Studies on the Nature of the Binding of Thiamine Pyrophosphate to Enzymes

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SUMMARY

The binding of thiamine pyrophosphate (TPP) to several enzymes has been determined by measuring cofactor-dependent activity after passage of each enzyme through a column of Sephadex G-25 to remove non-protein-bound cofactors. TPP was found to be bound irreversibly to yeast and Zymomonas pyruvate decarboxylases, Aerobacter α-acetolactate synthetase, and Escherichia glyoxylate carboligase. Cofactors were lost when Proteus pyruvate oxidase, Escherichia pyruvate dehydrogenase, and Micrococcus diacetyl carboligase were gel-filtered; the binding of TPP was strongest for diacetyl carboligase. A procedure has been devised for efficient resolution of enzymes for TPP and divalent cations. Resolved enzymes reconstituted for cofactors had properties similar to those of native enzymes. Resolved pyruvate decarboxylases from both yeast and Zymomonas mobilis failed to bind Mg++ in the absence of TPP. Cofactor reconstitution for yeast pyruvate decarboxylase was shown to be a slow process for low concentrations of TPP. TPP alone, in high concentrations, was able to activate and partially reconstitute TPP enzymes in the absence of added divalent cations. Zymomonas pyruvate decarboxylase, Aerobacter α-acetolactate synthetase, and Escherichia glyoxylate carboligase appear to be heterogeneous, in that part of each enzyme can bind TPP irreversibly; this cofactor dissociates reversibly for the remainder of the enzyme. When yeast pyruvate decarboxylase, saturated for cofactors, was gel-filtered at pH 8.0, 50% of the enzyme-bound TPP dissociated from the enzyme, the remainder being irreversibly bound. "Thiazole pyrophosphate," a potent inhibitor of resolved yeast pyruvate decarboxylase, acts by binding to coenzyme sites on the enzyme. Unlike TPP, thiazole pyrophosphate was shown to be bound reversibly to the enzyme and could be displaced by high concentrations of TPP. The results obtained question the validity of calculating dissociation constants for cofactors which do not dissociate from their enzymes.

Although there is now considerable knowledge concerning the chemical events whereby thiamine pyrophosphate acts as a coenzyme for several enzymes (2-4), very little is known about the interaction of TPP with its related apoenzymes. When originally studied (5), yeast pyruvate decarboxylase (2-oxo-acid carboxyl-lyase, EC 4.1.1.1) could not be inactivated by dialysis, and it was concluded that a coenzyme was not required to activate this enzyme. Subsequent studies by Auhagen (6) showed that alkaline-washed yeast (dried cells) was unable to decarboxylate pyruvate, but this activity could be restored by addition of boiled yeast juice. The dialyzable organic component was called cocarboxylase and was later shown to be identical with TPP (7). Auhagen also discovered that magnesium salts functioned as a second cofactor (8).

Once resolved for cofactors, pyruvate decarboxylase was shown to require a much higher concentration of TPP for cofactor reconstitution than was necessary to supply sufficient TPP to saturate all of the enzyme in a particular test system (9-11). It was thus considered that reconstituted enzyme might be different from native undissociated decarboxylase.

Subsequent work with other TPP enzymes revealed that several of these were resolved for cofactors upon isolation in crude extracts or during purification with procedures not involving alkaline conditions (12-15). It has become customary to report dissociation constants for TPP, such calculations assuming free dissociation of TPP from enzymes requiring this cofactor.

The present study was undertaken in order to obtain a better understanding of the nature of the binding of TPP to enzymes. Several TPP enzymes have been examined and we have found that, whereas the binding of TPP to some is irreversible, this is not invariably the case; other TPP enzymes freely dissociate this cofactor. The most complete previous study of TPP binding to pyruvate decarboxylase, as present in dried yeast preparations, is that of Steyn-Parè and Westenbrink (16).

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† The abbreviation used is: TPP, thiamine pyrophosphate.
EXPERIMENTAL PROCEDURE

Growth of Organisms—Baker's yeast was obtained commercially in 1-pound packages (Fleischmann). *Zymomonas mobilis* (ATCC 10988) was grown in Fernbach flasks which were almost completely filled with a medium consisting of 5% glucose, 0.3% proteose peptone, 0.3% yeast extract, and 0.2% K$_2$HPO$_4$. The culture was incubated at 30° for 3 days without shaking or aeration. *Proteus vulgaris* (strain OX-19) was grown on the surface of tryptose-phosphate agar (Difco) for 20 hours at 37°. *Aerobacter aerogenes* (strain J1) was grown in a medium consisting of 1% glucose, 0.3% proteose peptone, and 0.8% K$_2$HPO$_4$ for 12 hours at 30°. In order to obtain cells containing pyruvate dehydrogenase activity, *Escherichia coli* (strain Crooke's) was grown in 8-2 mineral medium (17) containing 0.5% glucose for 18 hours at 37° with vigorous aeration. Cells of *E. coli* containing glyoxylate carboligase were grown according to Kruskow, Barkulis, and Hayashi (18). A Gram-negative coccus, tentatively identified as *Micrococcus ureae*, was grown for the production of diacetyl carboligase, as previously described (19).

Cell-free Extracts—Freshly obtained bakers' yeast was crumbled into small fragments which were spread in a thin layer on a sheet of heavy wrapping paper and air-dried at room temperature for 2 to 3 days. Cell-free yeast extracts were prepared by suspending air-dried cells in ice-cold 0.1 m sodium phosphate, pH 7.1, with 4 ml of buffer for every gram of dried cells. This suspension was stored at 5° for 48 hours, after which the cells were removed by centrifugation. Bacterial extracts were prepared by suspending wet packed cells in water, with 4 volumes of water per volume of cells. The suspensions were cooled in an ice bath and exposed to sonic vibration for 30 min in an ice-water-cooled Raytheon 10-ke sonic oscillator. The resultant preparations were centrifuged at 30,000 × g for 45 min at 5° to remove unbroken cells and cell debris.

Standard Gel Filtration Procedure—Small columns of Sephadex G-25 (medium), equilibrated at room temperature with 0.1 m sodium phosphate, pH 6.5, were used routinely to remove non-protein-bound cofactors from enzyme preparations. In one of the experiments, in which several different pH values were used, the gel filtrations were made with columns which had been equilibrated with 0.1 m sodium phosphate buffers of the appropriate pH values. The recovery of enzyme after gel filtration was 95 to 100%.

Assay Systems—For the manometric assay of pyruvate decarboxylation, Warburg vessels contained 0.157 m phosphate buffer (pH 6.0), 6.25 mm MgSO$_4$, 1.3 × 10$^{-4}$ m TPP, 0.187 m sodium pyruvate, and an appropriate amount of enzyme, in a total volume of 1.6 ml. The gas phase was air. After temperature equilibration at 30°, the reaction was started by addition of sodium pyruvate from the side arm of the Warburg vessel. The activity of the enzyme being assayed was determined as the amount of CO$_2$ evolved during the first 15 min of the reaction.

Pyruvate oxidase activity was also determined manometrically with the same assay mixture described above, with the addition of 0.2 ml of 20% KOH solution to a strip of filter paper in the center well of the Warburg vessel to absorb the liberated CO$_2$. The activity of the enzyme preparation was determined as the amount of oxygen taken up during the first 35 min after addition of sodium pyruvate from the side arm of the Warburg vessel. A second alternative procedure was the pyruvate-dependent reduction of 2,6-dichloroiodophenol, as described by Moyed and O'Kane (14).

Pyruvate dehydrogenase activity was determined as the initial rate of CO$_2$ evolution from sodium pyruvate in the presence of potassium ferricyanide as electron acceptor. The complete assay mixture contained 0.157 m phosphate buffer (pH 6.0), 6.25 mm MgSO$_4$, 1.3 × 10$^{-4}$ m TPP, 0.0312 m K$_3$Fe(CN)$_6$, 0.125 m sodium pyruvate, and an appropriate amount of enzyme in a total volume of 1.6 ml. After temperature equilibration at 30°, sodium pyruvate and potassium ferricyanide were added from the side arms of the Warburg vessel. The activity of the enzyme was determined as the amount of CO$_2$ evolved in the first 20 min.

α-Acetolactate synthetase activity was determined as the amount of CO$_2$ evolved during the first 10 min of the reaction with the use of the assay mixture described above for pyruvate decarboxylase.

For the assay of glyoxylate carboligase activity, Warburg vessels contained 0.157 m phosphate buffer (pH 6.0), 6.25 mm MgSO$_4$, 1.3 × 10$^{-4}$ m TPP, 0.0625 m sodium glyoxylate, and an appropriate amount of enzyme in a total volume of 1.6 ml. The reaction was started by adding sodium glyoxylate from the side arm of the Warburg vessel after equilibration at 20°. Enzyme activity was determined as the amount of CO$_2$ evolved during the first 10 min of the reaction.

Diacetyl carboligase was assayed by means of the colorimetric procedure described by Junji and Heym (20).

Materials—"Thiazole pyrophosphate" (4-methyl-5-(pyrophosphoryl)-2-hydroxyethyl)-thiazole) was prepared from TPP as described by Weijlard and Tauber (21). TPP and sodium glyoxylate were obtained from Sigma. Sodium pyruvate was obtained from Nutritional Biochemicals Corporation.

Resolution of Yeast Pyruvate Decarboxylase for Cofactors—Ammonium sulfate was added with stirring to an ice-cold, cell-free yeast extract to bring the final concentration to 0.4 m. The pH of the mechanically stirred mixture was then adjusted to 8.5 by dropwise addition of 2.0 m Tris. Stirring was continued for 20 min after which the enzyme solution was centrifuged at 20,000 × g for 20 min at 5°. The sediment, which consisted of phosphates of magnesium, calcium, and other naturally occurring divalent cations, and some precipitated protein, was discarded. Ammonium sulfate was added in small portions to the mechanically stirred supernatant fraction to bring the final concentration to 70%. The pH of the suspension was again adjusted to 8.5 by dropwise addition of 0.1 m NaOH in 2.0 m Tris, and this was followed by centrifugation at 20,000 × g for 15 min at 5°. The supernatant fraction, which should contain TPP, was discarded. The sedimented protein was suspended in 0.1 m sodium phosphate, pH 6.0, and the pH was adjusted to 8.5 by dropwise addition of 2.0 m Tris. The material that did not go back into solution was removed by centrifugation at 20,000 × g for 20 min at 5°. Ammonium sulfate was added to the supernatant, the above centrifugation, to a concentration of 70%, at which time the pH was adjusted to 8.5 by dropwise addition of 0.1 m NaOH in 2.0 m Tris. The precipitated protein was sedimented by centrifugation at 20,000 × g for 15 min at 5° and suspended in a solution prepared by mixing equal volumes of 0.5 m sodium phosphate, pH 6.0, and saturated ammonium sulfate. This suspension, which contained resolved pyruvate decarboxylase, could be stored at 18° for periods longer than 6 months without significant loss of activity. All operations were performed at 0-5°.

The recovery of pyruvate decarboxylase prepared in this manner ranged from 75 to 80%.
when the resolution procedure of Green, Herbert, and Subrahmanyan (9) was used. It is important to note that resolved pyruvate decarboxylase prepared by the procedures of Kubowitz and Lüttgens (10) and Green et al. (9) probably contains small amounts of naturally occurring cations, since these cations form insoluble phosphates at pH 8.0 or higher, the pH used to precipitate so called resolved enzyme by these two methods.

RESULTS

Cofactor Binding of Crude Bakers' Yeast Pyruvate Decarboxylase—As isolated in crude cell-free extract, pyruvate decarboxylase was 80% saturated for cofactors. Addition of TPP alone or TPP and Mg++, increased pyruvate-decarboxylating activity by 20% (Table I, Experiment 1). Passage of crude extract through column of Sephadex G-25, at room temperature, to remove non-enzyme-bound cofactors, gave complete recovery of added enzyme, which was still 78% saturated for cofactors (Table I, Experiment 2).

To determine whether that part of the enzyme preparation not saturated for cofactors (20%) was nevertheless capable of binding TPP and Mg++, crude enzyme was first incubated with these cofactors (2.1 mM TPP, 0.01 M MgSO₄) in 0.1 M sodium phosphate, pH 6.5, for 30 min at 30°. Following this incubation, part of the mixture was gel-filtered through a column of Sephadex G-25 and assayed for possible cofactor requirements. It can be seen (Table I, Experiment 3) that the enzyme was 97% saturated following gel filtration.

Cofactor Binding of Pyruvate Decarboxylase Resolved for Cofactors—Pyruvate decarboxylase, which had been resolved for cofactors by the method described under "Experimental Procedure," was shown to be completely devoid of activity. Although addition of Mg++ alone did not restore any activity, addition of TPP in the absence of divalent cations was effective, in relatively high concentrations, in stimulating pyruvate decarboxylation (Table I, Experiment 4). To test for the stability of cofactor binding, resolved enzyme was incubated for 30 min at 30° in 0.1 M sodium phosphate, pH 6.5, together with either 0.01 M MgSO₄ or 2.1 mM TPP or a mixture of 0.01 M MgSO₄ and 2.1 mM TPP. These mixtures were then individually passed through separate Sephadex G-25 columns to remove non-protein-bound cofactors. When the enzyme was incubated with Mg++ alone, there was no evidence for protein-bound cation after gel filtration, since the filtered enzyme was no more active with added 1.3 × 10⁻⁴ M TPP than was untreated enzyme (Table I, Experiments 4 and 5). In the presence of excess Mg++, this concentration of TPP was sufficient to saturate resolved enzyme approximately 90% for decarboxylating activity (Fig. 1). The concentration of MgSO₄ used during incubation prior to gel filtration was in excess of that required to give optimal activity with low concentrations of TPP (Fig. 4).

When incubated with TPP alone, in a concentration of TPP sufficient to saturate resolved enzyme approximately 70% for activity (Fig. 1), the gel-filtered enzyme was found to be only 45% saturated for activity (Table I, Experiment 6). Furthermore, addition of Mg++ to the gel-filtered enzyme did not increase the activity of this preparation (Table I, Experiment 6).

Incubation of resolved enzyme with both cofactors prior to gel filtration resulted in a filtered product which was 90% saturated for activity (Table I, Experiment 7). Since this level of reconstitution (90%) was a little lower than that obtained with native enzyme (97%) (Table I, Experiment 3), the gel-filtered product of resolved enzyme previously incubated with both cofactors, was gel-filtered once again to detect any possible slow dissociation of cofactors. That this level of reconstitution (90%) represented an irreversible association of cofactors with apoenzyme was illustrated by the fact that the preparation that had been gel filtered a second time still remained 90% reconstituted (Table I, Experiment 8). In fact, the activity of any of the preparations in Table I was unaffected by a second gel filtration. It would thus appear that once TPP associates with yeast pyruvate decarboxylase the complex formed cannot dissociate freely.

Comparison of Resolved Pyruvate Decarboxylases Prepared in Different Ways—The procedure of Green et al. (9) for resolving yeast pyruvate decarboxylase involves precipitation of the enzyme with alkaline (NH₄)₂SO₄. Since divalent cations form insoluble phosphates under alkaline conditions, it is likely that the precipitated enzyme also contains some cations. A new method, described under "Experimental Procedure," was therefore devised in order to obtain a preparation free of divalent cations as well as of TPP. The data in Fig. 1 suggest that enzyme resolved according to Green et al. (9) may still contain divalent cations. When assayed in phosphate buffer, this resolved preparation could be activated for decarboxylating activity by relatively high concentrations of TPP (Fig. 1, Curve 8). In the presence of citrate buffer an even higher concentration of TPP was required to activate resolved enzyme (Fig. 1, Curve 9) than when phosphate buffer was used (Fig. 1, Curve 3). Since citrate chelates divalent

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<th>Addition</th>
<th>MgSO₄ 6.25 mM</th>
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* The activity is given as the percentage of the activity of the particular fraction when assayed in the presence of 6.53 × 10⁻⁴ M TPP and 6.25 mM MgSO₄.

Cofactor requirements of native yeast pyruvate decarboxylase

Before gel filtration, enzyme preparations were incubated in 0.1 M sodium phosphate, pH 6.4, together with cofactors (2.1 mM TPP, 10⁻⁴ M MgSO₄) as indicated, for 30 min at 30°.
The results of some of the experiments in which resolved enzymes were gel-filtered, shown in Table I, strongly suggest that the reconstitution of resolved enzyme for decarboxylating activity by TPP alone may be different from the reconstitution obtained in the presence of both TPP and Mg++. Whereas assay in 1.3 mM TPP was able to give 69% reconstitution of activity (Table I, Experiment 4), the same preparation after incubation in 2.1 mM TPP alone, followed by gel filtration, was only 45% activated (Table I, Experiment 6). By contrast, resolved enzyme incubated with TPP and Mg++ remained 90% activated even after two successive passages through columns of Sephadex G-25 (Table I, Experiments 7 and 8). It would appear that approximately 24% of the resolved enzyme that had been activated by TPP alone lost this coenzyme during gel filtration. When resolved enzyme was activated in the presence of TPP and Mg++, only 10% of the activated enzyme lost cofactors (measured as decarboxylating activity) after gel filtration (Table I, Experiment 7).

Effects of EDTA on Reconstitution of Resolved Pyruvate Decarboxylase—The effects of the order of addition of cofactors and EDTA to resolved yeast pyruvate decarboxylase are shown in Table II. The concentrations of TPP used in these experiments (1.3 x 10^{-5} M and 2.6 x 10^{-4} M) were only sufficient to activate resolved enzyme 88 and 91%, respectively, in the presence of excess Mg^{2+}. At higher TPP concentrations, the addition of EDTA at any time was without effect. In Experiment 3 (Table II) only partial activation was observed when EDTA was added to the reaction mixture 5 min after the addition of TPP. When the time interval between addition of TPP and EDTA was increased to 40 min, the rate of CO_{2} evolution was equal to that of the control (Table II, Experiment 2). This result suggests that reconstitution of holoenzyme from apoenzyme and cofactors is a

![Graph](attachment:image.png)

**Fig. 1.** Activity of resolved yeast pyruvate decarboxylase as a function of TPP concentration. Crude enzyme resolved according to the procedure of Green et al. (9) was assayed: Curve 1, in 0.156 M sodium citrate buffer, pH 6.0, with no added MgSO_{4}; Curve 2, in sodium citrate buffer and 6.25 mM MgSO_{4}; Curve 3, in 0.156 M sodium phosphate buffer, pH 6.0, with no added MgSO_{4}, Curve 4, in sodium phosphate buffer and 6.25 mM MgSO_{4}. Enzyme resolved according to the method described in “Experimental Procedure” was assayed as follows: Curve 5, in sodium phosphate buffer and no added MgSO_{4}; Curve 6, sodium phosphate buffer and 6.25 mM MgSO_{4}. Reaction mixtures were incubated for 30 min at 30° prior to addition of sodium pyruvate to start the assay.

The results of some of the experiments in which resolved enzymes...
relatively slow process. That this process is also irreversible is shown by the fact that EDTA was not inhibitory for enzyme that had been reconstituted. In Experiment 5 (Table II) in which EDTA was added before TPP and Mg++, the enzyme was severely inhibited. Experiment 6 (Table II) shows that addition of EDTA to a mixture of TPP and Mg++, before addition of enzyme, is very nearly equivalent to the effect of omitting Mg++ from the reaction mixture (Table II, Experiment 1). Addition of cofactors to resolved enzyme which had been previously treated with EDTA (Table II, Experiments 5 and 7) also resulted in severe inhibition. When the order of addition of cofactors to resolved enzyme was TPP, EDTA, Mg++, the same degree of inhibition was observed (Table II, Experiment 8).

To rule out the possibility that inhibition by EDTA is the result of blocking coenzyme sites on the apoenzyme by an EDTA-magnesium complex, a preincubated mixture of resolved pyruvate decarboxylase, 0.01 M MgSO4, and 0.06 M EDTA in 0.1 M sodium phosphate, pH 6.5, was passed through a Sephadex G-25 column as described under "Experimental Procedure." This gel-filtered enzyme behaved in a manner identical with that of the gel filtrate obtained after incubation of resolved enzyme with Mg++ prior to filtration (Table I, Experiment 5). Addition of cofactors resulted in full enzyme activation, showing that the coenzyme sites had not been blocked.

Kinetics of Reconstitution of Resolved Pyruvate Decarboxylase—
It was noted above that addition of EDTA was inhibitory if added before, or very shortly after, addition of cofactors to resolved pyruvate decarboxylase. EDTA was not inhibitory if added some time after TPP and Mg++. These results suggested that reconstitution of resolved enzyme was probably a slow process. The rate of reconstitution was determined by incubating resolved enzyme in 0.1 M sodium phosphate, pH 6.0, containing 0.01 M MgSO4, for various lengths of time at 23° after addition of a given amount of TPP. Reconstitution was stopped by dilution of the reaction mixture into an excess of EDTA (Fig. 2). It was shown, in control experiments, that this procedure prevented further reconstitution but was without effect on that part of the enzyme that was already saturated for cofactors. The data in Fig. 2 show that the rate of reconstitution at 23° was a function of TPP concentration. In order to achieve complete reconstitution of all the enzyme in about 1 hour, in the presence of excess Mg++, it was necessary to use TPP concentrations of $6.25 \times 10^{-4}$ M or higher. With TPP concentrations of $2 \times 10^{-4}$ M and higher, rates of enzyme reconstitution were too rapid to be determined at 23° with the present procedure. Fig. 3 shows the results of reconstitution experiments carried out with TPP concentrations greater than $2 \times 10^{-4}$ M at a temperature of 0°.

The requirement of resolved enzyme for Mg++ with the use of a given concentration of TPP ($6.25 \times 10^{-4}$ M) is shown in Fig. 4. It can be seen that concentrations of MgSO4 of 0.01 M or greater result in maximum activation.

Cofactor Binding as Function of pH—Since it is well known (9) that TPP dissociates from pyruvate decarboxylase under alkaline conditions, it was of some interest to determine the effect of pH on cofactor binding. Such information should also be useful in determining optimal conditions for reconstitution of resolved enzyme. A crude cell-free yeast extract was incubated with 1.04 mM TPP and 0.015 M MgSO4 in 0.1 M sodium phosphate, pH 6.5, for 10 min at room temperature. Samples of this fully constituted native enzyme were adjusted to the various pH values

![Fig. 2. Reconstitution of resolved yeast pyruvate decarboxylase as a function of time at 23° for several concentrations of TPP. Resolved enzyme was incubated at 23° in 0.1 M sodium phosphate, pH 6.0, containing $10^{-2}$ M MgSO4 and $2.1 \times 10^{-5}$ M TPP (\(\bigcirc\)), $4.2 \times 10^{-5}$ M TPP (\(\times\)), $6.3 \times 10^{-5}$ M TPP (\(\bigtriangledown\)), $8.4 \times 10^{-5}$ M TPP (\(\triangle\)), or $1.26 \times 10^{-4}$ M TPP (\(\Delta\)). At the indicated times, 0.05 ml of reaction mixture was added to 1.25 ml of 0.195 M sodium phosphate, pH 6.0, containing 0.04 M EDTA, in the main compartment of a Warburg vessel. After temperature equilibration at 30° for 15 min, the assay was started by tipping 0.3 ml of 0.1 M sodium pyruvate from the side arm of the vessel. Activity is expressed as the percentage of the activity of a similar quantity of enzyme assayed in the presence of an excess of MgSO4 and TPP.]
The pH of each filtrate was adjusted to 6.2, and aliquots were diluted in EDTA and assayed as in Fig. 2.

A similar procedure was used for the experiments with resolved pyruvate decarboxylase, but in this case the pH values of both resolved enzyme and a solution of TPP and Mg$^{++}$ were adjusted to the same value before mixing. For both native and resolved enzyme preparations, cofactor dissociation was apparent when incubation took place at pH values of 7.5 to 8.5 (Fig. 5). If the observed dissociation represented a reversible release of cofactors at the higher pH values, it would be expected that gel filtration at a high pH would result in complete loss of cofactors, since this procedure separates non-enzyme-bound cofactors from proteins. When crude cell-free extract was passed through a Sephadex G-25 column at pH 8.0, its activity was 50% of that observed in the presence of added cofactors. Passage of this gel filtrate through a second column of Sephadex G 25 at pH 8.0 gave a second filtrate which had 46% of the activity observed in the presence of added cofactors. Similar results were obtained in another experiment performed at pH 7.8. It is therefore clear that the loss of cofactors during gel filtration at pH values above 7.5 does not imply reversible dissociation of all cofactors, since that part of the enzyme which remained saturated for cofactors after a single passage through the alkaline Sephadex column must have bound the cofactors in an irreversible manner.

Inhibition of Resolved Pyruvate Decarboxylase by Thiazole Pyrophosphate—It is well known that thiamine and thiamine monophosphate do not serve as cofactors for pyruvate decarboxylase nor do they act as inhibitors when added to apoenzyme together with TPP (22). Although oxythiamine, pyrithiamine, N-methyl thiamine, deamino thiamine, and their pyrophosphate derivatives are all inactive in replacing TPP as coenzyme for pyruvate decarboxylase, the pyrophosphates all inhibit the activity of this enzyme, presumably by preventing association of TPP with apoenzyme (23-27). It would appear that the pyrophosphate group permits the analogues of TPP to bind to the coenzyme site on the enzyme. This evidence strongly implicates the pyrophosphate group of TPP as being that part of the coenzyme molecule involved in the apparent irreversible binding of TPP to pyruvate decarboxylase.

We have attempted to shed some light on the nature of the binding of TPP to apoenzyme with the use of the inhibitor thiazole pyrophosphate. Buchmann, Heekgaard, and Bonner (28) have shown that thiazole pyrophosphate is a good inhibitor of yeast pyruvate decarboxylase. When added to resolved enzyme, together with a fixed amount of TPP, increased concentrations of thiazole pyrophosphate resulted in increased inhibition of enzyme reconstitution, as shown in Table III. When resolved enzyme was reconstituted with TPP and Mg$^{++}$ prior to addition of thiazole pyrophosphate, however, subsequent addition of 1.61 $\times 10^{-3}$ M thiazole pyrophosphate resulted in only a 6% inhibition. This last result indicates that thiazole pyrophosphate probably inhibits pyruvate decarboxylase by occupying the TPP site on resolved enzyme. When this coenzyme site is bound up irreversibly with TPP, thiazole pyrophosphate cannot exert its inhibitory effect.

To determine whether the binding of thiazole pyrophosphate to the coenzyme site of resolved decarboxylase was reversible or irreversible, the following experiment was performed. After
thiazole pyrophosphate ($1.61 \times 10^{-4}$ M) was allowed to react with resolved enzyme in the usual test system (Table III). TPP was added to give a final concentration of $1.3 \times 10^{-3}$ M, an unusually high concentration, and the mixture was allowed to incubate at $30^\circ$C for 30 min. Upon addition of substrate the enzyme was shown to be fully activated for pyruvate decarboxylation. This result indicates that, unlike the case for TPP, the binding of thiazole pyrophosphate to resolved enzyme is reversible. In another experiment, resolved enzyme was incubated with $3.8 \times 10^{-3}$ M thiazole pyrophosphate and $0.01$ M MgSO$_4$ in 0.1 M sodium phosphate buffer, pH 6.5, for 30 min at $30^\circ$C and then passed through a Sephadex G-25 column which had been equilibrated in the same buffer. The gel-filtered enzyme showed a complete dependence for activity on addition of TPP and Mg$^{++}$, as was the case in Experiments 4 and 5 of Table I. This result shows, once again, that thiazole pyrophosphate binds reversibly to resolved pyruvate decarboxylase and that Mg$^{++}$ does not influence the nature of the binding.

To test the inhibitory effect of a fixed concentration of thiazole pyrophosphate on the reconstitution of resolved enzyme with TPP, over a wide range of TPP concentrations, the experiment of Fig. 6 was performed. It can be seen (Fig. 6) that the inhibitory effect of thiazole pyrophosphate can be overcome completely when the concentration of TPP is increased appropriately.

Cofactor Binding of Pyruvate Decarboxylase from *Zymomonas mobilis*—The activity of an unsupplemented, crude cell-free extract was found to be 84% of that obtained after addition of cofactors (Table IV, Experiment 1). Addition of either TPP or Mg$^{++}$ alone resulted in virtually no stimulation of activity. When this crude extract was passed through a column of Sephadex G-25, the unsupplemented gel filtrate showed 74% of the maximum activity obtained in the presence of added cofactors (Table IV, Experiment 2). When crude enzyme was incubated with an excess of cofactors prior to gel filtration, the activity of the filtrate was found to be 79% of the maximum activity measurable in the presence of added cofactors (Table IV, Experiment 3). A second passage of the gel filtrate of the crude extract

**Table III**

**Effect of increasing concentrations of thiazole pyrophosphate on cofactor activation of resolved yeast pyruvate decarboxylase**

Reaction mixtures contained 0.187 M sodium phosphate (pH 6.0), 6.25 mM MgSO$_4$, $0.5 \times 10^{-3}$ M TPP, thiazole pyrophosphate as indicated, 0.187 M sodium pyruvate, and an appropriate amount of resolved yeast pyruvate decarboxylase in a total volume of 1.6 ml. A mixture of TPP and thiazole pyrophosphate was added from one of the side arms to the main compartment of a Warburg vessel containing buffer, MgSO$_4$, and resolved enzyme. After incubation for 15 min at $30^\circ$C, the assay was started by addition of sodium pyruvate from the second side arm. The rate of the reaction was determined as the amount of CO$_2$ evolved during the first 15 min after addition of substrate.

<table>
<thead>
<tr>
<th>Thiazole pyrophosphate</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M \times 10^{-4}$</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1.55</td>
<td>58</td>
</tr>
<tr>
<td>3.1</td>
<td>43</td>
</tr>
<tr>
<td>6.2</td>
<td>27</td>
</tr>
<tr>
<td>10.1</td>
<td>13</td>
</tr>
<tr>
<td>32.2</td>
<td>7</td>
</tr>
</tbody>
</table>

**Fig. 6.** Activity of resolved yeast pyruvate decarboxylase as a function of TPP concentration in the presence and absence of thiazole pyrophosphate. Resolved enzyme was incubated at $30^\circ$C in 0.19 M sodium phosphate, pH 6.0, containing 6.25 mM MgSO$_4$, and either the indicated concentration of TPP alone (O) or both $4 \times 10^{-3}$ M thiazole pyrophosphate and the indicated concentration of TPP (□). After 30 min the assay was started by addition of sodium pyruvate. Activity is expressed as the percentage of the activity measured in the presence of excess TPP and MgSO$_4$.

**Table IV**

**Cofactor requirements of pyruvate decarboxylase from *Zymomonas mobilis***

Before gel filtration, enzyme preparations were incubated with cofactors as described in Table I.

<table>
<thead>
<tr>
<th>Experiment and enzyme preparation</th>
<th>Additions</th>
<th>% activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>MgSO$_4$</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>$6.5 \times 10^{-4}$ M TPP</td>
<td>89</td>
</tr>
<tr>
<td>1. Untreated extract...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Gel filtrate of Experiment 1...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Gel filtrate of (Experiment 1 + TPP + MgSO$_4$)...</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>4. Gel filtrate of Experiment 2...</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>5. Resolved enzyme...</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>6. Gel filtrate of (Experiment 5 + MgSO$_4$)...</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>7. Gel filtrate of (Experiment 5 + TPP)...</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>8. Gel filtrate of (Experiment 5 + TPP + MgSO$_4$)...</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

* The activity is given as the percentage of the activity of the particular fraction when assayed in the presence of $6.53 \times 10^{-4}$ M TPP and 6.25 mM MgSO$_4$. 

---

*The activity is given as the percentage of the activity of the particular fraction when assayed in the presence of $6.53 \times 10^{-4}$ M TPP and 6.25 mM MgSO$_4$.*
Activity is expressed as the percentage of the activity measured after 30 min. The assay was started by addition of sodium pyruvate. Activity is expressed as the percentage of the activity measured in the presence of excess TPP and MgSO₄.

The dependence of resolved Zymomonas pyruvate decarboxylase for TPP in the presence of an excess of Mg⁺⁺ is shown in Fig. 7. This curve is very similar to that obtained with resolved yeast decarboxylase (Fig. 1, Curve 6). Since resolved Zymomonas enzyme was partially activated by high concentrations of TPP alone (Table IV, Experiment 5), a study was made of the relationship between activity of resolved enzyme as a function of TPP concentration. The results of this experiment (Fig. 8) show that a maximum activation of 41% takes place when the TPP concentration is very high (6.2 to 10.4 mM).

Coactivator Binding of α-Acatolactate Synthetase from Aerobacter aerogenes—Crude, unsupplemented, cell-free extracts were found to have 61% of the maximum activity observed in the presence of added cofactors. Addition of Mg⁺⁺ alone did not influence the activity of this preparation, but TPP added alone did result in considerable stimulation of activity (Table V, Experiment 1). Passage of this cell-free extract through a column of Sephadex G-25 did not affect the activity of this preparation (Table V, Experiment 2). It would therefore appear that the cofactors are bound irreversibly to native enzyme. It was not possible to resolve the crude enzyme completely for cofactors (Table V, Experiment 3), but the partially resolved preparation was stimu-

![Graph](http://www.jbc.org/)

**Fig. 7.** Activity of resolved Zymomonas pyruvate decarboxylase as a function of the concentration of TPP. Resolved enzyme was incubated at 30° in 0.19 M sodium phosphate, pH 6.0, containing 6.25 mM MgSO₄ and the indicated concentration of TPP. After 30 min the assay was started by addition of sodium pyruvate. Activity is expressed as the percentage of the activity measured in the presence of excess TPP and MgSO₄.

![Graph](http://www.jbc.org/)

**Fig. 8.** Activity of resolved Zymomonas pyruvate decarboxylase as a function of the concentration of TPP in the absence of added divalent cations. Resolved enzyme was incubated at 30° in 0.19 M sodium phosphate, pH 6.0, containing the indicated concentration of TPP. After 30 min the assay was started by the addition of sodium pyruvate. Activity is expressed as the percentage of the activity measured in the presence of excess TPP and MgSO₄.

<table>
<thead>
<tr>
<th>Experiment and enzyme preparation</th>
<th>Addition</th>
<th>Activity %</th>
<th>Activity %</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>MgSO₄, 6.25 mM</td>
<td>3.9 x 10⁻⁴ M</td>
<td></td>
</tr>
<tr>
<td>1. Untreated extract</td>
<td>61</td>
<td>62</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>2. Gel filtrate of Experiment 1</td>
<td>61</td>
<td>61</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>3. Resolved enzyme</td>
<td>21</td>
<td>22</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>4. Gel filtrate of (Experiment 1 + TPP + MgSO₄)</td>
<td>67</td>
<td>67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The activity is given as the percentage of the activity of the particular fraction when assayed in the presence of 6.33 x 10⁻⁴ M TPP and 6.25 mM MgSO₄.
lated very markedly by TPP alone. When the preparation was incubated with TPP and Mg\(^{++}\) at concentrations adequate to saturate completely for activity, subsequent gel filtration resulted in a preparation that was only \(67\%\) saturated for cofactors (Table V, Experiment 2). These findings serve to show that \(\alpha\)-acetolactate synthetase is heterogeneous in that only about \(67\%\) of the enzyme is able to bind cofactors irreversibly.

**Cofactor Binding for Pyruvate Oxidase from Proteus vulgaris**—The results of our studies of cofactor binding of pyruvate oxidase of *Proteus vulgaris* are shown in Table VI. The crude extract was essentially devoid of activity but could be stimulated somewhat by addition of Mg\(^{++}\) alone or completely by TPP alone (Table VI, Experiment 1). When passed through a column of Sephadex G-25 after prior incubation with 0.01 M Mg\(^{++}\), however, the gel filtrate was completely inactive and could not be stimulated by addition of Mg\(^{++}\) alone (Table VI, Experiment 2); high concentrations of TPP alone gave partial activation. The gel filtrate obtained after incubation with an excess of TPP and MgSO\(_4\) was essentially inactive (Table VI, Experiment 3). The inability of this preparation to retain significant activity after gel filtration and assay, in the absence of added cofactors, may be taken as evidence for the reversible binding of cofactors to this enzyme.

**Cofactor Binding of Pyruvate Dehydrogenase from Escherichia coli**—The results of cofactor-binding studies of pyruvate dehydrogenase from *E. coli* (Table VII) are essentially similar to those presented for pyruvate oxidase from *P. vulgaris* (Table VI). Gel-filtered enzyme from *Escherichia*, however, was stimulated more significantly by addition of TPP alone. It would appear that the binding of cofactors to *E. coli* pyruvate dehydrogenase is also reversible.

**Cofactor Binding of Glyoxylate Carboligase from Escherichia coli**—Our studies of cofactor binding of glyoxylate carboligase of *E. coli* (Table VIII) yielded results very similar to those obtained with \(\alpha\)-acetolactate synthetase from *A. aerogenes* (Table V).
Apparently, it is only possible to saturate irreversibly about half of glyoxylate carboligase. Unlike the case for \( \alpha \)-acetolactate synthetase, however, gel filtration of glyoxylate carboligase results in a preparation that can be stimulated slightly by addition of \( Mg^{++} \) alone but only very little by addition of TPP alone (Tables V and VIII).

Cofactor Binding of Bacterial Diacetyl Carboligase—Crude unsupplemented cell-free extracts containing diacetyl carboligase were nearly saturated for cofactors (Table IX, Experiment 1). The gel filtrate of this extract displayed a marked drop in the percentage of cofactor saturation (Table IX, Experiment 2), suggesting possible cofactor dissociation during passage through Sephadex G-25. A second gel filtration resulted in another marked drop in the percentage of cofactor saturation (Table IX, Experiment 3). These results imply that there is a reversible cofactor binding for diacetyl carboligase but that cofactor dissociation is a relatively slow process.

**DISCUSSION**

The results of our studies with several TPP enzymes reveal certain characteristic differences in cofactor binding for the various preparations. Pyruvate dehydrogenases from yeast and *Zymomonas mobilis*, \( \alpha \)-acetolactate synthetase, and glyoxylate carboligase all bind TPP irreversibly. By contrast, pyruvate oxidase from *P. vulgaris*, pyruvate dehydrogenase from *E. coli*, and diacetyl carboligase from *M. ureae* reversibly dissociate TPP. Native yeast pyruvate decarboxylase was only 80% saturated for cofactors when isolated. Incubation with excess cofactors resulted in a preparation that was 97% saturated for cofactors after passage through a column of Sephadex G-25 (Table I). Yeast pyruvate decarboxylase resolved for cofactors could be reconstituted only 90% (Table I). The inability to saturate irreversibly 10% of the resolved enzyme may indicate that although capable of catalyzing in the presence of excess cofactors, 10% of the enzyme must have been altered during the resolution procedure, so that it was no longer capable of irreversibly.

**Crude Zymomonas pyruvate decarboxylase and Aerobacter \( \alpha \)-acetolactate synthetase** were partially capable of binding TPP irreversibly; the former enzyme could be saturated approximately 80%, whereas the latter enzyme could be saturated only 67% for cofactors (Tables IV and V). These results show that these enzyme preparations must be heterogeneous in their ability to bind cofactors. Ulrich, Wittorf, and Guber (29) have proposed recently that native yeast pyruvate decarboxylase exists as a tetramer. It is possible that some preparations may also contain part of the enzyme as dimer or monomer units, which may be catalytically active but unable to bind cofactors irreversibly. It is also possible that the various cofactor-binding sites in a unit such as a tetramer may bind cofactors with different strengths. The finding, reported above, that gel filtration of yeast pyruvate decarboxylase at alkaline pH values dissociated only part of its bound TPP, the remaining coenzyme being irreversibly linked, may be taken as further evidence for the presence of distinct differences in the kinds of cofactor-binding sites in a given enzyme preparation.

It has been proposed that divalent cations serve as bridges in the binding of TPP with the enzyme (9). If this were the case, it might be expected that resolved enzyme could possibly bind divalent cations at least as firmly as the enzyme binds TPP. The results of our studies with resolved pyruvate decarboxylases from yeast and *Zymomonas* show quite clearly that these resolved enzymes are unable to bind \( Mg^{++} \) at all (Tables I and IV).

The ability to activate resolved TPP enzymes in the presence of high concentrations of TPP, but in the absence of added divalent cations, may signify that the resolved preparation or some of the reagents used in the assay contain traces of divalent cations. If this were the explanation, it would be expected that resolved yeast pyruvate decarboxylase, which had been reconstituted 70% for decarboxylating activity by TPP alone, would retain most of this TPP at the enzyme sites after gel filtration. The finding that only 45% of the enzyme remained constituted for cofactors after gel filtration (Table I) would tend to verify that activation of resolved enzyme by high concentrations of TPP, in the absence of added divalent cations, is different in some way from activation by both cofactors. A similar and quantitatively more marked effect was observed with the use of resolved *Zymomonas* pyruvate decarboxylase (Table IV). In the case of *Proteus* pyruvate oxidase and *Escherichia* pyruvate dehydrogenase, where cofactors are reversibly bound and can be removed readily by gel filtration, TPP alone can stimulate gel-filtered preparations for their catalytic activities (Tables VI and VII).

It has been observed by other workers that various TPP enzymes, resolved for cofactors, can be stimulated by addition of TPP alone, in the absence of added divalent cations (15, 30–32). In the case of highly purified wheat germ pyruvate decarboxylase, Singer and Pensky (15) were able to activate this resolved enzyme 23% in the presence of a relatively low concentration of TPP (1.94 \( \times 10^{-4} \) M) but without added divalent cations. Spectroscopic examination of the enzyme and reagents used in the assay failed to reveal a sufficient amount of divalent cations to account for the stimulatory effect of TPP alone. It is also of interest to point out that Horecker, Smyrniotis, and Klenow (33) have shown that, unlike spinach transketolase, which requires TPP and divalent cations for reconstitution, resolved rat liver transketolase required only TPP for complete cofactor reconstitution; added divalent cations were without stimulatory effect. The mechanism by which divalent cations act as cofactors for TPP enzymes would seem to require much more detailed investigation.

The experiments with thiazole pyrophosphate, reported above, provide evidence that the pyrophosphate group is certainly of importance in the binding of TPP to enzymes. The inability of thiazole pyrophosphate to bind irreversibly to resolved yeast pyruvate decarboxylase, unlike the case of TPP in which binding is irreversible, indicates that the 2-methyl-4-amino-pyrimidine group of TPP must also play a role in TPP binding. The finding that large quantities of 2-hydroxymethyl-TPP could be obtained when pig heart pyruvate oxidase, but not yeast pyruvate decarboxylase, was incubated with pyruvate and TPP (34) indicates that the former enzyme binds TPP reversibly. Recent studies of Sable and Biaglow (35) show that thiamine can form complexes with indole derivatives in a model system, and the authors suggest that such complexes may be involved in the binding of TPP to enzymes.

With the use of resolved enzyme preparations, it has been customary to report dissociation constants for TPP (15, 30, 33, 36). In view of the irreversible nature of the binding of TPP to certain enzymes, as well as the very slow rate of reconstitution of resolved enzyme at low TPP concentrations (Fig. 2), the significance of such dissociation constants is questionable.
After the experiments described above were completed, several papers appeared (37-41) which shed further light on the nature of TPP binding to yeast pyruvate decarboxylase. Schellenberger et al. (37) have reported that 4-hydroxy-4-deamino-TPP remains bound to yeast pyruvate decarboxylase after gel filtration through a column of Sephadex G-25. These workers also report that this gel filtrate could be reactivated for pyruvate decarboxylation when a great excess of TPP was added to the assay mixture. This last result implies that the binding of 4-hydroxy-4-deamino-TPP to pyruvate decarboxylase, although strong, is nevertheless reversible. When resolved yeast pyruvate decarboxylase was activated with TPP and Ca" ions, in place of Mg" ions, and followed by gel filtration, none of the TPP was found to remain bound to the filtered enzyme (38). The nature of the divalent cations obviously plays an important role in TPP binding. Schellenberger and Hübner (39) have also shown that resolved enzyme does not bind Mg" in the absence of TPP with the use of the same technique that we have employed. A large series of TPP analogues has been studied by Schellenberger et al. (37, 40) and by Schellenberger, Heinroth, and Hübner (41) for the ability to act as cofactors as well as, in some cases, to bind to pyruvate decarboxylase in the presence of divalent cations.

REFERENCES

Studies on the Nature of the Binding of Thiamine Pyrophosphate to Enzymes
A. V. Morey and Elliot Juni