Stereoisomers of Tetrahydrothiamin Pyrophosphate, Potent Inhibitors of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*†

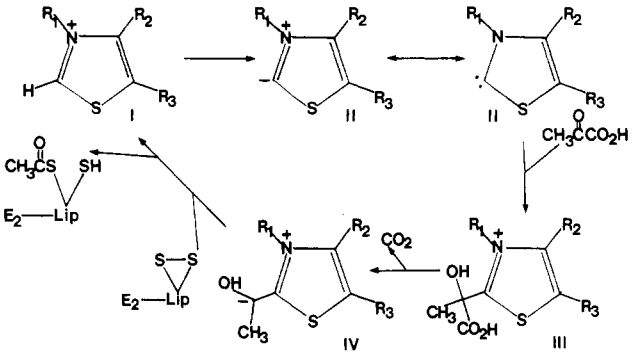
Peter N. Lowe, Finian J. Leeper, and Richard N. Perham*

ABSTRACT: Tetrahydrothiamin pyrophosphate, an analogue of thiamin pyrophosphate (TPP) in which the thiazolium ring has been reduced to a thiazolidine ring, was prepared by borohydride reduction of TPP. It consists of four stereoisomers, comprising two diastereomers each of which is a racemic mixture, generated by the introduction of two chiral centers on the thiazolidine ring. The major and minor diastereomers were separated and inferred to be of the cis and trans configurations, respectively, from a study of the nuclear Overhauser effects in the ^{1}H NMR spectrum of the simpler tetrahydrothiamin. Tetrahydro-TPP behaves as a mixture of potent inhibitors of the pyruvate decarboxylase (E1) component of the pyruvate dehydrogenase complex from *Escherichia coli*. The site of binding is probably the TPP-binding site on E1, and the K_d for each of the four stereoisomers was estimated. The cis isomers of tetrahydro-TPP bind more tightly than does TPP, whereas the trans isomers appear to bind with about the same K_d as TPP. Sodium borohydride caused a rapid inhibition of E1 activity in the presence of TPP, believed to be due to reaction of borohydride with enzyme-bound TPP. The experiments are consistent with an enhancement of the reactivity of the thiazole ring of TPP when bound to the catalytic site of E1, which could be due to polarization of the $>^+N{=}C$ bond near a hydrophobic or positively charged region of the protein. A spontaneous reactivation occurred after the initial inhibition by borohydride, attributable to a weakly binding inhibitor, not tetrahydro-TPP, being formed at the catalytic site.

The pyruvate dehydrogenase multienzyme complex contains multiple copies of three enzymes: pyruvate decarboxylase (E1), lipoate acetyltransferase (E2), and lipoamide dehydrogenase (E3) [reviewed by Reed (1974)]. The pyruvate decarboxylase component catalyzes the decarboxylation of pyruvate and the reductive acetylation of lipoic acid covalently bound to the E2 subunits (see Scheme I). Thiamin pyrophosphate (TPP)[1] (I) is an essential cofactor for this process. The mechanism of the reaction involves removal of H-2 of the thiazole ring to produce the ylide (II) followed by addition of pyruvate to give 1-carboxy-1-(hydroxyethyl)-TPP (III) (Breslow, 1958; Crosby et al., 1970; Krampitz, 1969). Decarboxylation follows, yielding the anion of 1-(hydroxyethyl)-TPP (IV) which can react further with lipoic acid covalently bound to E2 to give *S*-acetyllipoic acid residues and regenerate TPP. The initial reactions of pyruvate decarboxylase from yeast and of pyruvic oxidase from *Escherichia coli* are believed to be very similar although the fates of 1-(hydroxyethyl)-TPP and the enzyme structures are different. In the former enzyme, acetaldehyde is released whereas in the latter further oxidative reactions occur yielding acetic acid.

The negative charge on C-2 of the ylide, structure II, is stabilized by electrostatic interaction with the positively charged quaternary nitrogen atom and probably to a lesser extent by overlap with the d orbitals of the sulfur atom. It has been suggested that if the thiazole ring is bound to a hydrophobic region of the enzyme, the positive charge would have a greater electron-attracting effect than in free solution and would cause an enhancement of the reactivity of the thiazole ring (Crosby et al., 1970; Crosby & Lienhard, 1970). This would speed up the decarboxylation reaction, in addition to promoting the addition of pyruvate. Indeed, it has been demonstrated that the rates of decarboxylation of 1-carboxy-1-(hydroxyethyl)thiamin and of 2-[1-carboxy-1-(hydroxyethyl)]-3,4-dimethylthiazolium chloride, both analogues of structure III (Scheme I), are much faster in less polar solvents (Crosby et al., 1970; Kluger et al., 1981). Furthermore, there is now increasing evidence that TPP-binding sites on a number of enzymes have some hydrophobic character (Gutowski & Lienhard, 1976; Wittorf & Gubler, 1970; O'Brien & Gennis, 1980; Kluger & Smyth, 1981).

Scheme I



In this paper, we show that tetrahydro-TPP, an analogue of TPP in which the sulfur-containing ring is uncharged (Figure 1), can easily be prepared by borohydride reduction of TPP. Its four stereoisomers prove to be inhibitors of the pyruvate decarboxylase (E1) component of the pyruvate dehydrogenase complex from *Escherichia coli* with interesting differences in potency. Reduction with $NaBH_4$ occurs faster with TPP already bound to E1 than with TPP in free solution, suggesting that the reactivity of the thiazole ring in enzyme-bound TPP is enhanced. Tetrahydro-TPP is likely to be a

† From the Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, England (P.N.L. and R.N.P.), and the Department of Organic and Inorganic Chemistry, University Chemical Laboratory, Cambridge CB2 1EW, England (F.J.L.). *Received August 5, 1982.* This work was supported by a research grant from the Science Research Council to R.N.P.

[1] Abbreviations: TPP, thiamin pyrophosphate; tetrahydro-TPP, diphosphoric acid mono[2-[3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-4-methyl-5-thiazolidinyl]ethyl] ester; Dnp, 2,4-dinitrophenyl.

0006-2960/83/0422-0150$01.50/0

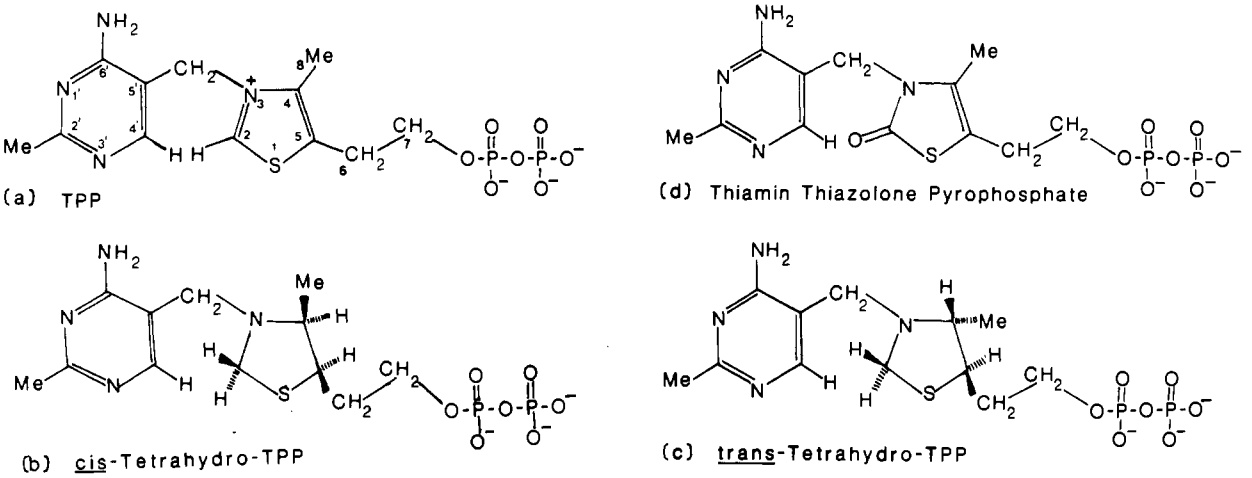


FIGURE 1: Structure of TPP and analogues.

useful probe of the stereochemical environment of TPP-binding sites.

Materials and Methods

Preparation and Characterization of Tetrahydro-TPP. Thiamin pyrophosphate chloride (898 mg, 1.95 mmol) was dissolved in 10 mL of water, and 0.8 M NaOH was added to raise the pH to 8. A solution of $NaBH_4$ (228 mg in 1 mL of water, 6.03 mmol) was added dropwise to the stirred solution at 0 °C over a period of 30 min. The mixture was then left at room temperature for 1 h after which 1 mL of acetone was added to react with any excess $NaBH_4$. After a further 30 min, the whole mixture was freeze-dried to yield crude tetrahydro-TPP.

A portion of crude tetrahydro-TPP (188 mg, approximately 310 μmol) was mixed with 350 μL of water. Part of the material dissolved. The mixture was centrifuged, and the supernatant, which contained all the UV-absorbing material, was retained. The purity of the extract was assayed by thin-layer electrophoresis at pH 3.5: 70% of the material moved as a negatively charged species with a mobility 0.39 that of Dnp-aspartic acid, and 25% had the mobility of unreacted TPP. A small amount of fluorescent material with an intermediate mobility was also present. The bulk of the extract was then electrophoresed at pH 2 on Whatman 3MM paper. The nonfluorescent UV-absorbing material near the origin was eluted with 3 mL of water and freeze-dried to yield 60 mg of pure tetrahydro-TPP. Elution of the material that had the mobility of TPP did not yield a substance capable of inhibiting the pyruvate dehydrogenase complex activity.

The purified tetrahydro-TPP ran as a single UV-absorbing spot on thin-layer electrophoresis at pH 2, 3.5, and 6.5. At all pH values, TPP has a similar mobility to that of N^6-Dnp-lysine, whereas tetrahydro-TPP has a greater mobility toward the positive electrode. At pH 2, tetrahydro-TPP has a mobility of 0.65, at pH 3.5 a mobility of 0.39, and at pH 6.5 a mobility of 0.20, all relative to Dnp-aspartic acid and taking N^6-Dnp-lysine as arbitrarily having zero mobility. The difference in mobilities between tetrahydro-TPP and TPP cannot be due to a difference in the pK values of the pyrophosphate group since a similar separation is obtained between tetrahydrothiamin and thiamin. The difference in mobilities is less than that expected for a charge difference of unity and may be due to adsorption chromatography which differs between the aromatic and saturated systems.

The UV spectrum of tetrahydro-TPP, recorded in 0.1 M potassium phosphate, pH 8.5, showed λ_{max} = 272, 233 nm; λ_{min} = 216, 253 nm (cf. TPP λ_{max} = 266, 232 nm; λ_{min} = 210, 249 nm). The UV spectrum recorded in 0.1 M HCl showed λ_{max} = 244 nm. The extinction coefficient at pH 8.5 was estimated as follows: $NaBH_4$ (30 mM) was added to an aqueous solution of TPP (final concentration 13.1 mM) at 22 °C, previously adjusted to pH 8 with NaOH. UV spectra were recorded at intervals on 10-μL samples diluted into 1 mL of 0.1 M K_2HPO_4. The reaction was complete within 60 min, and no further spectral change occurred on addition of more $NaBH_4$. The final absorbance at λ_{max} was measured and the molar extinction coefficient calculated by assuming a 100% conversion of TPP to tetrahydro-TPP. ϵ_{232} was 8430 L mol^{-1} cm^{-1}, and ϵ_{272} was 4900 L mol^{-1} cm^{-1}.

The ^1H NMR spectrum of tetrahydro-TPP (90 and 270 MHz, ^2H_2O, referenced to external Me_4Si) included the following signals: δ 8.3 (br s, H-4′), 3.5–4.5 (m, bridge methylene, H-2, H-4, H-5, CH_2CH_2OP), 2.54 (s, 2′-CH_3), 2.05 and 1.86 (m, CH_2CH_2OP), 1.45 (d, J = 4.5 Hz, 4-CH_3 of minor diastereomer), 1.25 (d, J = 4.5 Hz, 4-CH_3 of major diastereomer). The spectrum was not sufficiently well resolved to permit assignment of all the resonances, but it was very similar to that observed for tetrahydrothiamin in which all the resonances have been assigned (Leeper & Lowe, 1983). The spectrum does clearly show a number of features: (1) the resonance at very low field (δ 9.7) due to H-2 of TPP is absent and is replaced by a resonance at δ 3.5–4.5 [cf. 3-benzyl-4-methylthiazolidine; H-2 resonates at δ 4 (Clarke & Sykes, 1967)], confirming that the aromatic character of the ring has been destroyed; (2) the removal of the positive charge on the thiazolium nitrogen atom has caused the bridging methylene protons to move to higher field (δ 3.4–4.5 compared with δ 5.6 in TPP); (3) the reduction of both double bonds of the thiazolium ring has resulted in the production of two chiral centers. This is most clearly seen in the resonances of the 4-CH_3 group. In TPP, it resonates as a singlet at δ 2.6, whereas in tetrahydro-TPP, the 4-CH_3 is now attached to a saturated carbon with a single proton. This causes an upfield shift and doublet splitting. In addition, since two diastereomers are now present, two resonances are now evident at δ 1.25 and 1.45. The ratio of the intensities of the peaks at δ 1.25 and 1.45 is 3.2:1, which is presumed to be the ratio of the amounts of the two diastereomers present.

Preparation and Characterization of Tetrahydrothiamin. Tetrahydrothiamin and its major diastereomer were prepared from thiamin reduced with $NaBH_4$ as described by Clarke & Sykes (1967). For the enzymological experiments, the crude diastereomeric mixture was used. The ratio of diastereomers

was found by ^1H NMR spectroscopy to be ca. 2.6:1. The ^1H NMR spectrum of the diastereomeric mixture was examined in more detail (250 MHz, ^2H_2O, 65 °C), and the following signals were assigned separate from any others: for the major isomer, δ 1.10 (4-CH_3), 2.00 (CH_2CH_2OH), 3.42 (H-4); for the minor isomer, δ 1.34 (4-CH_3), 2.13 (CH_2CH_2OH), 2.95 (H-4), 3.29 (H-5). Further NMR experiments on the separated isomers which confirm these assignments and establish the preferred conformation of the major isomer are published elsewhere (Leeper & Lowe, 1983).

Preparation of Purified Diastereomers of Tetrahydro-TPP. The pure major diastereomer and the diastereomeric mixture of tetrahydrothiamin were pyrophosphorylated by adding 60 mg of each separately to 0.14 mL of 88% H_3PO_4 and 0.27 g of P_2O_5 and heating at 110 °C for 25 min (Matsukawa et al., 1970). The brown mass was stirred with 13 mL of ethanol, and the insoluble white material was collected by centrifugation. The pellet was washed with 13 mL of ethanol and 13 mL of diethyl ether and was then dried in vacuo. The powder, dissolved in 0.5 mL of water, was poured into 13 mL of ethanol. The precipitate was collected, dissolved in 0.1 mL of water, and applied to Whatman 3MM paper for electrophoresis at pH 2. Five major UV-absorbing spots were obtained with mobilities corresponding to those of the unreacted compound and the mono-, di-, tri-, and tetraphosphorylated species. Each was eluted with water and freeze-dried. Although chromatographically pure, these samples showed slight contamination in the UV spectrum, which could be removed by ethanol precipitation. The ratio of diastereomers was assumed not to change during the pyrophosphorylation procedure.

Since the minor diastereomer of tetrahydrothiamin was not available in sufficient amount for the above procedure, the diastereomeric mixture of tetrahydro-TPP was resolved by ion-exchange high-pressure liquid chromatography (HPLC) on a Varian MicroPak SAX-10 quaternary amine column (4 mm i.d. × 30 cm long) eluted with 56 mM potassium phosphate, pH 4.5. Tetrahydro-TPP (6 μmol of diastereomeric mixture) was applied to the column. The two diastereomers eluted as overlapping peaks with retention times of 10.8 (major peak) and 13.9 min (minor peak). The minor peak was collected and reapplied to the column twice more to yield the minor diastereomer contaminated with 1–5% of the major diastereomer. Before use in enzymological experiments, the solution was adjusted to pH 7 by addition of 4 M KOH.

Estimation of $NaBH_4$. $NaBH_4$ was assayed by adding small portions to 1 mL of 0.1 M potassium phosphate, pH 8.0, containing 0.2% (w/v) starch, 75 μM I_2, and 0.45 mM KI. The decrease in A_{590} was used to calculate the amount of $NaBH_4$, assuming that the molar extinction coefficient of the starch/iodine complex is 23 300 L mol^{-1} cm^{-1} and that 4 mol of I_2 reacts with 1 mol of $NaBH_4$.

Spectroscopic Measurements. UV spectra were obtained on a Perkin-Elmer 555 spectrophotometer. ^1H NMR spectra were obtained at 90 MHz on a EM-390 (University of Cambridge), at 250 MHz on a Bruker WM-250 (University of Cambridge), or at 270 MHz on a Bruker WH-270 (NIMR, London) spectrometer. The latter two instruments were operated in the Fourier-transform mode. The molar extinction coefficient of TPP at pH 8 at 266 nm was taken as 8300 L mol^{-1} cm^{-1} (Dawson et al., 1969).

Enzyme Assays. Overall pyruvate dehydrogenase complex activity was measured as described by Danson et al. (1978). Pyruvate decarboxylase (E1) activity was measured in two ways, either by reconstitution with methyl acetimidate modified E2E3 subcomplex, as described by Hale & Perham (1979), or by an assay involving reduction of 2,6-dichlorophenolindophenol (Khailova et al., 1977). In the latter assay, E1 was added to 1 mL of a mixture containing 0.1 M potassium phosphate, pH 7, 100 μM dichlorophenolindophenol, 0.2 mM TPP, and 1 mM $MgCl_2$ at 30 °C. The A_{600} was followed continuously. The inhibition induced by tetrahydro-TPP was found to be the same in both assays.

Enzyme Isolation. Pyruvate dehydrogenase complex was prepared from a constitutive mutant of *E. coli* as described by Danson et al. (1979) based on the method of Reed & Mukherjee (1969). The E2E3 subcomplex and the E1 component were prepared as described by Coggins et al. (1976).

Electrophoresis. Paper electrophoresis and thin-layer electrophoresis were carried out essentially as described by Perham (1978). The thin-layer electrophoresis was carried out on cellulose plates (Polygram CEL400, 0.1-mm layer thickness; Machery-Nagel). Thiamin-containing compounds were detected by absorption of UV light (254 nm).

HPLC. This was performed on a Varian 5020 liquid chromatograph operating at ambient temperature with a flow rate of 2 mL/min. Detection was by UV absorption at 250–288 nm, depending upon the sensitivity required.

Protein Determination. Protein was estimated routinely by precipitation with trichloroacetic acid by using the method of Peterson (1977) with bovine serum albumin as a standard. The assay was calibrated by amino acid analysis, using the amino acid composition of Harrison (1974). The relative molecular mass of the E1 polypeptide chain was taken as 100 000. All molar enzyme concentrations were calculated by using this value.

Binding Tetrahydro-TPP to E1. Tetrahydro-TPP consists of a mixture of four isomeric inhibitors, I_A, I_B, I_C, and I_D. The binding to a single site on the E1 component (E) can be described as follows:

$$E + I_A \overset{K_A}{\rightleftharpoons} EI_A \qquad E + I_B \overset{K_B}{\rightleftharpoons} EI_B$$

$$E + I_C \overset{K_C}{\rightleftharpoons} EI_C \qquad E + I_D \overset{K_D}{\rightleftharpoons} EI_D$$

K_A, K_B, K_C, and K_D are dissociation constants for these equilibria.

The concentration of free enzyme, [E], is then given by

$$[E] = \frac{[E]_0}{1 + \frac{[I_A]_0}{K_A + [E]} + \frac{[I_B]_0}{K_B + [E]} + \frac{[I_C]_0}{K_C + [E]} + \frac{[I_D]_0}{K_D + [E]}}$$

where $[E]_0$, $[I_A]_0$, $[I_B]_0$, $[I_C]_0$, and $[I_D]_0$ are the total concentrations of E1, I_A, I_B, I_C, and I_D, respectively.

This equation has been used to construct the theoretical curves shown in Figure 4.

Characterization of the Effects of $NaBH_4$ on the E1/TPP Complex. E1 (2.17 mg/mL, 21.7 μM) dissolved in 20 mM potassium phosphate and 1 mM $MgCl_2$, pH 7, was incubated with 69 μM TPP for 1 h at 22 °C in a volume of 3.5 mL. $NaBH_4$ was then added to a concentration of 5 mM. After 45 s, the enzyme activity was found to be inhibited by about 70%. After 5 min, 1.75 mL of 2 M $HClO_4$ was added. The mixture was left on ice for 10 min, and the precipitated protein was then removed by centrifugation. To the supernatant was added 1.1 mL of approximately 4 M KOH (standardized so as to neutralize the $HClO_4$). After 10 min on ice, the mixture was centrifuged and the supernatant freeze-dried. The dried material was dissolved in 0.88 mL of water, any precipitate was removed, and the pH was adjusted to pH 7–8, if necessary.

Alkaline phosphatase (8.8 μL, Boehringer grade 1; 220 units) was added, and the sample was incubated at 37 °C for 24 h. The solution was freeze-dried and redissolved in 0.3 mL of water. Samples were then analyzed by HPLC on a MicroPak MCH-10 column (4 mm i.d. × 30 cm long) eluted with a 7:3 (v/v) mixture of 50 mM ammonium formate and methanol. Thiamin eluted at 10–12 min, the minor diastereomer of tetrahydrothiamin at 38 min, and the major isomer at 43 min. Since base-line separation of the two isomers was not obtained, exact quantitation was not possible. Controls consisting of 69 μM TPP, and of E1 in the absence of TPP, were treated with $NaBH_4$ exactly as described above.

Results

Preparation and Structure of Tetrahydro-TPP. TPP was easily reduced by $NaBH_4$ in aqueous solution, producing tetrahydro-TPP (see Materials and Methods and Figure 1). The ^1H NMR spectrum showed that the thiazolium ring was fully reduced and that the isolated product was a mixture of diastereomers in the ratio of ca. 3.2:1.

Each diastereomer would be expected to exist as a racemic mixture. Measurements of nuclear Overhauser effects (NOE's) were therefore carried out with tetrahydrothiamin, for which the resonances were easier to assign. When the signal for the 4-CH_3 of the major diastereomer was irradiated for 3 s before each scan, an NOE was observed to H-6a and H-6b. On the other hand, irradiation of the 4-CH_3 signal of the minor diastereomer produced an NOE to H-5 but not to H-6a or H-6b. We conclude that in the major diastereomer the side chains are cis to one another (Figure 1b) and in the minor diastereomer they are trans (Figure 1c). In confirmation of this result, NOE's were observed for the major diastereomer, from H-6a to H-8, and for the minor diastereomer, from H-5 to H-8, from H-4 to H-6b, and from H-6a to H-4. The major and minor diastereomers of tetrahydro-TPP obtained by reduction of TPP or by pyrophosphorylation of tetrahydrothiamin have the same configuration as the major and minor diastereomers of tetrahydrothiamin.

Since the pyrimidine ring and the pyrophosphate side chain are unchanged and a five-membered sulfur–nitrogen heterocyclic ring is retained, it might be expected that tetrahydro-TPP would be a good analogue of TPP. It is known to be a tightly binding inhibitor of yeast pyruvate decarboxylase (Wittorf & Gubler, 1971) and of transketolase (Heinrich et al., 1972). Therefore, we investigated whether tetrahydro-TPP acts as an inhibitor of the pyruvate dehydrogenase complex of *E. coli*. The existence of four stereoisomers of tetrahydro-TPP was expected to be important in governing its interactions with a chiral binding site on an enzyme, and we took advantage of our ability to separate the diastereomers to study their effects more closely. The concentrations of tetrahydro-TPP described in this paper refer to the total concentration of stereoisomers in question.

Inhibitory Effects of Tetrahydro-TPP on E. coli Pyruvate Dehydrogenase Complex and Its Component Enzyme Activities. The overall reaction of *E. coli* pyruvate dehydrogenase complex can be completely inhibited in a time-dependent process by preincubation of the complex with low concentrations of tetrahydro-TPP (Figure 2). In contrast, tetrahydrothiamin (0.9 mM), tetrahydrothiamin monophosphate (0.2 mM), and tetrahydrothiamin tetraphosphate (0.2 mM) were without effect on the enzyme activity, and tetrahydrothiamin triphosphate (0.2 mM) led to an inhibition of only 15%. The inhibition was measured after dilution into the assay medium, and little or no increase in catalytic activity occurred during the time course of the assay. This indicated that the bound inhibitor was released only very slowly. The kinetics of inhibition were not pseudo first order with respect to remaining enzyme activity, even when a concentration of tetrahydro-TPP was used 30 times greater than the concentration of E1 subunits (Figure 2); the inhibition was initially fast, but the rate decreased with time.

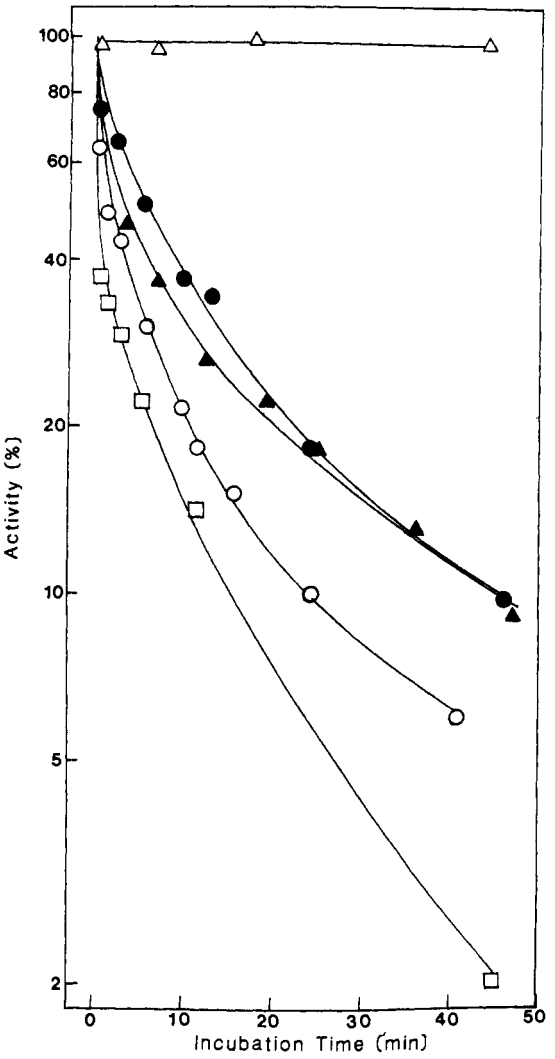


FIGURE 2: Inhibition of pyruvate dehydrogenase complex and E1 by tetrahydro-TPP. Pyruvate dehydrogenase complex (30 μg/mL) was incubated with 1.4 (▲), 2.8 (○), or 6.2 μM (□) tetrahydro-TPP. E1 (23 μg/mL) was incubated with 2.3 μM tetrahydro-TPP (●) or 920 μM tetrahydrothiamin (△). The incubation mixture contained 50 mM potassium phosphate and 1 mM $MgCl_2$, pH 7, and the temperature was 0 °C. Samples (20 μL) were removed at intervals and assayed for overall complex activity either directly or after reconstitution with E2E3 subcomplex (3 μg) in the case of E1.

Preincubation of the E2E3 subcomplex with tetrahydro-TPP which was followed by diluting the enzyme into assay medium and adding E1 did not produce any inhibition of enzyme activity, whereas preincubation of E1 with tetrahydro-TPP followed by addition of the E2E3 subcomplex led to inhibition of enzyme activity. We infer that the site of binding of tetrahydro-TPP is on the E1 subunits.

The kinetics of inhibition of isolated E1 by tetrahydro-TPP were similar to those observed with the intact complex (Figure 2), and the inhibition again did not follow pseudo-first-order kinetics. The rate of inhibition shows a very large dependence on the temperature of preincubation, increasing 30-fold when the temperature was raised from 0 to 22 °C (Figure 3). The rate of inhibition in the presence of tetrahydro-TPP was lowered by adding TPP. The kinetics were complex in that ini-

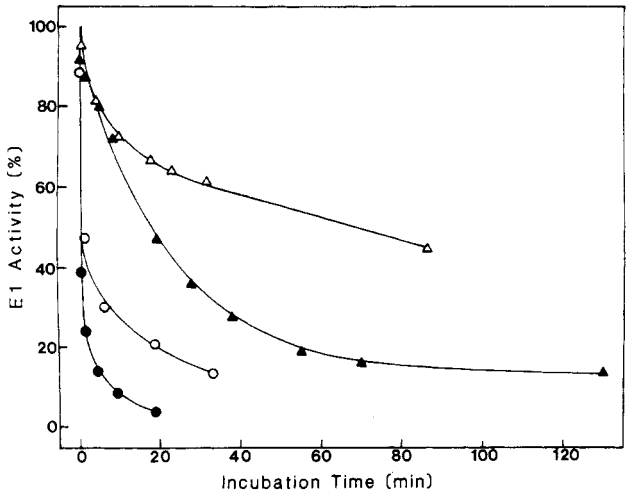


FIGURE 3: Inhibition of E1 by tetrahydro-TPP; protection by TPP and effects of temperature. E1 (30 μg/mL) was incubated with 0.92 μM tetrahydro-TPP in 50 mM potassium phosphate and 1 mM $MgCl_2$, pH 7. E1 activity was assayed at intervals by adding samples of 25 μL to 1 mL of assay medium containing 4 μg of E2E3 subcomplex. Incubations were at 0 °C in the absence of TPP (▲), 0 °C in the presence of 52 μM TPP (△), 22 °C without TPP (●), and 22 °C with 52 μM TPP (○).

tially no protection was evident, but protection became apparent with longer times of incubation (Figure 3). During the second phase, the rate of inhibition was inversely proportional to the concentration of TPP present. The concentration of TPP that reduced the rate of inhibition by 50% was 16 μM at 0 °C. This is similar to the reported K_d value for TPP at 4 °C of 9–24 μM (Moe & Hammes, 1974), which suggests that tetrahydro-TPP is binding at the same site as TPP. It should be noted that with longer incubation times (12 h) the enzymic activities of all four incubations shown in Figure 3 were inhibited by more than 96%; i.e., the presence of 52 μM TPP did not significantly affect the final degree of inhibition. This indicates that the K_d for tetrahydro-TPP is much smaller than that for TPP.

Estimation of the Stoichiometry and Binding Constant of Tetrahydro-TPP. Various concentrations of unresolved tetrahydro-TPP prepared from tetrahydrothiamin were incubated for prolonged times with a constant concentration of E1. Samples were then assayed for residual E1 activity. The results presented in Figure 4 were obtained with two different concentrations of E1. It can be seen that the degree of inhibition is not proportional to the concentration of inhibitor added, in contrast to the observations with thiamin thiazolone pyrophosphate (Gutowski & Lienhard, 1976; Stanley et al., 1981). Moreover, the relationship between the inhibitor concentration and the degree of inhibition is not consistent with a model in which tetrahydro-TPP binds at a single site with a unique K_d (see theoretical curves in Figure 4). The results are consistent, however, with the sample of tetrahydro-TPP consisting of several species binding at a single site but with differing binding constants. This could have a physical explanation in the existence of the four isomers (two diastereomers) of tetrahydro-TPP. In Figure 4, theoretical curves were fitted by assuming that the diastereomeric species are present in concentrations calculated from the ratio (2.6:1) observed in NMR spectra (see Materials and Methods). The two cis isomers were assigned K_d's of 0.02 and 0.15 μM and the two trans isomers a K_d of 5–10 μM each. Thus, the two cis isomers bind considerably more tightly than does TPP whereas the trans isomers have about the same K_d as TPP. Very similar results were obtained when the experiments were repeated with tetrahydro-TPP prepared by borohydride reduction of TPP, for which the ratio of diastereomers was 3.2:1.

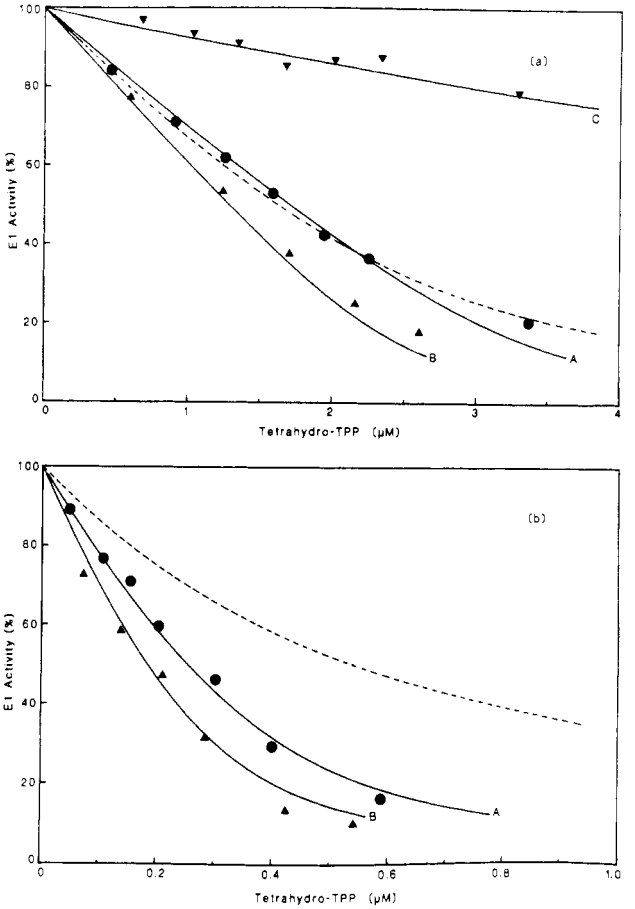


FIGURE 4: Plot of the relationship between tetrahydro-TPP concentration in the preincubation and the equilibrium amount of residual E1 activity. In (a), the E1 concentration was 0.24 mg/mL (2.4 μM) and in (b) 24 μg/mL (0.24 μM). A series of incubations were set up at 22 °C containing various concentrations of unresolved tetrahydro-TPP prepared by pyrophosphorylation of tetrahydrothiamin (●) or with various concentrations of the cis (▲) or trans (▼) diastereomers. After 7 h, the E1 activity was measured by reconstitution with E2E3. The same degree of inhibition was measured if the incubations were allowed to proceed for 20 h. The solid lines (—) indicate theoretical curves calculated by assuming one inhibitor binding site per E1 monomer. For line A, the unresolved tetrahydro-TPP was taken to consist of cis isomers (assigned K_d values of 0.01 and 0.15 μM) and trans isomers (assigned K_d values of 10 μM) in the ratio of 2.6:1. For line B, the cis isomers were used as inhibitors (assigned K_d values of 0.01 and 0.15 μM). For line C, the trans isomers were used as inhibitors (assigned K_d values of 10 μM). The dashed line (- - -) is the theoretical curve for one inhibitor binding site per E1 monomer and all inhibitors having a single K_d value of 0.42 μM.

It is clear that with the choice of four different binding constants, other fits to the data could be made. For example, the cis isomers could each have a K_d of 0.43 μM, and the trans isomers a K_d each of 0.001 μM, generating an equally good fit to that shown in Figure 4. The scheme was therefore tested by looking at the effects of the two separated sets of diastereomers individually (Figure 4). It can be seen that the isolated cis diastereomer behaved as a more potent inhibitor than the complete mixture and that a good fit to the experimental data was obtained with the binding constants used in Figure 4. Such a fit could not be obtained with an alternative scheme in which the trans isomers have a higher affinity than the cis isomers.

It can be seen from Figure 4a that 50% inhibition of E1 activity (enzyme concentration 2.4 μM) occurred at 1.3 μM tetrahydro-TPP (cis isomers). This gives an upper limit for

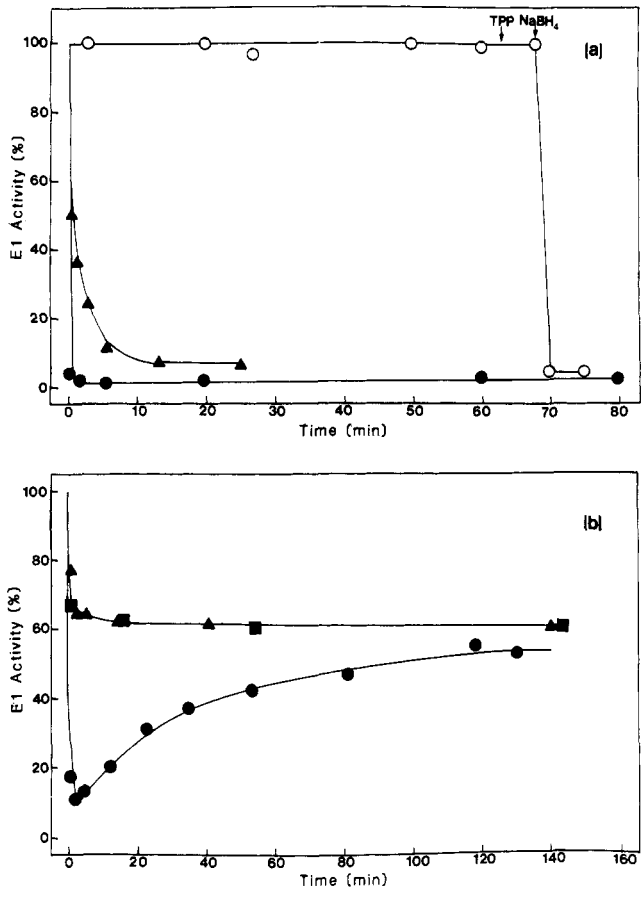


FIGURE 5: Inhibition of E1 activity by $NaBH_4$ in the presence of TPP. (a) E1 (0.27 mg/mL, 2.7 μM) dissolved in 50 mM potassium phosphate and 1 mM $MgCl_2$, pH 7, was incubated at 22 °C for 20 min in the absence (○) or presence (●) of 210 μM TPP. At zero time on the graph, $NaBH_4$ (5 mM) was added to each incubation. E1 activity was followed by diluting samples (2 μL) into 1 mL of assay medium and measuring the overall complex activity after reconstitution with E2E3 (3 μg). To the incubation in the absence of TPP was added TPP (210 μM) 63 min after $NaBH_4$ addition. 5 mM $NaBH_4$ was added 5 min later as indicated by the arrows. In a separate experiment, $NaBH_4$ (5 mM) was added to a solution containing 50 mM potassium phosphate, 1 mM $MgCl_2$, and 0.21 mM TPP. After 40 min, E1 was added to a concentration of 0.27 mg/mL. E1 activity was monitored at intervals (▲). (b) E1 (0.27 mg/mL, 2.7 μM) was incubated with 50 mM potassium phosphate, 21 μM TPP, and 1 mM $MgCl_2$, pH 7, for 20 min. $NaBH_4$ (5 mM) was added at zero time, and E1 activity (●) was followed as in (a). $NaBH_4$ (5 mM) was also added to a solution of 50 mM potassium phosphate, 21 μM TPP, and 1 mM $MgCl_2$, pH 7. After either 5 (▲) or 49 min (■) of preincubation, E1 was added at zero time to a concentration of 0.27 mg/mL, and E1 activity was followed as in (a).

the amount of tetrahydro-TPP bound to cause complete inhibition of E1 as 1.1 mol of inhibitor/mol of E1 chain.

Inhibition of the Enzymic Activity of E1 by TPP in the Presence of $NaBH_4$. The addition of $NaBH_4$ to a solution of E1 containing TPP resulted in a rapid inhibition of E1 activity, as measured by removing samples and assaying either by reconstitution with E2E3 subcomplex (Figure 5a) or by the dichlorophenolindophenol reduction assay. In the absence of TPP, incubation with $NaBH_4$ had no effect on the enzyme activity. Similarly, in the absence of $NaBH_4$, incubation with TPP had no effect.

This inhibition could be due either to the production of borohydride-reduced TPP in solution, which could then bind to E1, or to the reduction of TPP already bound in the catalytic site of the enzyme. We therefore compared the rate and extent of inhibition obtained by incubating E1 with TPP that had been treated with $NaBH_4$ with that obtained by adding $NaBH_4$ to a mixture of E1 and TPP. The amounts of enzyme and reagents were identical for the two experiments. It is important to realize that the pH of incubation (pH 7) was such that the half-time for the spontaneous hydrolysis of $NaBH_4$ was about 0.15 min. Thus, reduction of TPP in free solution would have ceased after 1.5 min. Under the conditions described in Figure 5, about 7% of the free TPP was reduced, as judged by the UV spectral change at 266 nm. It can be seen from Figure 5a that addition of $NaBH_4$ to a mixture of E1 and 0.21 mM TPP caused a rapid and complete inhibition of E1 activity, which was irreversible over the time course of the experiment. Addition to E1 of TPP that had been pretreated with $NaBH_4$ caused a slower inhibition but also led eventually (2 h) to total loss of enzyme activity. The more rapid inhibition observed for enzyme treated with $NaBH_4$ in the presence of TPP indicates that inhibitor is being produced in the active site, thereby eliminating the slower process of inhibitor binding to the active site from free solution.

In a further experiment (Figure 5b), the initial concentration of TPP was lowered 10-fold to 21 μM so that the system could be studied under conditions of incomplete enzyme inhibition. Addition of 21 μM TPP, pretreated with $NaBH_4$, led to a 40% inhibition of E1 activity. This would be consistent with the presence of about 1.4 μM tetrahydro-TPP in the mixture (see Figure 4). On the other hand, addition of $NaBH_4$ to a solution containing E1 and 21 μM TPP produced a 90% inhibition. Therefore, E1 appears to increase the inhibitory effect of $NaBH_4$ in the presence of TPP. However, on prolonged incubation, the catalytic activity of enzyme inhibited in this way recovered to a level similar to that obtained when the prereduced TPP was used as inhibitor (Figure 5b). The recovery of E1 activity occurred when all the $NaBH_4$ had decomposed. Comparable results were obtained with different initial concentrations of TPP. It should be noted that addition of TPP that had been treated with $NaBH_4$ for 5 or 49 min inhibited the enzyme identically, whereas the inhibition obtained by adding $NaBH_4$ to solutions of E1 containing TPP decreased between 5 and 49 min (Figure 5b). This eliminates the possibility that a potent but unstable inhibitor is produced during reduction of TPP by $NaBH_4$ in free solution. Similarly a direct effect of $NaBH_4$ on the enzyme is unlikely since inhibition was TPP dependent, and, second, addition of TPP (10 mM) at time 3 min caused a gradual reactivation to more than 95% activity under the conditions in Figure 5b (data not shown).

We attempted to characterize any product formed on the enzyme by adding $NaBH_4$ to E1 in the presence of TPP and, after denaturing the protein, examining the material that remained in solution. We analyzed the products directly by ion-exchange HPLC by using the system described under Materials and Methods for the isolation of the minor diastereomer of tetrahydro-TPP. The resolution was not as good as in the resolution of diastereomers, but it was clear that no major change in the total amount or diastereomeric ratio of tetrahydro-TPP had occurred in the presence of the enzyme. Better resolution was obtained after treatment with alkaline phosphatase to yield dephosphorylated compounds which could be analyzed by reverse-phase HPLC. The ratio of major to minor diastereomer was 4.3 (±0.6):1 in the presence of E1 and 2.9 (±0.2):1 in the absence of E1. No other species with retention times between those of tetrahydrothiamin and thiamin were observed.

Discussion

Borohydride reduction of TPP produces a mixture of four stereoisomers (two diastereomers each of which is a racemic mixture) of tetrahydro-TPP, in which both double bonds of

the thiazolium ring have been reduced (Figure 1). We have shown that the major diastereomer has the cis configuration. Tetrahydro-TPP had previously been prepared indirectly in a more laborious manner by pyrophosporylation of borohydride-reduced thiamin (Wittorf & Gubler, 1971; Heinrich et al., 1972), and its structure had not been confirmed. Tetrahydro-TPP resembles TPP (Figure 1), but it cannot function as a coenzyme since it no longer possesses the chemical features necessary to catalyze the decarboxylation of pyruvate. It is a potent inhibitor of the *E. coli* pyruvate dehydrogenase complex and of the isolated E1 component (Figures 2–4). We propose that it acts as an inactive analogue of TPP, binding at or near the TPP-binding site on E1. Thus, we have observed that TPP lowers the rate of inhibition brought about by tetrahydro-TPP, that the pyrophosphate group of tetrahydro-TPP is essential for inhibition, just as it is for binding TPP to the enzyme, and that the stoichiometry of binding tetrahydro-TPP is about 1.1 mol/mol of E1 chain (Figure 4a) compared with about 1 mol/mol of TPP per E1 chain (Moe & Hammes, 1974).

The curvature of the semilogarithmic plots of enzyme activity against time of incubation (Figure 2) could be due either to a mixture of inhibitors binding to the enzyme at different rates or to a single inhibitor binding to more than one site at different rates. The former explanation is more likely to be correct since tetrahydro-TPP does consist of a mixture of four stereoisomers. Thiamin thiazolone pyrophosphate (Figure 1d) is very similar in structure to tetrahydro-TPP but comprises a single isomer and shows pseudo-first-order kinetics of inhibition (Gutowski & Lienhard, 1976). There is no evidence at present for more than one type of TPP-binding site.

The binding data in Figure 4 can be explained with a model in which the four stereoisomers of tetrahydro-TPP are assigned K_d values of 0.2 and 0.15 μM (major diastereomers, cis configuration) and about 5–10 μM (minor diastereomers, trans configuration). These values should only be taken as estimates. In particular, we have fitted the data by assuming that the two minor isomers have the same binding constant. Equally good fits could be obtained if one was assumed to bind extremely weakly and the other slightly more tightly. More accurate estimates could not be made because the trans isomers are not bound tightly and were not isolated completely free of the tight-binding cis isomers. It would appear that both cis isomers bind very much more tightly than does TPP [K_d = 9 μM (Moe & Hammes, 1974; Gutowski & Lienhard, 1976)], although they do not bind as tightly as thiamin thiazolone pyrophosphate and thiamin thiothiazolone pyrophosphate (Gutowski & Lienhard, 1976; Butler et al., 1977).

It has been proposed that analogues of TPP that do not bear a charge on the sulfur-containing ring bind very tightly to the pyruvate dehydrogenase complex and pyruvate oxidase from *E. coli* because a hydrophobic region or positively charged region is present in the TPP-binding sites of those enzymes to stabilize the transition state (Crosby et al., 1970; Gutowski & Lienhard, 1976; O'Brien & Gennis, 1980). The observation that the cis isomers of tetrahydro-TPP bind extremely tightly to the TPP-binding site of *E. coli* pyruvate dehydrogenase complex is consistent with this view. However, the trans isomers bind no more strongly than does TPP, which proves that steric factors are important. It has been suggested that the high temperature dependence of the binding constant for TPP to *E. coli* pyruvate dehydrogenase complex (Moe & Hammes, 1974) and pyruvate oxidase (O'Brien et al., 1977) and of the rate of binding of thiamin thiazolone pyrophosphate to pyruvate oxidase (O'Brien & Gennis, 1980) is further evidence for the existence of a hydrophobic interaction in binding TPP. A similar temperature dependence governing the binding of tetrahydro-TPP to *E. coli* pyruvate dehydrogenase complex was observed (Figure 3).

The experiments in Figure 5a show that $NaBH_4$ caused a rapid inhibition of E1 activity in the presence of TPP, faster than that caused by adding tetrahydro-TPP to E1 free in solution. TPP was essential for the process, and the inhibition was reversed by subsequent addition of TPP. The most likely explanation appears to be the production of a reversible inhibitor at the active site of E1, presumably by reduction of TPP by $NaBH_4$. The experiment shown in Figure 5b also implies that reduction of TPP is facilitated by binding to the active site of the enzyme, since a greater extent of inhibition is produced by the action of a limited amount of $NaBH_4$ on the E1/TPP complex than by addition of the equivalent prereduced TPP to E1. This could be due to the polarization of the $>^{+}N{=}C$ bond of the thiazole ring on the enzyme, as predicted by Crosby et al. (1970) and Crosby & Lienhard (1970).

In the experiments depicted in Figure 5b, the inhibition of E1 caused by reaction of enzyme-bound TPP with $NaBH_4$ was found to reverse slowly, once the excess $NaBH_4$ was destroyed by spontaneous hydrolysis. Since the final level of inhibition was identical whether $NaBH_4$ was added to E1 in the presence of TPP or enzyme was added to prereduced TPP, the inhibitor formed at the active site must bind only weakly. It therefore cannot be simply the normal diastereomeric mixture of tetrahydro-TPP.

Possible explanations for this phenomenon are (a) that stereoselective reduction of enzyme-bound TPP favors a particular stereosiomer of tetrahydro-TPP (this would act as an inhibitor when bound at the TPP site but must have a higher K_d than those of either TPP or the normal diastereomeric mixture of tetrahydro-TPP, so that it is slowly displaced by these compounds causing reactivation) or (b) that reduction of enzyme-bound TPP generates dihydro-TPP, which is then oxidized back to TPP or dissociates from the enzyme to form a very weak inhibitor in free solution.

We attempted to look for products formed by the action of $NaBH_4$ on enzyme-bound TPP by adding $NaBH_4$ to the E1/TPP complex, denaturing the protein, and analyzing any products by HPLC. No great change in the total amount of tetrahydro-TPP was observed, when compared with that formed from TPP and $NaBH_4$ in the absence of E1. This suggests that tetrahydro-TPP was not being formed in large amounts on the enzyme. Moreover, the observed increase in the ratio of cis to trans diastereomers formed in the presence of E1 is the reverse of that expected if the reactivation process were due to the stereoselective production of one or both of the weakly binding trans diastereomers. We conclude that $NaBH_4$ does not cause reduction of enzyme-bound TPP solely to tetrahydro-TPP.

Borohydride reduction of thiamin passes through dihydrothiamin (Clarke & Sykes, 1967), and dihydrothiamin is slowly oxidized by air to thiamin (Hirano et al., 1959). However, we found no fall in the rate of reversal of inhibition of E1 treated with TPP and $NaBH_4$ in the presence of 1 mM dithiothreitol and little effect when the reaction was carried out under an atmosphere of N_2. It is possible that the reactivation observed was due to displacement of a dihydro-TPP by the excess TPP and that the former was only slowly oxidized back to TPP before the samples were ready for HPLC analysis. Addition of $NaBH_4$ to TPP bound at the E1 active site might cause a very rapid, enzyme-enhanced, reduction of the

$>^{+}N{=}C$ bond to yield a dihydro-TPP derivative in stoichiometric amount on the enzyme. A small portion of this could rearrange to the thiazolinium form and be reduced further to the tetrahydro derivative (Clarke & Sykes, 1967). This reduction could be stereoselective on the enzyme and produce the observed change in the ratio of diastereomers. Most of the dihydrothiamin would not be reduced further since the $NaBH_4$ is rapidly destroyed under the experimental conditions. It could then be displaced slowly by the excess of TPP, and the final level of activity would be determined by the diastereomeric mixture of tetrahydro-TPP. The released dihydro-TPP could then be oxidized back to TPP by air during subsequent attempts to isolate it.

We have described a simple method for preparing tetrahydro-TPP and for resolving its diastereomers. The stereoisomers show significant differences in their binding constants to the pyruvate dehydrogenase complex of *E. coli*, and we have demonstrated that the major and minor diastereomers are of the cis and trans configurations, respectively. Tetrahydro-TPP is a potent inhibitor of yeast pyruvate decarboxylase (Wittorf & Gubler, 1971) and of transketolase (Heinrich et al., 1972). The diastereomeric purity of the tetrahydro-TPP was not discussed in those studies, but further analysis of the inhibition of these important TPP-dependent enzymes by purified diastereomers could form a useful guide to the stereochemical properties of their active sites. The only other information on this subject available thus far is that a single enantiomer of 2-(α-hydroxyethyl)-TPP is formed by the action of pig heart pyruvate dehydrogenase complex on pyruvate (Ullrich & Mannschreck, 1967). The absolute configuration of this species is not yet known.

Acknowledgments

We are grateful to Dr. H. B. F. Dixon, Dr. H. W. Duckworth, and Dr. P. F. Leadlay for helpful discussion. We thank Dr. G. C. K. Roberts and Dr. P. D. Regan for recording NMR spectra and Dr. P. Rich for computer programs to calculate binding curves. One of us (F.J.L.) gratefully acknowledges the support of a research fellowship from St. John's College, Cambridge.

Registry No. TPP, 154-87-0; tetrahydro-TPP, 35728-83-7; (±)-*cis*-tetrahydro-TPP, 83704-57-8; (±)-*trans*-tetrahydro-TPP, 83704-58-9; (±)-*cis*-tetrahydrothiamin, 83704-59-0; E1, 9001-04-1; pyruvate dehydrogenase, 9014-20-4.

References

Breslow, R. (1958) *J. Am. Chem. Soc. 80*, 3719–3726.

Butler, J. R., Pettit, F. H., Davis, P. F., & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun. 74*, 1667–1674.

Clarke, G. M., & Sykes, P. (1967) *J. Chem. Soc. C*, 1411–1414.

Coggins, J. R., Hooper, E. A., & Perham, R. N. (1976) *Biochemistry 15*, 2527–2533.

Crosby, J., & Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 5707–5716.

Crosby, J., Stone, R., & Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 2891–2900.

Danson, M. J., Hooper, E. A., & Perham, R. N. (1978) *Biochem. J. 175*, 193–198.

Danson, M. J., Hale, G., Johnson, P., Perham, R. N., Smith, J., & Spragg, S. P. (1979) *J. Mol. Biol. 129*, 603–617.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd ed., p 210, Oxford University Press, London.

Gutowski, J. A., & Lienhard, G. E. (1976) *J. Biol. Chem. 251*, 2863–2866.

Hale, G., & Perham, R. N. (1979) *Eur. J. Biochem. 94*, 119–126.

Harrison, R. A. (1974) Ph.D. Thesis, University of Cambridge.

Heinrich, P. C., Steffen, H., Janser, P., & Wiss, O. (1972) *Eur. J. Biochem. 30*, 533–541.

Hirano, H., Yonemoto, H., & Hara, Y. (1959) *Chem. Pharm. Bull. 7*, 545.

Khailova, L. S., Bernhardt, R., & Huebner, G. (1977) *Biokhimiya (Moscow) 42*, 113–117.

Kluger, R., & Smyth, T. (1981) *J. Am. Chem. Soc. 103*, 1214–1216.

Kluger, R., Chin, J., & Smyth, T. (1981) *J. Am. Chem. Soc. 103*, 884–888.

Krampitz, L. O. (1969) *Annu. Rev. Biochem. 38*, 213–240.

Leeper, F. J., & Lowe, P. N. (1983) *Heterocycles* (in press).

Matsukawa, T., Hirano, H., & Yurugi, S. (1970) *Methods Enzymol. 18A*, 141–162.

Moe, O. A., & Hammes, G. G. (1974) *Biochemistry 13*, 2547–2552.

O'Brien, T. A., & Gennis, R. B. (1980) *J. Biol. Chem. 255*, 3302–3307.

O'Brien, T. A., Blake, R., & Gennis, R. B. (1977) *Biochemistry 16*, 3105–3109.

Perham, R. N. (1978) *Tech. Life Sci. B110*, 1–39.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.

Reed, L. J., & Mukherjee, B. B. (1969) *Methods Enzymol. 13*, 55–61.

Stanley, C. J., Packman, L. C., Danson, M. J., Henderson, C. E., & Perham, R. N. (1981) *Biochem. J. 195*, 715–721.

Ullrich, J., & Mannschreck, A. (1967) *Eur. J. Biochem. 1*, 110–116.

Wittorf, J. H., & Gubler, C. J. (1970) *Eur. J. Biochem. 14*, 53–60.

Wittorf, J. H., & Gubler, C. J. (1971) *Eur. J. Biochem. 22*, 544–550.