TPP} \rightarrow \text{CO}_2 + E_1\cdot\text{hydroxyethyl-TPP} \]  
\[ E_1\cdot\text{hydroxyethyl-TPP} + E_1\cdot\text{lip} \rightarrow \]  
\[ E_1\cdot\text{TPP} + E_1\cdot\text{lip}(\text{SH})\cdot\text{S-acetyl} \]  

primarily in catalyzing Reaction 5, and we refer to it here as the "over-all reaction." Equations 1 to 4 outline the proposed molecular pathway of the over-all reaction indicating the roles played by the five coenzymes involved and by the three enzyme components of the complex (1, 2). In these equations, \( E_1 \) is pyruvate dehydrogenase, which catalyzes the TPP-dependent decarboxylation of pyruvate and the dehydrogenation of hydroxyethyl-TPP; \( E_2 \) is dihydrolipoyl transacetylase, which catalyzes the transfer of acetyl groups from acetyl dihydrolipoamide to CoASH, and \( E_3\cdot\text{FAD} \) is dihydrolipoyl dehydrogenase, which catalyzes the dehydrogenation of dihydrolipoamide dehydrogenase.

The individual components of the complex, and the complex itself, catalyze several other reactions which have been used as the bases of assay methods for purifying the individual enzymes (4). These include the reactions described by Equations 6 to 8.

\[ \text{Pyruvate} + \text{H}_2\text{O} + 2\text{Fe(CN)}_6^{3-} \xrightarrow{E_1\cdot\text{TPP}} \text{acetate} + \text{CO}_2 + 2\text{Fe(CN)}_6^{4+} + 2\text{H}^+ \]  
\[ \text{Acetyl-} - \text{CoA} + \text{lipoamide(SH)}_2 \xrightarrow{E_2} \text{lipoamide(SH)} - \text{S-acetyl} + \text{CoASH} \]  
\[ \text{H}^+ + \text{DPNH} + \text{lipoamide} S_2 \xrightarrow{E_3\cdot\text{FAD}} \text{DPN}^+ + \text{lipoamide(SH)}_2 \]  

The pyruvate dehydrogenase catalyzes Reaction 6, dihydrolipoyl transacetylase catalyzes Reaction 7, and dihydrolipoyl dehydrogenase catalyzes Reaction 8. The pyruvate dehydrogenase

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1 The abbreviations used are: TPP, thiamine pyrophosphate; lip(SH)_2, lip(SH)_3, and lip(SH)_S—acetyl refer to lipoic acid, dihydrolipoic acid, and 5-acetyl dihydrolipoic acid covalently bonded to dihydrolipoyl transacetylase by amide linkage to a lysino-ε-amino group; lipoamide S_2, lipoamide(SH)_2, and lipoamide (SH)_S—acetyl are the similar forms of lipoamide which are utilized as substrates or produced as products in the partial reactions.

* The naming of the components of this complex has not been consistent. We are using the names recently suggested by Reed et al. (1, 3). This nomenclature differentiates between the names pyruvate dehydrogenase, which refers to the TPP-dependent component, and pyruvate dehydrogenase complex, which refers to the whole complex.
complex itself catalyzes all of these reactions, and we refer to them as the "partial reactions" catalyzed by the complex.

Reed and his coworkers have conducted extensive research on the purification and physical characterization of this complex (5), on the resolution of the complex into its individual enzyme components and its reconstitution from the purified components (6), and on its regulatory properties (7). In this paper we report on the effect of bromopyruvate, a substrate analog, on the catalytic activities of this complex in the over-all and partial reactions and on the TPP-dependent irreversible binding of [2-14C]bromopyruvate.

EXPERIMENTAL PROCEDURE

Materials—E. coli K-12 cells (1 log, washed) were obtained from Grain Processing Corp., Muscatine, Iowa. Coenzymes and substrates were commercial preparations obtained from the following sources: TPP, Calbiochem and Nutritional Biochemicals; CoASH, Calbiohem and Sigma; oxidized lipoamide, Nutritional Biochemicals and Sigma; DPN+ and DPNH, Sigma and Boehringer Mannheim, respectively; acetyl-P, Boehringer Mannheim; and bromopyruvate and potassium pyruvate, Calbiochem. Rabbit muscle lactate dehydrogenase was purchased from Calbiochem and Boehringer Mannheim and phosphotransacetylase from Kluyveraer. Sodium [2-14C]pyruvate was purchased from New England Nuclear. Dihydrolipoamide was prepared from oxidized lipoamide as described (8).

Assays—During purification of the pyruvate dehydrogenase complex we measured its activity by the dismutation assay procedure described by Reed and Willms (4), which is based on Equation 9.

\[
2\text{CH}_3\text{C}-\text{CO}_2^+ + \text{HPO}_4^{2-} + \text{H}^+ \rightarrow \text{CH}_3\text{C}-\text{OP}_2\text{O}^- + \text{CO}_2 + \text{CH}_2\text{CH}^- + \text{CO}_2
\]

This is a coupled assay in which the production of DPNH is coupled to the reduction of pyruvate by lactate dehydrogenase and the production of acetyl—S—CoA is coupled to the production of acetyl-P by phosphotransacetylase. Acetyl-P produced is measured as the Fe+3 complex of acetylhydroxamate.

We assayed the purified enzyme directly by measuring the initial rate of DPNH production in Reaction 5. The complete reaction mixture in this uncoupled assay consisted of 0.05 M potassium phosphate buffer, pH 8.1, 5.0 \times 10^{-3} M CoASH, 3.0 \times 10^{-5} M cystaine, 2.33 \times 10^{-5} M DPN+, 2.0 \times 10^{-4} M TPP, 1.0 \times 10^{-4} M MgSO4, 2.0 \times 10^{-4} M pyruvate, and enzyme in a total volume of 1.0 ml. We monitored the increase in \(A_{340}\) continuously at 27°C using a Norelco-Unicam SP-800 spectrophotometer equipped with an external recorder and scale expansion accessory. One unit of activity produced 1 pmole of DPNH per min under these conditions.

We assayed the pyruvate dehydrogenase activity of the complex spectrophotometrically by measuring the disappearance of Fe(CN)_{6}^{4-} in Reaction 6. The complete reaction mixture in this assay was that described by Schwartz and Reed (7) and consisted of 2.0 \times 10^{-3} M Tricine (N-tris(hydroxymethyl)methylglycine) buffer, pH 7.7, 5.0 \times 10^{-3} M pyruvate, 1.2 \times 10^{-3} M MgCl2, 1.0 \times 10^{-4} M TPP, 1.8 \times 10^{-3} M K_{3}Fe(CN)_{6}, and enzyme in the sample cuvette. The decrease in \(A_{420}\) was monitored continuously versus a reference cuvette containing 0.6 \times 10^{-3} M K_{3}Fe(CN)_{6} at 27°C. We observed a biphasic decrease in absorbance at 420 nm as the reaction proceeded, in which the initial non-zero order rate decreased within a few minutes to a constant zero order rate. We have not as yet established the basis for the biphasic behavior. It was not changed by initiating the reaction with TPP or pyruvate instead of by enzyme. Both the initial rate and the final zero order rate were proportional to enzyme concentration. We used the zero order rate in our activity calculations because it was more easily and accurately measured. One unit of activity consumed 2 \(\mu\)moles of Fe(CN)_{6}^{4-} per hour under the assay conditions.

We measured the dihydrolipoyl transacetylase and the dihydrolipoyl dehydrogenase activities of the pyruvate dehydrogenase complex according to the assay procedures described by Reed and Willms (4). These assays were based on Reactions 7 and 8, respectively.

Protein concentrations were measured by the method of Lowry using crystalline bovine serum albumin as the standard (9).

Radiochemical assays were performed by liquid scintillation counting in a Packard model 3110 Tri-Carb liquid scintillation spectrometer. The scintillation solvent contained 7 g of 2,5-diphenyloxazole, 300 mg of p-bis[2-(5-phenylloxazolyl)]benzene, and 100 g of naphthalene per liter of dioxane solution. For each assay 1.0 ml of aqueous sample was combined with 15 ml of scintillation solvent.

Purification of Pyruvate Dehydrogenase Complex—We purified pyruvate dehydrogenase complex from E. coli K-12 according to the procedure of Koike, Reed and Carroll (5), as described by Reed and Willms (4), for purification of the enzyme from Crookes strain. Our purification data were similar to those described by Reed and Willms (4). We have not conducted extensive physical studies on the purity and molecular weight of the complex; however, the catalytic activities of our preparations were similar to those of preparations obtained by Reed and coworkers from Crookes strain. The activities of our preparations in the uncoupled assay based on Reaction 5 were 25 to 35 units per mg of protein as compared with 18 to 20 reported for the Crookes strain enzyme in similar assays (2, 7). The activities of our preparations in the d.hydrolipoyl transacetylase and d.hydrolipoyl dehydrogenase assays were 180 to 230 and 20 units per mg of protein, respectively, compared with the corresponding values of 120 to 130 and 20 units per mg of protein reported for the complex isolated from Crookes strain (5). Koike et al. (5) measured the pyruvate dehydrogenase activity of their preparations under assay conditions which differed from those we have routinely used. The specific activities of their preparations of the complex were about 12 units per mg of protein. The activities of our preparations ranged between 8.5 and 9.5 units per mg of protein when assayed by the same procedure (4). In the dissimuta
tion assay based on Reaction 9 our preparations were significantly less active, 450 to 600 units per mg of protein as compared with 900 to 1300 units per mg of protein quoted by Koike, Reed, and Carroll for the complex they isolated (5).

Preparation of Bromopyruvic Acid—We synthesized bromopyruvic acid by direct bromination of pyruvic acid in the presence of H2SO4 as the catalyst as described in (10). We prepared [2-14C]bromopyruvic acid on a small scale by a similar procedure described by Meloche (11) with one modification. We did not add one drop of H2SO4 catalyst because it interfered in our
enzymatic experiments and was difficult to remove. We found that bromine stored under H₂SO₄ was effective in brominating pyruvic acid in acetic acid solvent. Occasionally we added 0.5 μl of H₂SO₄ catalyst when the reaction was very slow.

RESULTS

TPP-dependent Inactivation of Pyruvate Dehydrogenase Complex—In a TPP-dependent process, bromopyruvate destroys the activity of the E. coli pyruvate dehydrogenase complex as a catalyst for the over-all reaction (Equation 5). As illustrated in Fig. 1, 0.001 M bromopyruvate inactivates the complex in the presence of 2 × 10⁻⁴ M TPP with a half-time of about 9 min, whereas in the absence of TPP the activity decreases at less than one-tenth of that rate. Fig. 1 shows first order kinetics for 80% of the reaction, but in other experiments we have observed first order kinetics for over 70% of the reaction. The effect of bromopyruvate in this reaction appears to be irreversible, as indicated by the fact that gel filtration over Sephadex G-25 does not reactivate the complex. It is not reactivated by substrates under the conditions of the uncoupled assay, in which the bromopyruvate concentration has been reduced by dilution to 2 × 10⁻⁴ M, nor is it reactivated by lipoylamine S₃ or lipoylamine(SH)₂ after isolation from bromopyruvate by gel filtration over Sephadex G-25.

Bromopyruvate is an unstable compound in the physiological pH range (11), and it is not noted for its purity under any conditions. Melting points between 58 and 75° have been reported, always with a broad melting point range (10-12). It undergoes hydrolysis at a significant rate above pH 6 and at pH 8 it is completely hydrolyzed within 1 hour, as indicated by the volume of base consumed by a pH-stat set at pH 8. Our synthetic samples of bromopyruvate acid and those obtained commercially from Calbiochem are similar and are not improved, with respect to melting point range, even by repeated recrystallization from chloroform. The possibility therefore exists that the TPP-dependent inactivation depicted in Fig. 1 may be caused by some molecule other than bromopyruvate which is present in all samples of bromopyruvate. We consider this to be only a remote possibility; we find that none of the materials used to synthesize bromopyruvic acid and none of the compounds known to be produced in the hydrolysis of bromopyruvate, including hydroxybromopyruvate (10), exerts a similar effect upon the activity of the pyruvate dehydrogenase complex in Reaction 5.

Effect of Bromopyruvate on Individual Enzymes—In order to gain information about the site of action of bromopyruvate in the TPP-dependent inactivation of the complex, we have studied its effects upon the activities of the individual enzymes within the complex. We obtained the data of Figs. 2 and 3 and of Table I, in which all of the reaction mixtures were similar to those of Fig. 1 with the exception that the experiments were conducted at pH 6.75 instead of at pH 7.

In Fig. 2 we measured the pyruvate dehydrogenase (E₃) ac-
The reaction mixtures were identical with those of Fig. 2 except that the concentration of pyruvate dehydrogenase complex was 8 µg per ml. Aliquots were removed at various times and diluted into ice-cold buffer, and the samples were assayed for dihydrolipoyl transacetylase activity as described (4). The data were obtained in five experiments, two with TPP present and three without TPP present. Similar data were obtained at 1, 4 and 12 min.

The reaction mixtures contained 0.10 m potassium phosphate, pH 6.75, 0.01 m MgSO₄, 2.0 × 10⁻⁴ m TPP, 2.8 × 10⁻³ m bromopyruvate, and 0.001 m pyruvate dehydrogenase complex in a total volume of 0.50 ml at 27°. In each experiment the activities of pyruvate dehydrogenase complex and of one of the component enzymes were measured at the indicated times. Data in Table I suggest but do not clearly show that, in the presence of TPP, the activity of the complex in the over-all reaction could be essentially completely destroyed by bromopyruvate, whereas its activity in the partial reactions would not be reduced to zero. Specifically, Fig. 1 is a semilog plot of specific activity versus time, and the data on TPP-dependent inactivation produce a straight line. This is consistent with the interpretation that inactivation is first order in enzyme and that the activity at infinite time would be zero or nearly zero. If it did not approach zero the plot should show upward curvature. The data do not exclude other more complex, in our opinion less likely, interpretations. On the other hand, the data of Figs. 2 and 3 and Table I show that the activities of the individual enzyme components would not approach zero in the presence of TPP, certainly not at a rate comparable to that of Fig. 1.

The data given in Table II more clearly show that, under experimental conditions in which the catalytic activity of the complex in the over-all reaction is nearly completely destroyed, the catalytic activities of the component enzymes in the partial reactions are reduced but do not closely approach zero. Table II compiles data from three experiments in which pyruvate dehydrogenase complex was permitted to react with 0.028 m bromopyruvate, 28 times the concentration employed in Figs. 1 to 3 and Table I, in the presence and absence of 2 × 10⁻⁴ m TPP. In each experiment we measured the catalytic activity of the complex in the over-all reaction and the activity of one component of the complex in the corresponding partial reaction after 1-min and 15-min incubation. Our data on the activity of the complex in the over-all reaction in the three experiments are averaged in the first line of Table II. The data show that under conditions in which the complex has lost 99% of its activity as a catalyst for the over-all reaction it retains 15 to 90% of its activity as a catalyst for the partial reactions. The data also confirm that, at least within the 1st min, most of the destruction of activity in the over-all reaction is TPP-dependent, whereas TPP has little or no influence on the effect of bromopyruvate in reducing the activities of the individual components. The modest reduction in the activity of dihydrolipoyl transacetylase in this experiment as contrasted with the data of Table I we attribute to the much larger concentration of bromopyruvate.

**TPP-dependent Binding of [2-14C]Bromopyruvate.—**We have performed experiments with [2-14C]bromopyruvate in order to learn whether the TPP-dependent inactivation of the complex is accompanied by irreversible binding of bromopyruvate. The

### Table I

**Activity of dihydrolipoyl transacetylase in presence of bromopyruvate**

The complete reaction mixtures contained 0.10 m potassium phosphate, pH 6.75, 0.01 m MgSO₄, 2.0 × 10⁻⁴ m TPP, 2.8 × 10⁻³ m bromopyruvate, and 0.001 m pyruvate dehydrogenase complex in a total volume of 0.50 ml at 27°. In each experiment the activities of pyruvate dehydrogenase complex and of one of the component enzymes were measured at the indicated times. Data on pyruvate dehydrogenase complex in the three experiments are given in the first line.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Activity of dihydrolipoyl transacetylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+TPP</td>
</tr>
<tr>
<td>0</td>
<td>174</td>
</tr>
<tr>
<td>2</td>
<td>173  ± 2</td>
</tr>
<tr>
<td>8</td>
<td>173  ± 2</td>
</tr>
<tr>
<td>15</td>
<td>172  ± 4</td>
</tr>
<tr>
<td>30</td>
<td>177  ± 3</td>
</tr>
</tbody>
</table>

### Table II

**Relative effects of bromopyruvate on activities of complex and its components**

The complete reaction mixtures contained 0.10 m potassium phosphate, pH 6.75, 0.01 m MgSO₄, 2.0 × 10⁻⁴ m TPP, 2.8 × 10⁻³ m bromopyruvate, and 0.001 m pyruvate dehydrogenase complex in a total volume of 0.50 ml at 27°. In each experiment the activities of pyruvate dehydrogenase complex and of one of the component enzymes were measured at the indicated times. Data on pyruvate dehydrogenase complex in the three experiments are given in the first line.

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Percentage of residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>+TPP</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase complex</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>23</td>
</tr>
<tr>
<td>Dihydrolipoyl transacetylase</td>
<td>100</td>
</tr>
<tr>
<td>Dihydrolipoyl dehydrogenase</td>
<td>84</td>
</tr>
</tbody>
</table>

The data of Table I, obtained under similar conditions, show that 0.001 m bromopyruvate has no detectable effect upon the dihydrolipoyl transacetylase (E₃) activity of the complex either in the presence or absence of 2 × 10⁻⁴ m TPP.

The data in Figs. 2 and 3 show that the effect of bromopyruvate upon the activities of two of the individual enzymes within the complex in their partial reactions (Equations 6 and 8) does not parallel its effect upon the activity of the complex in the over-all reaction (Equation 5) shown in Fig. 1. In particular, neither of those enzymes within the complex are subject to TPP-dependent inactivation by bromopyruvate. TPP exerts an effect only upon the activity of the pyruvate dehydrogenase component (Fig. 2); in that case it is a protective effect.

Relative Degrees of Inactivation by Bromopyruvate—Figs. 1 to 3 and Table I suggest but do not clearly show that, in the presence of TPP, the activity of the complex in the over-all reaction could be essentially completely destroyed by bromopyruvate, whereas its activity in the partial reactions would not be reduced to zero. Specifically, Fig. 1 is a semilog plot of specific activity versus time, and the data on TPP-dependent inactivation produce a straight line. This is consistent with the interpretation that inactivation is first order in enzyme and that the activity at infinite time would be zero or nearly zero. If it did not approach zero the plot should show upward curvature. The data do not exclude other more complex, in our opinion less likely, interpretations. On the other hand, the data of Figs. 2 and 3 and Table I show that the activities of the individual enzyme components would not approach zero in the presence of TPP, certainly not at a rate comparable to that of Fig. 1.

The data given in Table II more clearly show that, under experimental conditions in which the catalytic activity of the complex in the over-all reaction is nearly completely destroyed, the catalytic activities of the component enzymes in the partial reactions are reduced but do not closely approach zero. Table II compiles data from three experiments in which pyruvate dehydrogenase complex was permitted to react with 0.028 m bromopyruvate, 28 times the concentration employed in Figs. 1 to 3 and Table I, in the presence and absence of 2 × 10⁻⁴ m TPP. In each experiment we measured the catalytic activity of the complex in the over-all reaction and the activity of one component of the complex in the corresponding partial reaction after 1-min and 15-min incubation. Our data on the activity of the complex in the over-all reaction in the three experiments are averaged in the first line of Table II. The data show that under conditions in which the complex has lost 99% of its activity as a catalyst for the over-all reaction it retains 15 to 90% of its activity as a catalyst for the partial reactions. The data also confirm that, at least within the 1st min, most of the destruction of activity in the over-all reaction is TPP-dependent, whereas TPP has little or no influence on the effect of bromopyruvate in reducing the activities of the individual components. The modest reduction in the activity of dihydrolipoyl transacetylase in this experiment as contrasted with the data of Table I we attribute to the much larger concentration of bromopyruvate.
Fig. 4. TPP-dependent irreversible binding of [2-14C]bromopyruvate. The complete reaction mixture for A contained, at 27°, 0.1 m potassium phosphate buffer, pH 6.75; 0.01 m MgSO4, 2.0 × 10⁻⁴ m TPP; 0.038 m [2-14C]bromopyruvate (0.1 × 10⁶ cpm per mole), and 4.0 mg of pyruvate dehydrogenase complex in a total volume of 0.50 ml. TPP was omitted in B. In each experiment an aliquot was removed for assay after 1 min and the remainder was immediately passed into a column of Sephadex G-25 and eluted with 0.02 m potassium phosphate buffer, pH 7.0. Fractions of 1-ml volume were collected and analyzed for protein content, A₂₈₀, and radioactivity. Symbols: O——O, A; ——, radioactivity.

The above data lead us to conclude that bromopyruvate exerts at least three classes of effects upon the catalytic activities of E. coli pyruvate dehydrogenase complex. The first is TPP-dependent destruction of its catalytic activity in the over-all reaction. The second is TPP-independent reduction of its catalytic activities in two of the partial reactions. Inasmuch as the time dependence for its effect on dihydrolipoyl dehydrogenase differs from that for pyruvate dehydrogenase, the second class probably includes more than one reaction. The third class is the slow secondary inactivation of the pyruvate dehydrogenase component in the absence of TPP shown in Fig. 2 to be reduced in rate by 2 × 10⁻⁴ m TPP.

The data on binding of [2-14C]bromopyruvate reveal at least two classes of sites at which 14C is irreversibly bound, one independent of TPP and the second TPP-dependent. The data as presented do not exclude the possibility that the complex might retain some TPP throughout the purification, so that TPP-dependent and TPP-independent binding might be identical molecular processes. However, our preparations of complex require added TPP for activity in the over-all reaction, as measured by the uncoupled assay. In addition it has been reported that TPP binding to this complex is freely reversible (14). We therefore interpret the results to mean that there are two classes of irreversible binding interactions.

TPP dependence in the destruction of catalytic activity for the over-all reaction and in irreversible binding of 14C from [2-14C]bromopyruvate suggests that bromopyruvate interacts with the pyruvate dehydrogenase component of the complex in place of pyruvate. This interpretation is strengthened by the fact that bromopyruvate is a structural analog of pyruvate. In addition, bromopyruvate is probably not too bulky to interact as a substrate since α-ketobutyrate is known to be a substrate for this complex (4). This initial TPP-dependent interaction with pyruvate dehydrogenase probably does not block the active site of this component because, as shown in Fig. 2, the activity of the complex in catalyzing partial Reaction 6 is not subject to TPP-dependent destruction. It appears likely that this initial interaction introduces the bromopyruvate molecule into the normal sequence of reaction steps experienced by substrates and that bromopyruvate undergoes one or more of these reactions. How-
ever, at some point it is either unable to proceed to the next step or its chemical disposition diverges from the normal sequence of catalytic steps in such a way as to block catalytic activity for the over-all reaction and lead to irreversible binding of $^{14}$C from [2-\textsuperscript{14}C]bromopyruvate.

The present data do not identify the site or sites at which $^{14}$C from [2-\textsuperscript{14}C]bromopyruvate is bound to pyruvate dehydrogenase complex in its TPP-dependent reaction(s). However, on the basis of the present results and the known properties of the component enzymes in the partial reactions they catalyze, we are tempted to speculate that the lipoyl moieties covalently attached to dihydrolipoyl transacetylase may be involved. The speculation is interpretive and tentative, since it is not supported by direct evidence. Two facts established here appear to us to be significant in this connection. First, no component of the complex is subject to TPP-dependent inactivation. Second, the catalytic activities of the complex in the partial reactions are not destroyed under conditions in which its activity in the over-all reaction is essentially completely destroyed, although the dihydrolipoyl dehydrogenase and pyruvate dehydrogenase activities are reduced in TPP-independent reactions. We conclude that TPP-dependent inactivation results from the blocking of some site or sites essential to activity in the over-all reaction but not required for or involved in catalyzing the partial reactions. Such a site could be the lipoyl moieties covalently bonded to the dihydrolipoyl transacetylase component, since it is known that they are not essential for and probably not involved in catalyzing the partial reactions. That they are not required for the partial reaction activities is established by the work of others as follows. Pyruvate dehydrogenase and dihydrolipoyl dehydrogenase retain their activities as catalysts for reactions 6 and 8 after being resolved from dihydrolipoyl transacetylase (6). The activity of dihydrolipoyl transacetylase component in catalyzing Reaction 7 is reported to be unaffected by enzymatic removal of covalently bonded lipoyl moieties from the complex (15). In Equation 7 an acetyl group is transferred from acetyl-CoA to a thiol group of lipoamide($\text{SH}_2$). Since removal of the prosthetic lipoyl groups from dihydrolipoyl transacetylase does not affect activity in Reaction 7, it appears likely that they are not involved in this reaction and that lipoamide($\text{SH}_2$) accepts the acetyl group directly by binding at the lipoyl site of dihydrolipoyl transacetylase in place of the prosthetic lipoyl moiety (15). This interpretation, and similar interpretations of the fact that the activity of dihydrolipoyl dehydrogenase within the complex is also unaffected by the removal of prosthetic lipoyl moieties (15), are consistent with the current view of the mobility of the prosthetic lipoyl moieties, which is that they do not occupy fixed sites within the complex but instead oscillate among the active sites of the three enzyme components (1). It is presumably this mobility which enables the lipoyl moieties to mediate transfer of acetyl groups and electrons among the several active sites within the complex.

It appears, therefore, that if the lipoyl moieties were blocked the complex would be inactive as a catalyst for the over-all reaction, which requires their participation. However, given the mobility of the lipoyl moieties, the complex might retain activity as a catalyst for the partial reactions. This is what we observe in the case of TPP-dependent inactivation. Given the complexity of this system, however, further work is required to identify the sites at which bromopyruvate acts.

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