The Elementary Reactions of the Pig Heart Pyruvate Dehydrogenase Complex

A STUDY OF THE INHIBITION BY PHOSPHORYLATION

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1. A method was devised for preparing pig heart pyruvate dehydrogenase free of thiamin pyrophosphate (TPP), permitting studies of the binding of [^{35}S]TPP to pyruvate dehydrogenase and pyruvate dehydrogenase phosphate. The K_d of TPP for pyruvate dehydrogenase was in the range 6.2–8.2 μM , whereas that for pyruvate dehydrogenase phosphate was approximately 15 μM ; both forms of the complex contained about the same total number of binding sites (500 pmol/unit of enzyme). EDTA completely inhibited binding of TPP; sodium pyrophosphate, adenylyl imidodiphosphate and GTP, which are inhibitors (competitive with TPP) of the overall pyruvate dehydrogenase reaction, did not appreciably affect TPP binding. 2. Initial-velocity patterns of the overall pyruvate dehydrogenase reaction obtained with varying TPP, CoA and NAD^+ concentrations at a fixed pyruvate concentration were consistent with a sequential three-site Ping Pong mechanism; in the presence of oxaloacetate and citrate synthase to remove acetyl-CoA (an inhibitor of the overall reaction) the values of K_m for NAD^+ and CoA were $53 \pm 5 \mu\text{M}$ and $1.9 \pm 0.2 \mu\text{M}$ respectively. Initial-velocity patterns observed with varying TPP concentrations at various fixed concentrations of pyruvate were indicative of either a compulsory order of addition of substrates to form a ternary complex (pyruvate–Enz–TPP) or a random-sequence mechanism in which interconversion of ternary intermediates is rate-limiting; values of K_m for pyruvate and TPP were $25 \pm 4 \mu\text{M}$ and $50 \pm 10 \text{ nM}$ respectively. The $K_{\text{in}}^{\text{TPP}}$ (the dissociation constant for Enz–TPP complex calculated from kinetic plots) was close to the value of K_d^{TPP} (determined by direct binding studies). 3. Inhibition of the overall pyruvate dehydrogenase reaction by pyrophosphate was mixed non-competitive versus pyruvate and competitive versus TPP; however, pyrophosphate did not alter the calculated value for $K_{\text{in}}^{\text{TPP}}$, consistent with the lack of effect of pyrophosphate on the K_d for TPP. 4. Pyruvate dehydrogenase catalysed a TPP-dependent production of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]pyruvate in the absence of NAD^+ and CoA at approximately 0.35% of the overall reaction rate; this was substantially inhibited by phosphorylation of the enzyme both in the presence and absence of acetaldehyde (which stimulates the rate of $^{14}\text{CO}_2$ production two- or three-fold). 5. Pyruvate dehydrogenase catalysed a partial back-reaction in the presence of TPP, acetyl-CoA and NADH. The K_m for TPP was $4.1 \pm 0.5 \mu\text{M}$. The partial back-reaction was stimulated by acetaldehyde, inhibited by pyrophosphate and abolished by phosphorylation. 6. Formation of enzyme-bound [^{14}C]acetylhydrolipoate from [$3\text{-}^{14}\text{C}$]pyruvate but not from [$1\text{-}^{14}\text{C}$]acetyl-CoA was inhibited by phosphorylation. Phosphorylation also substantially inhibited the transfer of [^{14}C]acetyl groups from enzyme-bound [^{14}C]acetylhydrolipoate to TPP in the presence of NADH. 7. The enzyme-bound carbanion of 2- α -hydroxyethyl-TPP was estimated by retention of the protein-hydroxy[^{14}C]ethyl-TPP complex on Millipore membranes after incubation of pyruvate dehydrogenase with [$3\text{-}^{14}\text{C}$]pyruvate and TPP. The number of enzyme-bound 2- α -hydroxyethyl-TPP groups in pyruvate dehydrogenase was approximately half the total number of TPP-binding sites. Phosphorylation of pyruvate dehydrogenase decreased the number of hydroxyethyl-TPP-binding sites by greater than 90%. 8. Glyoxylate was a poor substrate for the overall dehydrogenase reaction (K_m $747 \pm 226 \mu\text{M}$; V_{max} approximately 1% of that with pyruvate) and was a competitive inhibitor with respect to pyruvate (K_i 500 μM). Glyoxylate also

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inhibited the partial back-reaction. Phosphorylation of pyruvate dehydrogenase inhibited decarboxylation of [1- ^{14}C]glyoxylate and incorporation of ^{14}C from [U- ^{14}C]glyoxylate into enzyme-bound lipoate. 9. It is concluded that phosphorylation of pyruvate dehydrogenase inhibits all reactions in which the carbanion of 2- α -hydroxyethyl-TPP is formed. These include the overall reaction, decarboxylation of pyruvate, formation of enzyme-linked acetylhydrolipoate from pyruvate, oxidation of acetoin, the partial back-reaction and the transfer of acetyl groups from enzyme-linked acetylhydrolipoate to TPP. Possible mechanisms of action of pyrophosphate are also discussed.

The pyruvate dehydrogenase complex (EC 1.2.4.1) performs key and central functions in cellular fuel utilization and fatty acid biosynthesis, and its regulation to a large extent dictates the relative metabolic fates of carbohydrates and fats in energy homeostasis. In the presence of TPP (thiamin pyrophosphate) and Mg^{2+} the complex catalyses a co-ordinated sequence of reactions that can be represented by:

This reaction is catalysed by three enzymes, pyruvate decarboxylase, dihydrolipoate acetyltransferase and dihydrolipoate dehydrogenase, which act sequentially as indicated in Scheme 1 (Gunsalus, 1954; Reed, 1960; Ullrich & Mannschreck, 1967; Hayakawa *et al.*, 1969; Linn *et al.*, 1972). The initial product of pyruvate decarboxylation (reaction 1) is probably the enzyme-bound carbanion form of 2- α -hydroxyethyl-TPP (Ullrich & Mannschreck, 1967). In the absence of CoA and NAD^+ , crude preparations of 'pyruvic oxidase' (Jagannathan &

Schweet, 1952) or purified pyruvate dehydrogenase (Cooper *et al.*, 1974) catalyse the decarboxylation of pyruvate at approx. 1–5% of the rate in their presence. The reactions involved are designated 1f and 6r (Scheme 1) and the products include 2- α -hydroxyethyl-TPP (protonated form) and acetoin, and possibly acetolactate (Jagannathan & Schweet, 1952; Juni & Heym, 1956; Ullrich & Mannschreck, 1967). Acetaldehyde stimulates pyruvate decarboxylation by enhancing acetoin production (Juni & Heym, 1956).

The composition and organization of the bovine heart and kidney complexes have been studied by Reed and co-workers (Reed & Oliver, 1968; Linn *et al.*, 1972; Barrera *et al.*, 1972; Roche & Reed, 1972; Reed, 1974). The complex has a mol.wt. of approx. 1000000 and contains a core of approx. 60 identical acetyltransferase units, each containing one moiety of covalently bound lipoate. As isolated, pyruvate decarboxylase has a mol.wt. of approx. 154000 and appears to be a tetramer of subunit structure $\alpha_2\beta_2$ (mol.wt. of α subunit approx. 41000; mol.wt. of β

Scheme 1. Reactions catalysed by the component enzymes of the pyruvate dehydrogenase complex

Enz₁, Enz₂, Enz₃ are pyruvate decarboxylase, dihydrolipoyl acetyltransferase and dihydrolipoyl dehydrogenase respectively. The NADH-dependent, CoA-dependent oxidation of pyruvate is designated in the text as the 'overall reaction'. Individual reactions proceeding in the direction of pyruvate (or acetoin) utilization and oxidation are designated in the text by the suffix 'f', i.e. 1f, 2f, 3f etc.; reactions in the reverse direction are designated 2r, 3r, 4r etc.

subunit approx. 36000). It has been suggested that the α subunit of pyruvate decarboxylase may catalyse reaction 1f, and the β subunit may catalyse reaction 2f (Scheme 1) (Roche & Reed, 1972). Approximately 30 pyruvate decarboxylase tetramers have been isolated per bovine heart complex. Dihydrolipoyl dehydrogenase is a flavin-containing enzyme, and each complex contains about 12 flavoprotein chains (Reed, 1974).

The complete reaction sequence is inhibited by acetyl-CoA (competitive with CoA) and NADH (competitive with NAD^+) (Garland & Randle, 1964; Randle *et al.*, 1966; Tsai *et al.*, 1973). Acetoin is a competitive inhibitor (against pyruvate) of the full reaction sequence (Cooper *et al.*, 1974). A number of compounds containing the pyrophosphate or imidodiphosphate moieties are competitive inhibitors of the overall pyruvate dehydrogenase reaction with respect to TPP (Cooper *et al.*, 1974).

In addition to the three catalytic components, the mammalian pyruvate dehydrogenase complex contains pyruvate dehydrogenase kinase which catalyses the phosphorylation and inactivation of pyruvate decarboxylase (Linn *et al.*, 1969a; Hucho *et al.*, 1972; Roche & Reed, 1972). Phosphorylation covalently modifies the α subunit of pyruvate decarboxylase, but the mechanism whereby this leads to inhibition of the overall pyruvate dehydrogenase reaction is not well understood. Roche & Reed (1972) have presented some evidence that phosphorylation inhibits decarboxylation of pyruvate in the absence of CoA and NAD^+ (reaction 1f), but not the subsequent 2- α -oxidation of hydroxyethyl-TPP (reactions 2f, 3f, 4f, 5f). A fifth enzyme, pyruvate dehydrogenase phosphate phosphatase catalyses the dephosphorylation and reactivation of pyruvate decarboxylase (Linn *et al.*, 1969a,b). It is more loosely bound in the complex and may be separated from the other components by ultracentrifugation. These phosphorylation and dephosphorylation reactions are potentially regulated by the concentration ratios of [ATP]/[ADP], [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] and by pyruvate, Ca^{2+} , Mg^{2+} and pyrophosphate compounds such as PPi and TPP (Linn *et al.*, 1969b; Denton *et al.*, 1972; Wieland *et al.*, 1973; Cooper *et al.*, 1974; Cooper *et al.*, 1975; Kerbey *et al.*, 1976).

To elucidate the mechanism whereby phosphorylation affects the pyruvate dehydrogenase reaction, its effects on the elementary (partial) reactions catalysed by the complex, together with the binding of TPP and 2- α -hydroxyethyl-TPP to the enzyme, have been investigated. The results of these studies are reported here in association with data defining the effects of PPi , pyrophosphate analogues and other inhibitors of the pyruvate dehydrogenase-catalysed reaction on these partial reactions. Several of these studies became possible when conditions were established to purify pyruvate dehydrogenase free of TPP. Obser-

vations made during these studies have also given some insight into aspects of the mechanism of the pyruvate dehydrogenase reaction and its regulation by product inhibition.

Experimental

Materials

Chemicals. Nucleotides, coenzymes, sodium pyruvate, TPP and citrate synthase were from Boehringer (London) Corp., London W.5, U.K. Thiamin, sodium glyoxylate, bovine serum albumin and Norit A were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Norit A was activated by stirring with 1M-HCl for 2h (7.5g/500ml), followed by centrifugation at 2500g and resuspension in water (1 litre). This washing procedure was repeated four times and the suspension was adjusted to pH 7.0 with NH_3 . After one further wash the Norit A was resuspended in water (approx. 50mg/ml). Acetyl-CoA was synthesized by the method of Simon & Shemin (1953). Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Butyl-PBD scintillator [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Other chemicals were Analytical-Reagent grade or the highest grade available.

Pig heart pyruvate dehydrogenase. This was prepared as described by Cooper *et al.* (1974) and was stored at a concentration of 30–60 units/ml in small batches at -20°C . The specific activity was in the range 7–9 units/mg. Preparations were low in phosphatase activity, and the activities of lactate dehydrogenase and 2-oxoglutarate dehydrogenase were negligible (<0.1% of that of pyruvate dehydrogenase).

Pyruvate dehydrogenase phosphate. Except where specifically indicated pyruvate dehydrogenase phosphate was prepared by the following procedure. Pyruvate dehydrogenase (30–40 units/ml) was incubated with 20mM-potassium phosphate, pH 7.0, containing 0.4mM-ATP, 2mM- MgCl_2 and 5mM-2-mercaptoethanol for 15min at 30°C . EDTA was added to a final concentration of 1mM, and the mixture layered on to a sucrose gradient (0.5ml of 10% sucrose below 5ml of 2% sucrose in 20mM-potassium phosphate, pH 7.0, containing 5mM-2-mercaptoethanol). After centrifugation at 150000g for 90min at 4°C , the pyruvate dehydrogenase phosphate was dispersed into the 10% sucrose layer, diluted to approx. 1ml, and stored at -20°C .

Pyruvate dehydrogenase phosphate phosphatase. This was prepared from pig heart muscle as described by Denton *et al.* (1972).

Methods

Assay of pyruvate dehydrogenase. This was assayed spectrophotometrically by the initial rate of ΔE_{340} at

30°C on addition of pyruvate dehydrogenase to 1.5 ml of medium containing 100 mM-Tris/HCl, 0.5 mM-EDTA, 1 mM-MgCl₂, 5 mM-2-mercaptoethanol, 1 mM-pyruvate, 1 mM-TPP, 0.1 mM-CoA, 0.5 mM-NAD⁺, pH 7.8 (assay system I). In certain experiments, to permit direct comparison of results with those obtained for the binding of TPP to the enzyme, the pH of the assay medium was 7.0, EDTA was omitted, and MgCl₂ was increased to 2 mM (assay system II).

Assay of pyruvate decarboxylase activity. Decarboxylation of pyruvate in the absence of CoA and NAD⁺ was assayed by the release of ¹⁴CO₂ from [1-¹⁴C]pyruvate essentially as described by Cooper *et al.* (1974). Except where otherwise indicated pyruvate dehydrogenase (0.2 to 1.0 units/ml) was incubated at 30°C in 100 mM-Tris/HCl, pH 7.4, with 2 mM-MgCl₂, 10 mM-2-mercaptoethanol, 10 μM-TPP and 100 μM-[1-¹⁴C]pyruvate in a total volume of 40 μl. The reaction was terminated by injection of 100 μl of 0.5 M-HCl and the liberated CO₂ was absorbed into 2-phenethylamine/methanol (1:1 v/v; 0.5 ml) injected into the system just before addition of HCl.

Assay of acetylation of enzyme-bound lipoyl groups in pyruvate dehydrogenase. This was based on the method described by Barrera *et al.* (1972). Incubations were at 21°C in Beckman 152 Microfuge tubes. Pyruvate dehydrogenase (approx. 280 munits) was incubated with 2.5 μM-TPP, 1 mM-MgCl₂, 20 mM-potassium phosphate, 5 mM-2-mercaptoethanol, pH 7.0, and other additions as stated in the text or Tables, for 30 s before initiation of the reaction with either [3-¹⁴C]pyruvate (0.125 mM final concn., 10.5 mCi/mmol) or with [1-¹⁴C]acetyl-CoA (0.125 mM final concn., approx. 4 mCi/mmol). The total volume of incubation mixture was 40 μl. After incubation (usually for 1 min), the reaction was terminated by applying 30 μl to a 1 cm square of Whatman 31 ET chromatography paper, which was immediately plunged into ice-cold 10% (w/v) trichloroacetic acid (5–10 ml/paper) and gently stirred for 15 min. The papers were subsequently washed twice for 15 min with 5% trichloroacetic acid, and with ethanol for 5 min; finally they were rinsed with ether and dried. Radioactivity was assayed by liquid-scintillation spectrometry in toluene-based scintillator (4 g of butyl PBD/litre of toluene). A correction for incorporation of radioactivity into non-thioester linkage was made as follows. Papers were placed in a desiccator over performic acid (2 vol. of 98% formic acid; 0.1 vol. of 30% H₂O₂) for 18 h at room temperature, and then over solid KOH for a further 24 h to remove [¹⁴C]acetic acid. The radioactivity remaining on the papers (about 5–10% of maximum ¹⁴C incorporation) was taken to be in non-thioester linkages and was subtracted from the total incorporation. There was no incorporation of radioactivity from [1-¹⁴C]-pyruvate into thioester linkage.

Assay of the formylation of enzyme-bound lipoyl

groups in pyruvate dehydrogenase. Techniques similar to those described for the assays of acetylation of enzyme-bound lipoyl groups were used after incubation of pyruvate dehydrogenase or pyruvate dehydrogenase phosphate with [U-¹⁴C]glyoxylate.

Assay of pyruvate dehydrogenase-bound 2-α-[¹⁴C]-hydroxyethyl-TPP formed from [2-¹⁴C]pyruvate or from [1-¹⁴C]acetyl-CoA. Pig heart pyruvate dehydrogenase complex (both phosphorylated and non-phosphorylated forms) is completely retained by Millipore filtration (Millipore membrane type IEA HAWP 00010; 0.45 μm pore size; 2.4 cm diam.) based on enzyme or ³²P assays. Enzyme-bound 2-α-hydroxyethyl-TPP, formed by incubation with [2-¹⁴C]-pyruvate or with [1-¹⁴C]acetyl-CoA, may be determined from membrane-associated radioactivity after correction for the radioactivity present as acetylhydrolipoate. Pyruvate dehydrogenase (250 munits) or pyruvate dehydrogenase phosphate (equivalent to 250 munits of pyruvate dehydrogenase) was incubated at 20°C for 1 min in 35 μl of 20 mM-potassium phosphate, pH 7.0, containing 5 mM-2-mercaptoethanol, 1 mM-MgCl₂ and other additions as indicated in the text and Tables. [2-¹⁴C]pyruvate (0.125 mM final concn., 10.5 mCi/mmol) or [1-¹⁴C]acetyl-CoA (0.125 mM final concn., approx. 4 mCi/mmol) was then added to give a final volume of 40 μl and incubation continued, usually for a further 1 min. A sample (30 μl) was applied to a Millipore filter under gentle suction and the filter (under continuous suction) was immediately washed with four successive 1 ml samples of 20 mM-potassium phosphate buffer, pH 7.0. For the combined assay of enzyme-bound hydroxyethyl-TPP and acetylhydrolipoate the membranes were immediately transferred to 5 ml of methoxyethanol/toluene-based scintillator (Severson *et al.*, 1974) and allowed to dissolve. For the assay of acetylhydrolipoate alone, the membranes were transferred to ice-cold 10% (w/v) trichloroacetic acid and treated as in the acetylation procedure, except for the omission of the ether wash. Radioactivity was determined in methoxyethanol/toluene-based scintillator. This technique gave results essentially similar to those obtained by using paper squares, as described in the acetylation procedure, but had the advantage that radioactivity was assayed under conditions identical with those for the combined assay of hydroxyethyl-TPP and acetylhydrolipoate. The dissolved filters caused little or no quenching when determined using an external or internal standard; a small correction was made for binding of pyruvate to the membrane by measurement in the absence of enzyme.

Removal of TPP from pyruvate dehydrogenase. Pyruvate dehydrogenase preparations contain variable amounts of TPP; enzyme activities between 2 and 30% of maximum were observed in the absence of added TPP (Cooper *et al.*, 1974).

TPP was removed from pyruvate dehydrogenase

by treatment with acid-washed Norit A. All operations were conducted at 4°C. Pyruvate dehydrogenase (0.5–1.0 ml; approx. 30 units/ml) in 20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol was dialysed against 100 mM-Tris/HCl, pH 7.4, containing 5 mM-2-mercaptoethanol for 1.5 h. The enzyme was added to a pellet containing 3–7 mg of Norit A (prepared as described previously; since considerable variation is observed between batches prepared from the same lot no., the amount required was characterized for each preparation) and mixed by gentle rotation for 30 min. After centrifugation at 16000g for 2 min, the supernatant was removed and subjected to two further identical Norit A treatments.

The amount of TPP remaining in the pyruvate dehydrogenase preparation after Norit A treatment was extrapolated from measurements of dehydrogenase activity as a function of TPP concentration. A typical experiment is shown in Fig. 1; the intercept on the TPP concentration axis was taken to be equal to the negative value of the residual TPP in the preparation. The possibility that the slow rate of NAD⁺ reduction in the absence of TPP was due to a TPP-independent enzyme-catalysed reaction was excluded by the observation that the supernatant from Norit A-treated pyruvate dehydrogenase which had been boiled for 1 min contained sufficient TPP to account for the residual rate. Norit A treatment yielded pyruvate dehydrogenase that contained less than 0.013 nmol of TPP per unit of enzyme. Such enzyme is referred to as 'TPP-free' in the present paper.

Preparation and purification of [³⁵S]TPP. This was prepared from [³⁵S]thiamin and ATP by using thiamin pyrophosphokinase (ATP-thiamin pyrophosphotransferase, EC 2.7.6.2) isolated from rat liver by an extensive modification of the methods of Mano (1960) and Deus *et al.* (1969). Unless stated otherwise, operations were carried out at 4°C. Livers (approx. 50 g total), removed from six rats after stunning and decapitation, were diced and homogenized in 0.25 M-sucrose (60 ml/liver) in a Teflon/glass Potter-Elvehjem homogenizer. The cytosol obtained by centrifugation at 110000g for 2 h (MSE 65 centrifuge; 8 × 50 ml rotor) was adjusted to pH 5.8 with 1% (v/v) acetic acid and divided into three batches. Each batch was warmed to 49°C for 5 min, and then cooled in ice to 0°C and centrifuged at 32000g for 50 min. The supernatant was adjusted to 52% saturation with (NH₄)₂SO₄ (32.8 g/100 ml), stirred for 30 min, and then centrifuged at 15000g for 15 min. The supernatant was adjusted to 68% saturation with (NH₄)₂SO₄ (10.3 g/100 ml), stirred for 30 min and then centrifuged at 15000g for 15 min. The precipitate was resuspended in 10 mM-potassium phosphate, pH 5.8 (13.5 ml) and dialysed for 14 h against two changes of the same buffer. After centrifugation at 10000g for 15 min, the supernatant was adjusted to pH 4.4 with 1% (v/v) acetic acid, incubated for 20 min

at 37°C and adjusted to pH 5.8 with 1 M-KOH. The supernatant obtained by centrifugation at 32000g for 15 min was adjusted to 68% saturation with (NH₄)₂SO₄ (42.8 g/100 ml). After 15 min, the precipitate was collected by centrifugation (32000g for 15 min), resuspended in 100 mM-potassium phosphate, pH 5.8 (0.8 ml), and applied to a column (0.9 cm × 55 cm) of Sephadex G-200, previously equilibrated with the same buffer. Fractions (1.0 ml) were collected at a flow rate of 2.5 ml/h and were assayed for protein by absorption at 280 nm. Fractions 25–29 inclusive, containing thiamin pyrophosphokinase, were pooled and stored at –15°C. The activity was approx. 2 munits/ml and was stable for several months. For thiamin pyrophosphokinase assay the reaction mixture contained 10 mM-ATP, 20 mM-MgCl₂, 0.25 mM-thiamin and thiamin pyrophosphokinase, in 100 mM-glycylglycine buffer, pH 8.0 (total volume 0.2 ml). After incubation at 30°C, the reaction was terminated by boiling for 1 min, and the suspension centrifuged at 16000g for 2 min; the supernatant was assayed for TPP content as in Fig. 1. Formation of TPP in this assay was linear with time for up to 40 min; there was no interference in the pyruvate dehydrogenase reaction by any of the components of the thiamin pyrophosphokinase assay.

[³⁵S]TPP was prepared as follows: [³⁵S]thiamin (0.33 mM; 100 mCi/mmol), 13.3 mM-ATP (adjusted

Fig. 1. Estimation of the residual TPP content of Norit A-treated pyruvate dehydrogenase

Pyruvate dehydrogenase (208 munits) which had been treated with Norit A (see the Experimental section) was added to 1.5 ml of assay system I (see the Experimental section) containing the amounts of TPP indicated, and the rate of ΔE₃₄₀ measured over a period of 4 min. Each point is the mean of duplicate measurements. This standard curve also served to determine the amount of TPP produced in the thiamin phosphokinase assay.

to pH 7.0 with triethylamine), 26.6 mM-magnesium acetate and thiamin pyrophosphokinase (2 munits) were incubated in 133 mM-triethylamine/ NaHCO_3 buffer, pH 8.0, in a total volume of 3.0 ml at 37°C for 6 h. The reaction was terminated by boiling for 1 min and the suspension centrifuged at 16000g for 2 min. [^{35}S]TPP was separated by DEAE-cellulose chromatography (Whatman DE52; 2.2 cm $\times$ 5 cm column) using a linear gradient (200 ml) of triethylamine/ NaHCO_3 , pH 7.5 (25–400 mM). Complete resolution of [^{35}S]TPP from [^{35}S]thiamin and ATP was achieved. Fractions containing [^{35}S]TPP were pooled, and acetic acid (0.1 M final concn.) was added before rotary evaporation to dryness. The [^{35}S]TPP was resuspended in 10 ml of water, re-evaporated to dryness, and stored at a concentration of 0.25 mM at –15°C (overall yield of ^{35}S approx. 20–30%). The calculated specific radioactivity of [^{35}S]TPP was within 5% of that of [^{35}S]thiamin from which it was prepared.

The purity of [^{35}S]TPP was checked by two t.l.c. systems; (1) plates of cellulose (Polygram Cel 300; Macherey-Nagel and Co., Düren, West Germany), developed with the pyridine/isobutanol/water (16:24:25, by vol.) solvent system described by Rossi-Fanelli *et al.* (1952), and (2) plates of anion-exchange cellulose (Polygram Cel 300 PE1/UV; Macherey-Nagel and Co.), developed with 1 M-triethylamine/ NaHCO_3 , pH 7.5. The latter plates were washed for 60 h with the elution buffer before use. Samples of [^{35}S]TPP and unlabelled thiamin and TPP (2 μg with system 1, 20 μg with system 2) were developed in systems (1) and (2) by ascending chromatography for approx. 4 h at room temperature. Thiamin and TPP were detected in system (1) by thiochrome fluorescence by spraying with a mixture of 2.5% $\text{K}_3\text{Fe}(\text{CN})_6$, 10% NaOH and 55% ethanol (Rossi-Fanelli *et al.*, 1952), and in system (2) by u.v. fluorescence. With both t.l.c. systems all the radioactivity (>98%) coincided with authentic TPP.

Detection of pyrophosphate hydrolysis after incubation with pyruvate dehydrogenase. This was assayed as P_i by the method of Fiske & Subbarow (1925), after deproteinization with trichloroacetic acid (5% final concn.). NaF (at the concentration used in the incubation with pyrophosphate and pyruvate dehydrogenase) had no effect on the determination of phosphate by this method. Values were corrected for blanks which had a small amount of phosphate present in the pyruvate dehydrogenase after dialysis, and for non-enzyme catalysed breakdown of pyrophosphate.

Ligand binding measurements by using equilibrium microdialysis cells. The microdialysis cells described by Englund *et al.* (1969) were made in the workshop of the Medical School, University of Bristol. The membrane was Visking 36/32 dialysis tubing (obtained from Medicell International, London EC4N 5SA, U.K.) which had been boiled for 2 h in double-

distilled water (two changes) and stored in double-distilled water. The cells were assembled by placing a small glass bead (approx. 1 mm diam.) in each chamber to aid mixing, placing a piece of membrane (1 cm $\times$ 2 cm) between the two chambers in each set, and then clamping the assembly in a metal holder. Three cells, each containing a twin set of dialysis chambers, could be accommodated in one holder. The cells were loaded with 25 μl of protein solution on one side of the membrane, and 25 μl of medium containing radioactive ligand on the other side, by using a microsyringe (S.G.E., Melbourne, Vic. 3051, Australia). For satisfactory filling of the chambers, it was essential to ensure that all parts of the chamber (especially the entry ports), and the tip of the microsyringe, were completely dry. The cells were sealed with tape and rotated at approx. 5 rev./min; at the end of equilibrium dialysis the contents of each chamber were removed by using a micro-syringe.

Expression of results. A unit of enzyme activity is the amount of enzyme catalysing the conversion of 1 μmol of substrate in 1 min at 30°C. For pyruvate dehydrogenase phosphate, a unit of enzyme activity is that amount which yields one unit of pyruvate dehydrogenase activity after complete conversion into the non-phosphorylated form with phosphatase in the presence of Mg^{2+} and Ca^{2+} .

Enzyme-kinetic data were analysed by using a Hewlett-Packard desk-top computer (Model 9821 A). Two-substrate enzyme-kinetic data were fitted to the equations for sequential or Ping Pong mechanisms (Cleland, 1963a) by using the weighting factors described by Cleland (1967); the most significant fit was calculated on the basis of a variance-ratio (F) test. Enzyme-inhibition data were analysed by fitting to the equation for mixed non-competitive inhibition, and comparing the significance of fit (F-test) with that obtained by using the equations for competitive, simple linear non-competitive and uncompetitive inhibition (for definitions of terms used and for details of analysis see Cleland, 1963a,b). Equilibrium-binding data were analysed by linear least-squares regression.

Results

Pig heart pyruvate dehydrogenase, as freshly prepared (Cooper *et al.*, 1974) contains oxidized lipoate and oxidized FAD (>90%) and is free of 2- α -hydroxyethyl-TPP and acetylhydrolipoate. Thus when incubated with NAD^+ , but in the absence of pyruvate, CoA and TPP, an amount of NAD^+ equivalent to less than 10% of the total lipoate and flavin content of the enzyme was reduced. The addition of CoA to the system produced no further formation of NADH. Results obtained from the studies of the acetylation of pyruvate dehydrogenase presented elsewhere in the present paper support

these conclusions. On storage of the enzyme at -20°C in the presence of 5 mM-2-mercaptoethanol the content of reduced lipoate increased significantly. Treatment of the enzyme with Norit A as described in the Experimental section yielded pyruvate dehydrogenase that was entirely dependent (99.6–99.9%) on the addition of TPP for activity.

Kinetic studies of the overall pyruvate dehydrogenase reaction

Tsai *et al.* (1973) have examined the kinetic mechanism of the overall pyruvate dehydrogenase reaction of the bovine kidney complex. Their results,

based on initial-velocity patterns with varying concentrations of pyruvate, CoA and NAD^+ , and on product-inhibition patterns with acetyl-CoA and NADH, are consistent with the patterns predicted by Cleland (1973) for a three-site Ping Pong mechanism. The availability of TPP-free pyruvate dehydrogenase has permitted an extension of these kinetic studies. Initial-velocity patterns obtained at a constant concentration of pyruvate with varying concentrations of TPP at either fixed concentrations of NAD^+ and a saturating concentration of CoA, or at a fixed concentration of CoA and a saturating concentration of NAD^+ , are presented in Figs. 2(a) and 2(b) respectively. The parallel reciprocal plots obtained

Fig. 2. Initial-velocity patterns for pyruvate dehydrogenase with TPP, NAD^+ and CoA as the variable substrates

(a) Reciprocal velocities are plotted as a function of TPP concentration at the concentrations of NAD^+ indicated on the Figure and saturating concentrations of pyruvate (0.9 mM) and CoA (90 μM). (b) Reciprocal velocities are plotted as a function of TPP concentration at the fixed concentrations of CoA indicated on the Figure and saturating concentrations of pyruvate (0.9 mM) and NAD^+ (0.44 mM). Assays were conducted at 30°C in system II, plus 0.9 mM-oxaloacetate and 10 μg of pig heart citrate synthase (110 units/mg) in a final volume of 1.7 ml, by using 8.8 munits (a) or 6.2 munits (b) of pyruvate dehydrogenase. Statistical analysis indicated a significant fit to a Ping Pong type mechanism for each experiment. Calculated K_m values were: NAD^+ , $53 \pm 5 \mu\text{M}$; CoA, $1.95 \pm 0.2 \mu\text{M}$; TPP, $60 \pm 6 \text{ nM}$ for experiment (a), $47 \pm 7 \text{ nM}$ for experiment (b).

Fig. 3. Initial-velocity patterns for pyruvate dehydrogenase with TPP and pyruvate as the variable substrates

Reciprocal velocities are plotted as a function of TPP concentration at the fixed concentrations of pyruvate indicated on the Figure. The 1.72 ml reaction mixtures of assay system II contained saturating concentrations of (a) NAD^+ (0.44 mM) and (b) CoA (90 μM) and 16 munits of pyruvate dehydrogenase. Calculated kinetic constants were K_m^{TPP} $50 \pm 10 \text{ nM}$, K_m^{Pyruvate} $25 \pm 4 \mu\text{M}$, K_{ia}^{TPP} $1.6 \pm 0.3 \mu\text{M}$. Assays were conducted at 30°C .

in both experiments are consistent with the three-site Ping Pong mechanism deduced for the bovine kidney complex by Tsai *et al.* (1973). The K_m for NAD^+ calculated from the data of Fig. 2(a) is $53 \pm 5 \mu\text{M}$, in close agreement with that obtained by Tsai *et al.* (1973). By contrast, the K_m for CoA calculated from the data of Fig. 2(b) is $1.9 \pm 0.2 \mu\text{M}$ and this value is an order of magnitude lower than that determined by Tsai *et al.* (1973). However, in the experiment presented in Fig. 2(b) 0.9 mM-oxaloacetate and citrate synthase were present to convert the acetyl-CoA produced into citrate and CoA, and thus avoid product inhibition of pyruvate dehydrogenase by acetyl-CoA. By this procedure, linear initial reaction rates for pyruvate dehydrogenase could be obtained even at an acetyl-CoA concentration of 0.88 μM . In the presence of the trapping system for acetyl-CoA, product inhibition by NADH was also less severe, and hence reaction mixtures containing oxaloacetate and citrate synthase were used in the experiment in Fig. 2(a).

The initial-velocity pattern obtained with various concentrations of TPP at fixed concentrations of pyruvate and saturating concentrations of NAD^+ and of CoA is shown in Fig. 3. The converging plots

indicate that there is either a compulsory order of addition of substrates to form a ternary complex (pyruvate-Enz-TPP) or a random-sequence mechanism in which interconversion of ternary intermediates is rate-limiting. The calculated K_m for pyruvate is $25 \pm 4 \mu\text{M}$, in close agreement with the value determined by Tsai *et al.* (1973). The K_m for TPP as determined from Fig. 3 is $50 \pm 10 \text{ nM}$, consistent with the values of $60 \pm 5 \text{ nM}$ and $47 \pm 6 \text{ nM}$ that can be calculated from the data of Figs. 2(a) and 2(b), respectively. As indicated in the subsequent section, TPP can bind to pyruvate dehydrogenase in the absence of pyruvate. Assuming either a compulsory sequence of reactions in which TPP binds first or a rapid-equilibrium random mechanism, then the dissociation constant of TPP is equal to the negative TPP concentration at the point of intersection of the plots in Fig. 3 (Frieden, 1957; Segel, 1975). With this assumption, the K_d for TPP is equivalent to the K_{ia} (Fig. 3) and equals $1.6 \pm 0.3 \mu\text{M}$.

Binding of TPP to pyruvate dehydrogenase: calculation of K_d

The binding of TPP to pyruvate dehydrogenase was established by equilibrium dialysis by using

[^{35}S]TPP and the micro-dialysis cells described by Englund *et al.* (1969). Equilibrium was attained within 4 h at 21°C, and the enzyme was stable throughout the duration of the experiment. After 4 h equilibrium dialysis with 8 μM -TPP, more than 85% of ^{35}S chromatographed as TPP in the t.l.c. systems described under 'Methods'; no other ^{35}S -labelled component was detected. From the extent of binding of [^{35}S]TPP to pyruvate dehydrogenase as a function of ligand concentration (Fig. 4), a single binding site with $K_d = 8.3 \mu\text{M}$ was identified. This value is similar to that calculated from the kinetic experiments (Fig. 3), which were performed at 30°C. The maximum binding capacity of this preparation was 490 pmol/unit of enzyme activity; with two other preparations of pyruvate dehydrogenase, values for K_d were 6.2 and 6.9 μM , and for maximum binding capacity 346 and 462 pmol/unit. This indicates a stoichiometry of between one and two TPP-binding sites per $\alpha_2\beta_2$ decarboxylase subunit, assuming 30 of these subunits per complex. The variability obtained may be associated with the decline in specific activity of pyruvate dehydrogenase on storage at -20°C, the latter resulting in an increase in the experimental value.

Binding of TPP to pyruvate dehydrogenase phosphate; calculation of K_d

The binding of [^{35}S]TPP to pyruvate dehydrogenase phosphate (Fig. 4) was performed in the presence of 50 mM-NaF to inhibit dephosphorylation of the enzyme during the 4 h equilibrium dialysis by traces of endogenous phosphatase. Under these conditions less than 1% dephosphorylation occurred. NaF does not affect the binding of TPP to the non-phosphorylated enzyme (Table 1). The total number of TPP binding sites on pyruvate dehydrogenase phosphate was close to that found for the non-phosphorylated enzyme. The K_d of TPP for pyruvate dehydrogenase phosphate was 15.1 μM ; this value is approximately twice that obtained for the non-phosphorylated enzyme, but would not appear to be sufficient to account for the two orders of magnitude difference in activity of the two forms of the enzyme. Roche & Reed (1972) reported a 12-fold difference in the TPP affinity, but in their experiments the enzyme had not been freed of endogenous TPP, and binding was investigated by a method in which equilibrium with free ligand was not maintained.

Potential modifiers of TPP binding to pyruvate dehydrogenase

Table 1 shows the effects on the binding of TPP to pyruvate dehydrogenase of reagents which modify either pyruvate dehydrogenase or pyruvate dehydrogenase kinase activities; the latter is significant, since

TPP inhibits pyruvate dehydrogenase kinase activity (Cooper *et al.*, 1974). In the present studies the concentration of TPP used was no greater than the K_d , so that effects on either binding affinity or binding capacity would be manifest. EDTA completely inhibited the binding of TPP to pyruvate dehydrogenase, presumably reflecting the requirement for Mg^{2+} for the overall pyruvate dehydrogenase reaction (Sanadi, 1963; Wieland *et al.*, 1969). Sodium pyrophosphate, which is an inhibitor of the overall pyruvate dehydrogenase reaction competitive with TPP (Cooper *et al.*, 1974), did not inhibit the binding of TPP to the enzyme. Approx. 25% of the pyrophosphate was hydrolysed during the equilibrium dialysis; NaF (50 mM), which completely inhibited this breakdown, did not affect binding of TPP in the presence or absence of PP_i . GTP and adenylyl imido-

Fig. 4. The binding of [^{35}S]TPP to pyruvate dehydrogenase and pyruvate dehydrogenase phosphate as a function of TPP concentration

Samples (25 μl) of TPP-free pyruvate dehydrogenase (see the Experimental section) were injected with a micro-syringe into one side of each microdialysis cell; the other side of each cell contained 25 μl of 100 mM-Tris/HCl, pH 7.4, with 4 mM-MgCl₂, 5 mM-2-mercaptoethanol, and the concentrations of [^{35}S]TPP (10–20 mCi/ μmol) indicated. The cells were rotated, and after 4 h samples were removed from both sides of each cell and assayed for [^{35}S]TPP content. Results show binding to pyruvate dehydrogenase (●) obtained in two separate series of experiments; the mean enzyme concentrations in the two experiments were 34.5 and 38.3 units/ml of the same preparation of pyruvate dehydrogenase. The binding of [^{35}S]TPP to pyruvate dehydrogenase phosphate (□), derived from the same preparation of pyruvate dehydrogenase and containing less than 1% residual pyruvate dehydrogenase activity, was in the presence of NaF (50 mM) at a mean enzyme concentration of 29.7 units/ml. Statistical analysis indicated a K_d for TPP of $8.2 \pm 0.3 \mu\text{M}$ for the non-phosphorylated complex and $15.1 \pm 2.1 \mu\text{M}$ for the phosphorylated form. The maximum number of binding sites were 490 ± 74 and 543 ± 36 pmol/unit of enzyme for pyruvate dehydrogenase and pyruvate dehydrogenase phosphate respectively.

Table 1. *Effects of EDTA, sodium pyrophosphate, GTP, adenyl imidodiphosphate, NAD, CoA, glyoxylate, acetoin, acetaldehyde and dichloroacetate on the binding of TPP to pyruvate dehydrogenase*

The experimental details were as described in the legend to Fig. 4, except that the additions shown were present. Results are means $\pm$ s.e.m. for the number of determinations in parentheses.

Expt. no.	Addition	[TPP] (μ M)	TPP bound (pmol/unit)
1	None	2.88 $\pm$ 0.18	215 $\pm$ 6 (3)
	EDTA (1 mM)	6.39 $\pm$ 0.39	0 $\pm$ 10 (3)
2	None	3.34 $\pm$ 0.08	159 $\pm$ 12 (3)
	Sodium pyrophosphate (0.5 mM)	3.14 $\pm$ 0.37	185 $\pm$ 15 (2)
	KCl (50 mM)	3.66 $\pm$ 0.44	174 $\pm$ 2 (2)
	KCl (50 mM)+sodium pyrophosphate (0.5 mM)	3.61 $\pm$ 0.43	155 $\pm$ 14 (3)
	Dichloroacetate (0.1 mM)	3.49 $\pm$ 0.38	182 $\pm$ 35 (2)
3	None	2.14 $\pm$ 0.08	229 $\pm$ 14 (3)
	NaF (25 mM)	1.93 $\pm$ 0.02	249 $\pm$ 22 (3)
	NaF (25 mM)+sodium pyrophosphate (0.5 mM)	2.13 $\pm$ 0.07	219 $\pm$ 4 (3)
4	None	2.45 $\pm$ 0.28	143 $\pm$ 8 (3)
	Adenyl imidodiphosphate (0.5 mM)	2.41 $\pm$ 0.15	142 $\pm$ 14 (2)
5	None	1.23 $\pm$ 0.04	86.3 $\pm$ 0.2 (2)
	GTP (0.5 mM)	1.17 $\pm$ 0.03	90.6 $\pm$ 0 (2)
	Thiamin (0.5 mM)	1.34 $\pm$ 0.05	84.6 $\pm$ 12 (2)
	Acetoin (0.5 mM)	1.08 $\pm$ 0.04	88.2 $\pm$ 2 (2)
6	None	5.11 $\pm$ 0.06	261 $\pm$ 12 (3)
	NAD ⁺ (0.13 mM)	5.90 $\pm$ 0.44	275 $\pm$ 18 (3)
	CoA (0.46 mM)	5.74 $\pm$ 0.53	273 $\pm$ 6 (3)
7	None	1.88 $\pm$ 0.10	188 $\pm$ 11 (3)
	Acetaldehyde (0.5 mM)	2.10 $\pm$ 0.26	167 $\pm$ 6 (3)
8	None	1.89 $\pm$ 0.08	241 $\pm$ 17 (3)
	Glyoxylate (1 mM)	1.83 $\pm$ 0.01	224 $\pm$ 17 (3)

diphosphate, which also inhibit pyruvate dehydrogenase competitively with respect to TPP (Cooper *et al.*, 1974), were also without effect on TPP binding. Thiamin did not influence the binding of TPP to pyruvate dehydrogenase, apparently indicating that the binding of TPP to pyruvate decarboxylase requires interactions with both the thiamin and pyrophosphate moieties. There was no effect on TPP binding of CoA and NAD⁺ (substrates for reactions 3f and 5f respectively), glyoxylate (substrate analogue for pyruvate), acetoin or acetaldehyde (inhibitors of the overall pyruvate dehydrogenase reaction), dichloroacetate (an inhibitor of pyruvate dehydrogenase kinase) and K⁺ ions (which alter the sensitivity of the kinase to ADP inhibition; Roche & Reed, 1974).

Kinetic analysis of the effect of inhibitors of the overall pyruvate dehydrogenase reaction

Since sodium pyrophosphate is a competitive inhibitor of the overall pyruvate dehydrogenase reaction with respect to TPP (Cooper *et al.*, 1974), but does not appear to affect TPP binding, a detailed kinetic investigation of the effects of pyrophosphate was undertaken. It was confirmed that pyrophosphate

was a competitive inhibitor against TPP over a wide range of pyruvate concentrations and it was shown that the K_i of pyrophosphate was independent of pyruvate concentration (K_i values of 255 ± 80 , 202 ± 19 and $218 \pm 41 \mu$ M were obtained at pyruvate concentrations of 10, 50 and 500μ M respectively). At less than saturating concentrations of TPP (0.27μ M), pyrophosphate showed mixed non-competitive inhibition against pyruvate (Fig. 5a). In Fig. 6 is presented the initial-velocity pattern versus TPP concentration for the pyruvate dehydrogenase reaction, at various concentrations of pyruvate in the presence of saturating concentrations of NAD⁺ and CoA, and of 500μ M-sodium pyrophosphate. The double-reciprocal plots were linear over the TPP concentration range 0.15 – 2μ M, but digressed from linearity at TPP concentrations of 0.05μ M and below. Rates observed in the absence of pyrophosphate are given in Fig. 3. Analysis of the linear portion of the plots in Fig. 6 indicates that, in the presence of 500μ M-pyrophosphate, the K_m for TPP is 200 ± 20 nM. This is fourfold higher than that calculated from the control data (Fig. 3), an observation which is compatible with competitive inhibition. The K_m for pyruvate in the presence of pyrophosphate is $44 \pm 3 \mu$ M; this value is slightly higher than that observed in the absence of

Fig. 5. Initial-velocity patterns for pyruvate dehydrogenase in the presence of the inhibitors sodium pyrophosphate, glyoxylate, and acetoin

(a) Initial rates in the presence (●) and absence (○) of 0.6 mM-sodium pyrophosphate as a function of pyruvate concentration. Enzyme activity was determined at pH 7.4 in assay system II (1.7 ml containing 7.2 munits of pyruvate dehydrogenase) except that the TPP concentration was 0.27 μM. Statistical analysis indicated a mixed non-competitive inhibition pattern, $K_i^1 = 0.64 \pm 0.2$ mM, $K_i^2 = 1.4 \pm 0.2$ mM, $K_m^{pyruvate} = 0.23 \pm 0.02$ mM (at a TPP concentration of 0.27 μM). (b) Initial rates in the presence (●) and absence (○) of 0.925 mM-glyoxylate as a function of variable pyruvate concentration determined as in (a) with 5.2 munits of enzyme. Statistical analysis indicated a competitive inhibition, $K_i = 0.56 \pm 0.05$ mM. (c) Initial rates in the presence (●) and absence (○) of 0.925 mM-glyoxylate as a function of variable TPP concentration determined as in (a) with 6.1 munits of enzyme. Statistical analysis indicated a mixed non-competitive pattern. $K_i^1 = 0.34 \pm 0.07$ mM, $K_i^2 = 2.7 \pm 0.4$ mM; $K_m^{TPP} = 0.082$ μM. (d) Initial rates in the presence (●) and absence (○) of 0.71 mM-acetoin as a function of variable TPP concentration determined as in (a) except that pyruvate concentration was 89 μM. Statistical analysis indicated an uncompetitive inhibition pattern, $K_i = 0.82 \pm 0.05$ mM, $K_m^{TPP} = 0.48 \pm 0.03$ μM (pyruvate = 89 μM).

inhibitor and is compatible with the results presented in Fig. 5(a). In the presence of pyrophosphate, the K_d for TPP, as calculated from the negative TPP concentration at the point of intersection of the reciprocal plots (K_{ia} , Fig. 6), is 1.3 ± 0.1 μM, which is not significantly different from the K_d of 1.6 ± 0.3 μM determined in the absence of PP_i (see previous discussion relating to Fig. 3). This is entirely consistent with the finding obtained by using equilibrium dialysis, that pyrophosphate does not alter TPP binding.

Glyoxylate, when incubated with pyruvate dehydrogenase, has been shown to form 2-α-hydroxy-methyl-TPP (Kohlhaw *et al.*, 1965). In the present study glyoxylate was shown to be a poor substrate for the overall dehydrogenase reaction; in the presence of pyruvate dehydrogenase (187 munit/ml), NAD⁺ (0.5 mM), CoA (0.1 mM) and TPP (1 mM) the K_m for glyoxylate was 747 ± 226 μM; the V_{max} of NAD⁺ reduction was approx. 1% of that with pyruvate. Glyoxylate was a competitive inhibitor against

Fig. 6. Effect of sodium pyrophosphate on the initial-velocity pattern for pyruvate dehydrogenase with TPP and pyruvate as the variable substrates

Reciprocal initial velocities are plotted as a function of TPP concentration at the fixed concentrations of pyruvate indicated. All reaction mixtures contained 500 μM -sodium pyrophosphate and saturating concentrations of NAD^+ (0.44 mM) and CoA (90 μM). Calculated kinetic constants from the linear portions of the double reciprocal plot ($>0.1 \mu\text{M}$ -TPP) were $K_m^{\text{TPP}} = 0.20 \pm 0.02 \mu\text{M}$, $K_m^{\text{Pyruvate}} = 44.2 \pm 2.7 \mu\text{M}$, $K_{iA} = 1.3 \pm 0.1 \mu\text{M}$.

pyruvate in the overall pyruvate dehydrogenase reaction [$K_i = 0.5 \text{ mM}$; Fig. 5(b)] but a mixed non-competitive inhibitor against TPP ($K_i^1 = 0.3 \text{ mM}$; $K_i^2 = 2.7 \text{ mM}$; Fig. 5c).

Acetoin, which may be formed when pyruvate is incubated with pyruvate dehydrogenase in the presence of TPP (Jagannathan & Schweet, 1952), was shown to reduce NAD^+ slowly in the presence of CoA (0.1 mM), NAD^+ (0.5 mM), TPP (1 mM) and pyruvate dehydrogenase (380 units/ml). The K_m was $0.43 \pm 0.04 \text{ mM}$ and the V_{max} of NAD^+ reduction was 0.6% of that with pyruvate. Pyruvate contamination of acetoin would not explain these rates (less than 0.001% on a molar basis when assayed with lactate dehydrogenase; assay of authentic pyruvate by lactate dehydrogenase was not affected by the presence of acetoin). No oxidation of acetoin was observed with pyruvate dehydrogenase phosphate. Acetoin is a competitive inhibitor of pyruvate dehydrogenase against pyruvate (Cooper *et al.*, 1974); the inhibition against TPP is uncompetitive (Fig. 5d).

Decarboxylation of [1- ^{14}C]pyruvate and [1- ^{14}C]glyoxylate in the absence of NAD^+ and CoA: effects of inhibitors of the overall forward reaction and of phosphorylation of pyruvate dehydrogenase

The NAD^+ and CoA-independent decarboxylation of pyruvate (Scheme 1; reactions 1f and 6r), as catalysed by the purified pig heart complex under the standard assay conditions (see the Experimental section), is a linear function of enzyme concentration (up to 1 unit/ml) and of time for at least a 30% utilization of 100 μM pyruvate (data not shown). This extent of decarboxylation is some 50 times that which would be observed on a single turnover of enzyme to yield enzyme-bound 2- α -hydroxyethyl-TPP, and up to four times the total TPP added. This indicates that, even in the absence of acetaldehyde, appreciable formation of acetoin and/or acetalactate must occur; no free acetaldehyde has been detected under these conditions (Cooper *et al.*, 1974). The rate, however, is only about 0.35% of the rate through the entire sequence in the presence of NAD^+ and CoA

at saturating concentrations. The rate-limiting step of decarboxylation in the absence of NAD^+ and CoA is unlikely to be the formation of the enzyme-bound carbanion of hydroxyethyl-TPP, but rather one of the steps in the formation of acetoin and/or acetolactate from this carbanion. Acetaldehyde (5 mM) which stimulates the decarboxylation two- to three-fold (Table 2) presumably accelerates or circumvents this rate-limiting step. The concentration-dependence for pyruvate decarboxylation (K_m = approx. $25 \mu\text{M}$; data not shown) in the absence of NAD^+ and CoA is close to that of the overall reaction (Fig. 3). Use of TPP-free enzyme demonstrated that the decarboxylation is dependent on TPP (Table 2).

Incubation of pyruvate dehydrogenase with arsenite and NADH leads to the formation of the lipoyl dithioarsenite derivative which does not catalyse the overall reaction (Reiss, 1958). Preincubation of pyruvate dehydrogenase with 0.5 mM-NADH and 0.1 mM-sodium arsenite had little or no influence on the rates of pyruvate decarboxylation in the absence of NAD^+ and CoA (Table 2), although the rate of the overall reaction with NAD^+ and CoA was inhibited by more than 99%. This indicates that the reactions involved in acetoin and/or acetolactate formation do not require the participation of enzyme-bound acetyl-hydroliipoate. The rate of pyruvate decarboxylation was not affected appreciably by preincubation of the enzyme with NAD^+ , CoA, NADH or acetyl-CoA,

added separately, but addition of NADH plus acetyl-CoA may have caused some inhibition (Table 2). Potassium ferricyanide did not stimulate decarboxylation, in agreement with Sanadi (1963). Acetoin, which inhibits the overall reaction, did not inhibit decarboxylation (Table 2), whereas inhibition was observed with sodium pyrophosphate. In the presence of acetaldehyde (Fig. 7), the apparent K_m for TPP at a pyruvate concentration of $100 \mu\text{M}$ was $0.11 \mu\text{M}$; this is close to the value for the overall reaction at the same pyruvate concentration. The inhibition by sodium pyrophosphate was mixed non-competitive against TPP (Fig. 7). Phosphorylation of pyruvate dehydrogenase inhibited the NAD^+ and CoA-independent decarboxylation of pyruvate by greater than 85% (Fig. 8a). A similar inhibition was observed for the acetaldehyde-stimulated decarboxylation (Table 2).

The initial rate of decarboxylation of glyoxylate by pyruvate dehydrogenase in the absence of NAD^+ and CoA was less than 10% of that observed with pyruvate and the rate rapidly declined to zero after 5–10 min of incubation (Fig. 8b). Addition of acetaldehyde (5 mM) did not affect the rate of decarboxylation of glyoxylate (not shown). By contrast with the results obtained with pyruvate, glyoxylate decarboxylation does not exceed a utilization of substrate beyond a stoichiometric amount of enzyme; presumably the side-reactions equivalent to those presented in reaction 6r (Scheme 1) do not occur

Table 2. *Effects of coenzymes, arsenite, ferricyanide, acetoin and phosphorylation on the rate of decarboxylation of $[1-^{14}\text{C}]$ pyruvate by pyruvate dehydrogenase*

Pyruvate dehydrogenase or pyruvate dehydrogenase phosphate (0.5 unit/ml) was incubated at 30°C with the additions indicated for 1 min within serum-capped flasks as described in the Experimental section. $[1-^{14}\text{C}]$ Pyruvate ($100 \mu\text{M}$) was then injected, and the $^{14}\text{CO}_2$ evolved over the next 10 min determined. Results are the mean of up to six determinations in four separate experiments and are expressed as the percentage of the net $^{14}\text{CO}_2$ evolved when only TPP ($10 \mu\text{M}$) was added. This rate was $3.42 \pm 0.68 \text{ nmol of CO}_2/\text{min}$ per unit of pyruvate dehydrogenase (mean $\pm$ S.E.M. for four separate determinations).

Enzyme	Additions	Rate of decarboxylation (% of control)	
		Without acetaldehyde	With acetaldehyde (5 mM)
Pyruvate dehydrogenase	TPP ($10 \mu\text{M}$) (control)	100	234
	TPP ($10 \mu\text{M}$), arsenite (0.25 mM)	94	224
	TPP ($10 \mu\text{M}$), arsenite (0.25 mM), NADH (0.5 mM)	103	248
	TPP ($10 \mu\text{M}$), NAD^+ (0.5 mM)	90	—
	TPP ($10 \mu\text{M}$), CoA (0.5 mM)	84	—
	TPP ($10 \mu\text{M}$), NADH (0.5 mM)	99	252
	TPP ($10 \mu\text{M}$), acetyl-CoA (0.5 mM), NADH (0.5 mM)	75	198
	TPP ($10 \mu\text{M}$), acetoin (1 mM)	91	244
	TPP ($10 \mu\text{M}$), $\text{K}_3\text{Fe}(\text{CN})_6$ (2 mM)	83	—
	None*	5	—
Pyruvate dehydrogenase phosphate	TPP ($10 \mu\text{M}$)	<2	8

* In this experiment TPP-free dehydrogenase was used.

Fig. 7. Inhibition by sodium pyrophosphate of NAD^+ - and CoA-independent pyruvate decarboxylation

$^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]$ pyruvate (initial concentration $100\text{ }\mu\text{M}$) was measured over a period of 10 min in the presence of 5 mM acetaldehyde, with (●) or without (○) 0.5 mM sodium pyrophosphate. The concentration of pyruvate dehydrogenase was approx. 2 units/ml; for other details see the Experimental section. Statistical analysis indicated a mixed non-competitive inhibition: $K_m^{\text{TPP}} = 0.11 \pm 0.03\text{ }\mu\text{M}$, $K_i = 0.12 \pm 0.05\text{ mM}$.

and formylhydrolipoate is the major product (see the Discussion section). No appreciable rate of glyoxylate decarboxylation was observed with pyruvate dehydrogenase phosphate (Fig. 8b).

Partial back-reaction of pyruvate dehydrogenase: effect of inhibitors of the complete forward reaction and of phosphorylation of the enzyme

Purified pyruvate dehydrogenase catalyses a TPP-dependent, acetyl-CoA-dependent oxidation of NADH, which is stimulated by acetaldehyde (Table 3). In the presence of assay buffer alone, purified pyruvate dehydrogenase oxidizes NADH. This rate of NADH oxidation is not appreciably modified by the addition of TPP ($17\text{ }\mu\text{M}$), or acetyl-CoA ($85\text{ }\mu\text{M}$), or acetaldehyde (1.7 mM), or TPP plus acetaldehyde, or acetyl-CoA plus acetaldehyde (Table 3). The addition of TPP plus acetyl-CoA markedly enhances the rate of NADH oxidation, and this reaction is further stimulated by acetaldehyde. All subsequent experimental values have been corrected for the basal rate of NADH oxidation (i.e. acetyl-CoA-independent rate).

This TPP-dependent, acetyl-CoA-dependent, oxidation of NADH is a partial reversal of the pyruvate dehydrogenase reaction (reactions 5r, 4r, 3r, 2r and 6r, Scheme 1) and is referred to as the 'partial back reaction' (see also Schreiber *et al.*, 1963). The re-

Fig. 8. Time-course of decarboxylation of (a) $[1\text{-}^{14}\text{C}]$ pyruvate, and (b) $[1\text{-}^{14}\text{C}]$ glyoxylate by pyruvate dehydrogenase and by pyruvate dehydrogenase phosphate

Pyruvate dehydrogenase [124 munits (a); 496 munits (b); ●] and, pyruvate dehydrogenase phosphate [124 munits (a); 496 munits (b); ○] were preincubated for 30 s at 20°C with 20 mM potassium phosphate, $\text{pH } 7.0$, containing 5 mM 2-mercaptoethanol, 2 mM MgCl_2 , and $100\text{ }\mu\text{M}$ TPP. The reaction was initiated by addition of (a) $[1\text{-}^{14}\text{C}]$ pyruvate ($100\text{ }\mu\text{M}$ final concn., 10.5 mCi/mmol) or (b) $[1\text{-}^{14}\text{C}]$ glyoxylate (final concn. $20\text{ }\mu\text{M}$; 6.67 mCi/mmol) in a total volume of $40\text{ }\mu\text{l}$. Reaction was terminated at the times indicated and $^{14}\text{CO}_2$ production measured as described in the Experimental section. Results are the means of duplicate observations. The pyruvate dehydrogenase phosphate contained 1.9% of pyruvate dehydrogenase; $^{14}\text{CO}_2$ evolution by pyruvate dehydrogenase phosphate was corrected for $^{14}\text{CO}_2$ evolution by this amount of pyruvate dehydrogenase.

Table 3. *Catalysis of NADH oxidation by pyruvate dehydrogenase*

The rate of oxidation of NADH was determined spectrophotometrically at 340 nm and 30°C. The reaction mixture contained 9.2 mM-Tris/HCl-buffer, pH 7.8, with 0.92 mM-MgCl₂, 0.46 mM-EDTA, 4.6 mM-2-mercaptoethanol, 0.25 mM-NADH and 103 munits of pyruvate dehydrogenase (total vol. 0.6 ml). The rate of NADH oxidation (*a*) was determined for 4 min. Acetyl-CoA (80 μ M) was then added to the cuvette, and the NADH-oxidation rate (*b*) determined for the subsequent 4 min. Acetyl-CoA-dependent oxidation rate was calculated as (*b*)–(*a*). Results are the means of duplicate determinations.

Additions	NADH oxidation (nmol/min)		
	Without acetyl-CoA(<i>a</i>)	With acetyl-CoA(<i>b</i>)	Stimulated by acetyl-CoA(<i>b</i> – <i>a</i>)
None	0.29	0.27	<0
Acetaldehyde (1.7 mM)	0.36	0.31	<0
TPP (17 μ M)	0.35	0.63	0.28
TPP (17 μ M) plus acetaldehyde (1.1 mM)	0.40	0.95	0.55

generation of free enzyme from the enzyme–hydroxyethyl-TPP carbanion complex is presumed to occur by a mechanism(s) analogous to that occurring when pyruvate is decarboxylated in the absence of NAD⁺ and CoA, a process which is also stimulated by acetaldehyde. The rate of the partial back-reaction in the absence of acetaldehyde (0.25% of that of the complete forward reaction) is close to that of NAD⁺ and CoA-independent pyruvate decarboxylation and the rate-limiting step in both cases may be the same, perhaps involving some step in the formation of products (e.g. acetoin) from the enzyme-bound hydroxyethyl-TPP.

The K_m for TPP for the partial back reaction is $4.1 \pm 0.5 \mu\text{M}$ (Fig. 9). Sodium pyrophosphate, a competitive inhibitor against TPP in the forward reaction, appears to act as a non-competitive inhibitor of the partial back-reaction (Fig. 9; $K_i = 0.16 \text{ mM}$). A similar value was evident from a plot of enzyme activity versus sodium pyrophosphate concentration, but complete inhibition at high pyrophosphate concentrations was not achieved (Fig. 9, insert). At less-than-saturating concentrations of TPP (4.5 μM), both GTP and adenylyl imidodiphosphate are also strong inhibitors (>90%) of the partial back-reaction (Table 4). Table 4 also indicates that glyoxylate (0.75 mM) completely inhibits (>90%) the partial back-reaction at either saturating (27 μM) or non-saturating (4.5 μM) concentrations of TPP, and that acetoin (0.75 mM) results in 46 and 35% inhibition at these respective TPP concentrations. The latter results are compatible with an uncompetitive form of inhibition, as is observed for the forward reaction with acetoin (Fig. 5d), but a full kinetic evaluation has not been made.

Phosphorylation of pyruvate dehydrogenase abolished the ability of the enzyme to catalyse the partial back reaction. Fig. 10 depicts the progress curve for the formation of pyruvate dehydrogenase phosphate

as catalysed by endogenous pyruvate dehydrogenase kinase. A very close correlation was observed between the inhibition of both the complete forward reaction and the partial back reaction.

Acetylation of enzyme-linked lipoyl groups in pyruvate dehydrogenase: effect of phosphorylation of the enzyme

The acetylation of enzyme-linked lipoyl groups in pyruvate dehydrogenase was assayed by the incorporation of ¹⁴C from [3-¹⁴C]pyruvate or [1-¹⁴C]acetyl-CoA into trichloroacetic acid-precipitable protein that was sensitive to performic acid oxidation (Barrera *et al.*, 1972). The cofactor requirements for formation of enzyme-linked acetylhydrolipoate from either pyruvate or acetyl-CoA are presented in Table 5. Maximum acetylhydrolipoate was formed to an equal extent from [3-¹⁴C]pyruvate or from [1-¹⁴C]acetyl-CoA. Full incorporation from the latter required NADH, compatible with the observation that in freshly prepared enzyme lipooate was primarily in the oxidized state. The NADH requirement could not be substituted for by NAD⁺ but on prolonged storage of the enzyme in 2-mercaptoethanol the extent of incorporation in the absence of NADH increased (compare Expts. 1 and 2, and Table 5). It is suggested that this may be due to a slow reduction of lipooate by 2-mercaptoethanol. The amount of acetylhydrolipoate formed from pyruvate was not affected by the addition of NADH or NAD⁺. Presumably the high free-energy change associated with pyruvate decarboxylation (Scheme 1, reaction 1f) can readily modify the position of equilibrium of reactions 2, 4 and 5. Addition of CoA to the incubation resulted in a decreased amount of acetylhydrolipoate through discharge of the acetyl moiety to produce acetyl-CoA.

Acetylation from [3-¹⁴C]pyruvate, but not from [1-¹⁴C]acetyl-CoA, requires TPP, as indicated by the use of TPP-free enzyme (results not shown). Acetyla-

Fig. 9. Inhibition of the partial back reaction by sodium pyrophosphate

Reciprocal initial velocities of the partial back-reaction of pyruvate dehydrogenase are plotted as a function of TPP concentration in the presence (○) or absence (●) of 0.14 mM-sodium pyrophosphate. The experimental details were as given in Table 3, except that acetaldehyde (1.7 mM) was present, the concentration of TPP was varied and sodium pyrophosphate was as indicated. Rates of NADH oxidation were corrected for the basal rate in the absence of acetyl-CoA. Statistical analysis indicates a simple linear non-competitive inhibition; $K_m^{TPP} = 4.1 \pm 0.5 \mu\text{M}$, $K_i = 0.16 \text{ mM}$, $V_{\text{max}} = 11.1 \text{ nmol of NAD}^+/\text{min per unit of pyruvate dehydrogenase}$. The insert indicates the inhibition of the partial back-reaction as function of sodium pyrophosphate concentration in the presence of $33 \mu\text{M}$ -TPP.

Table 4. Effects of GTP, adenylyl imidodiphosphate glyoxylate and acetoin on the partial back reaction catalysed by pyruvate dehydrogenase

Experimental details were as described in Table 3, except that all assays were performed in the presence of 1.7 mM-acetaldehyde, and the TPP concentrations were as indicated. Data have been corrected for the basal rate of NADH oxidation in the absence of acetyl-CoA, which was not altered by any of the additions shown. Results are the means of at least duplicate determinations.

Additions	NADH oxidation (nmol/min)	
	4.5 μM -TPP	27 μM -TPP
None	0.375	0.580
Glyoxylate (0.75 mM)	<0.003	0.019
Acetoin (0.75 mM)	0.244	0.313
GTP (2.25 mM)	<0.003	—
Adenylyl imidodiphosphate (1.25 mM)	0.039	—

tion of pyruvate dehydrogenase from acetyl-CoA was completely inhibited by arsenite (Table 5), consistent with the reduced form of lipoate serving as acceptor for the acetyl moiety. By contrast, in the absence of NADH acetylation from pyruvate was inhibited by arsenite only to an extent commensurate with the amount of reduced lipoate in the enzyme preparation. As illustrated in Table 5, Expt. 1, in the absence of added NADH, arsenite inhibited acetylation from pyruvate by 30% in the pyruvate dehydrogenase preparation used. This correlates with the observation that in the absence of NADH, acetylation from acetyl-CoA only occurred to approx. 40% the extent of that in the presence of NADH.

Acetylation of pyruvate dehydrogenase from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ was not observed if pyruvate dehydrogenase was incubated with $[^{12}\text{C}]\text{acetyl-CoA}$ (fourfold excess) with or without NADH. By contrast, incubation with an excess of $[^{12}\text{C}]\text{pyruvate}$ did not alter the extent of ^{14}C incorporation from

[1-¹⁴C]acetyl-CoA in the presence of NADH. Further, the presence of [¹²C]pyruvate permitted maximum formation of [¹⁴C]acetylhydrolipoate from [¹⁴C]acetyl-CoA in the absence of NADH. These observations suggest that exchange can occur between enzyme-linked acetylhydrolipoate and acetyl-CoA, but that there is no exchange between enzyme-linked acetylhydrolipoate and pyruvate.

The maximum extent of acetylation from [3-¹⁴C]-pyruvate and from [1-¹⁴C]acetyl-CoA was approx. 600pmol/unit of pyruvate dehydrogenase. Assuming a mol.wt. of 10⁷ this corresponds to 60 acetylation sites, in agreement with studies of Barrera *et al.* (1972) with bovine heart enzyme.

Fig. 11 presents the time-course of acetylation of pyruvate dehydrogenase and of pyruvate dehydrogenase phosphate from [3-¹⁴C]pyruvate and [1-¹⁴C]-acetyl-CoA. At the earliest time examined (30s), acetylation of pyruvate dehydrogenase from either pyruvate or acetyl-CoA was essentially complete. Similarly, the rate of acetylation of pyruvate dehydrogenase phosphate from acetyl-CoA was very rapid and, within the limits of the experimental technique, indistinguishable from that of the non-phosphorylated form. By contrast, the rate of acetylation of pyruvate dehydrogenase phosphate from [3-¹⁴C]-pyruvate was considerably slower (<14%) than that of the non-phosphorylated enzyme. The characteristics of the acetylation of pyruvate dehydrogenase phosphate were otherwise similar to those of the non-phosphorylated enzyme; acetylation from [1-¹⁴C]-acetyl-CoA required NADH and was not diminished in the presence of pyruvate, and there was no acetylation from [3-¹⁴C]pyruvate in the presence of acetyl-CoA.

Fig. 10. Correlation of the inhibition by phosphorylation of the partial back-reaction, the activity of acetylhydrolipoyl-pyruvate dehydrogenase and the overall pyruvate dehydrogenase reaction

Pyruvate dehydrogenase (6.6 units/ml) was incubated at 30°C in 20mm-potassium phosphate pH7.0, containing 5mm-2-mercaptoethanol, 0.2mm-ATP and 0.4mm-MgCl₂. At the times indicated, samples were removed for assay of pyruvate dehydrogenase activity (●), by system I (see the Experimental section) and the partial back-reaction (○), as described in Table 3, with 0.6mm-TPP. Rates of NADH oxidation have been corrected for the basal rate in the absence of acetyl-CoA, which was not modified by phosphorylation. Results are means ± S.E.M. for a minimum of three separate determinations. The activity of acetylhydrolipoyl-pyruvate dehydrogenase (□), prepared as described in the text and incubated at 30°C with 0.1mm-ATP and 0.2mm-MgCl₂ in 20mm-potassium phosphate, pH7.0, containing 0.5mm-2-mercaptoethanol, was assayed at the times indicated by using system I. The rate of inactivation of the control pyruvate dehydrogenase with 0.1mm-ATP (not shown) was identical to that occurring with 0.2mm-ATP (●).

Table 5. Characteristics of the formation of enzyme-linked acetylhydrolipoate from pyruvate and from acetyl-CoA

Pyruvate dehydrogenase (approx. 280munits) was incubated with either 0.125mm-[3-¹⁴C]pyruvate (10.5mCi/mmol) or 0.125mm-[1-¹⁴C]acetyl-CoA (approx. 4mCi/mmol) with 20mm-potassium phosphate buffer, pH7.0, containing 5mm-2-mercaptoethanol, 1mm-MgCl₂, 2.5μm-TPP and other additions as indicated. After incubation for 1 min at 21°C, the incorporation of ¹⁴C was determined as described in the Experimental section. In Expts. 1 and 2 the enzyme had been stored at -20°C for 8 and 26 days respectively before the experiment. Results are the means of duplicate determinations.

Other additions	Acetylation of enzyme-linked lipoyl groups (pmol/unit) by			
	[3- ¹⁴ C]Pyruvate		[1- ¹⁴ C]Acetyl-CoA	
	Without NADH	With NADH (0.125mm)	Without NADH	With NADH (0.125mm)
Expt. 1				
None	498	512	230	535
NAD ⁺ (0.125mm)	548	—	269	528
[¹² C]Pyruvate (0.5mm)	—	—	570	573
[¹² C]Acetyl-CoA (0.5mm)	0	0	—	—
CoA (0.125mm)	91	120	—	—
Sodium arsenite (1mm)	385	0	—	0
Expt. 2				
None	520	—	450	670

Fig. 11. Time-course of the acetylation of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate from $[1-^{14}\text{C}]$ acetyl-CoA and $[3-^{14}\text{C}]$ pyruvate

The acetylation of pyruvate dehydrogenase (a) and pyruvate dehydrogenase phosphate (b) from either $[3-^{14}\text{C}]$ pyruvate (●) or $[1-^{14}\text{C}]$ acetyl-CoA (○) was determined as indicated in the legend of Table 5. The enzyme concentration was 2.62 units/ml. Acetylation from acetyl-CoA was performed in the presence of 0.125 mM-NADH.

Deacetylation of enzyme-linked acetylhydrolipoate groups in pyruvate dehydrogenase: effect of phosphorylation of pyruvate dehydrogenase

The observations that phosphorylation of pyruvate dehydrogenase does not inhibit the acetylation of enzyme-linked lipoyl groups by acetyl-CoA, but does inhibit the partial back reaction suggested that phosphorylation may affect the transfer reaction that forms Enz_1 -2- α -hydroxyethyl-TPP from Enz_2 -acetylhydrolipoate and TPP (Scheme 1; reaction 2r). Accordingly, the TPP-dependent rates of release of ^{14}C from $[^{14}\text{C}]$ acetylhydrolipoyl pyruvate dehydrogenase and from $[^{14}\text{C}]$ acetylhydrolipoyl pyruvate dehydrogenase phosphate were compared.

$[^{14}\text{C}]$ Acetylhydrolipoyl pyruvate dehydrogenase was prepared by incubating pyruvate dehydrogenase (24 units) in 17 mM-potassium phosphate, pH 7.0, containing 1 mM- MgCl_2 , 0.2 mM-NADH, 0.125 mM- $[1-^{14}\text{C}]$ acetyl-CoA (4.2 mCi/mmol) and 5 mM-2-mercaptoethanol in a total volume of 1.57 ml for 1 min at 21°C. The reaction was terminated by immersing the reaction tube in ice, and $[1-^{14}\text{C}]$ acetyl-CoA, NADH and enzyme-bound TPP were removed by three successive treatments with 8.4 mg of Norit A for 1, 30 and 30 min respectively (see the Experimental section). By following this procedure, the pyruvate dehydrogenase still contained approx. 60% of non-protein bound radioactivity (probably acetate formed by acetyl-CoA hydrolase activity). This was removed by gel filtration. The supernatant from the final Norit A treatment was applied to a column (8.5 cm $\times$ 0.9 cm) of Sephadex G-25 equilibrated in 20 mM-potassium phosphate, pH 6.8, containing 0.5 mM-2-mercaptoethanol. The protein fraction eluting in the void volume contained approx. 500 pmol of $[^{14}\text{C}]$ acetyl groups/unit of pyruvate dehydrogenase. The radioactivity was over 90% trichloroacetic acid-precipitable and over 90% performic acid-labile. The acetyl-protein bond had a half-life of approx. 4 h at 0°C under these conditions of preparation, possibly reflecting slow acetylation of free thiol (2-mercaptoethanol). The extent of phosphorylation of acetylated pyruvate dehydrogenase in the presence of ATP (0.1 mM) and MgCl_2 (0.2 mM) was similar to the native enzyme (Fig. 10).

De-acetylation of $[^{14}\text{C}]$ acetylhydrolipoyl pyruvate dehydrogenase was followed by procedures similar to those used for acetylation. Complete release of protein-bound $[^{14}\text{C}]$ acetyl occurred on addition of CoA (results not shown) or of TPP plus NADH (Table 6). TPP alone was considerably less effective in promoting release. The effect of phosphorylation on the rate of deacetylation was investigated by first preparing $[^{14}\text{C}]$ acetylhydrolipoyl-pyruvate dehydrogenase, and, after treatment with Norit A, half of the preparation was incubated with ATP (0.1 mM) and MgCl_2 (0.2 mM) for 5.5 min to give $[^{14}\text{C}]$ acetylhydrolipoyl-pyruvate dehydrogenase phosphate. Because of the limited stability of acetylhydrolipoyl-pyruvate dehydrogenase, it was not practical to remove ATP and ADP from the preparation of acetylhydrolipoyl-pyruvate dehydrogenase phosphate. Pyrophosphate compounds (including ADP and potentially ATP) are competitive inhibitors (against TPP) of the overall pyruvate dehydrogenase reaction (Cooper *et al.*, 1974) and are thus potential inhibitors of TPP-dependent deacetylation of acetylhydrolipoyl-pyruvate dehydrogenase. However, a high concentration of TPP (1 mM) was used in the deacetylation reaction, and control experiments (with 1 mM-TPP) showed that neither ATP (100 μM) nor ADP (100 μM) affect the initial velocity of the pyruvate dehydrogenase reaction

Table 6. Stimulation of the de-acetylation of [1-¹⁴C]acetylhydrolipoyl-pyruvate dehydrogenase by TPP and NADH

[1-¹⁴C]Acetylhydrolipoyl-pyruvate dehydrogenase was prepared as described in the text. The extent of de-acetylation was examined by incubating [1-¹⁴C]acetylhydrolipoyl-pyruvate dehydrogenase (84 munits) with 30 mM-potassium phosphate, pH 7.0, containing 5 mM-2-mercaptoethanol, TPP and NADH as indicated (total volume 40 μ l), for 1 min at 21°C. Thereafter the procedure for determination of the extent of acetylation of enzyme-linked lipoyl groups was as described in the Experimental section. Results are the means of duplicate observations.

Additions		Protein-bound [¹⁴ C]acetate released (%)
TPP (mM)	NADH (mM)	
0	0	0
0.125	0	7
2.5	0	11
0	0.125	8
0	2.5	9
0.125	0.125	34
2.5	0.125	44
0.125	2.5	42
2.5	2.5	44

under these conditions. Moreover, adenylyl imidodiphosphate (0.2 mM) did not affect the rate of the pyruvate dehydrogenase reaction under these conditions, nor did it affect the rate of deacetylation of acetylhydrolipoyl-pyruvate dehydrogenase. In comparing the rates of deacetylation of acetylhydrolipoyl-pyruvate dehydrogenase and of acetylhydrolipoyl-pyruvate dehydrogenase phosphate, adenylyl imidodiphosphate (0.2 mM) was added to both series of incubations. EGTA [ethanedioxybis(ethylamine)-tetra-acetate, 0.1 mM] and NaF (25 mM) were also added to inhibit the small amount of pyruvate dehydrogenase phosphate phosphatase present.

The rates of loss of ¹⁴C from enzyme-bound acetylhydrolipoyl groups during incubation in the presence and absence of TPP plus NADH are shown in Fig. 12. Incubation for 8 min at 21°C resulted in little or no loss of ¹⁴C from either preparation in the absence of added TPP plus NADH. In the presence of TPP plus NADH, de-acetylation of the non-phosphorylated enzyme was almost complete at 8 min. There was also appreciable deacetylation of the phosphorylated enzyme, but the rate was only approx. 10% of that with the non-phosphorylated enzyme. Moreover much of this rate could have been due to pyruvate dehydrogenase present in the pyruvate dehydrogenase phosphate preparation, since after the short incubation (5.5 min) with ATP this amounted to about 6%. Phosphorylation thus appears to inhibit directly some aspect of the reactions involved in deacetylation (Scheme 1; reaction 2r).

Fig. 12. Time-courses of deacetylation of pyruvate dehydrogenase and of pyruvate dehydrogenase phosphate

[1-¹⁴C]Acetylhydrolipoyl-pyruvate dehydrogenase (approx. 500 pmol of acetate/unit) was prepared as described in the text; part of the preparation was phosphorylated by incubation with ATP-Mg²⁺ for 5.5 min (see Fig. 10). The extent of deacetylation was examined by incubation of 4.35 units/ml of either [1-¹⁴C]acetylhydrolipoyl-pyruvate dehydrogenase (closed symbols) or [1-¹⁴C]acetylhydrolipoyl-pyruvate dehydrogenase phosphate (open symbols) in 30 mM-potassium phosphate, pH 6.8, containing 0.2 mM-MgCl₂, 25 mM-NaF, 0.1 mM-EGTA, 1.5 mM-2-mercaptoethanol, 0.1 mM-adenylyl imidodiphosphate, in the absence (●, ○) or presence (□, ■) of 0.5 mM-TPP and 0.6 mM-NADH. For further experimental details, see Table 6. Acetylated pyruvate dehydrogenase phosphate contained 6.1% active enzyme; no dephosphorylation occurred during deacetylation reaction (8 min). Data have been corrected for bound radioactivity that is not performic acid-labile.

Formylation of enzyme-linked lipoyl groups in pyruvate dehydrogenase: effect of phosphorylation of the enzyme

In the absence of NAD⁺ and CoA, glyoxylate was decarboxylated by pyruvate dehydrogenase, but not by pyruvate dehydrogenase phosphate (Fig. 8b). The effect of phosphorylation of pyruvate dehydrogenase on the rates of formylation of enzyme-linked lipoyl groups was therefore examined. Table 7 shows that the incorporation of ¹⁴C from [U-¹⁴C]glyoxylate into acyl bound groups in pyruvate dehydrogenase was decreased by the presence of CoA, presumably owing to production of formyl-CoA. Formylation of pyruvate dehydrogenase by glyoxylate was markedly decreased by preincubation of pyruvate dehydrogenase with [¹²C]acetyl-CoA in accord with the effect of [¹²C]acetyl-CoA on the acetylation of pyruvate dehydrogenase from [3-¹⁴C]pyruvate (Table 5). As

Table 7. Characteristics of the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ into acyl-bound groups in pyruvate dehydrogenase

Pyruvate dehydrogenase (400munits) was preincubated with 20mM-potassium phosphate, pH 7.0, containing 1mM- MgCl_2 , 0.1mM-TPP and other additions as indicated, for 30s at 19° . Reaction was initiated by addition of $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ ($50\mu\text{M}$ final concn.; 7.5mCi/mmol), and at the times indicated, $30\mu\text{l}$ samples were analysed for protein-bound radioactivity (see the Experimental section). Results are the means of duplicate observations. Performate oxidation was as described in the Experimental section.

Additions	Time of incubation (min)	Incorporation of ^{14}C formate (pmol/unit)
None	1	244
Acetyl-CoA (4mM)	1	17
None	10	546
None (performate treated)	10	380
CoA (1mM)	10	106

shown in Table 7, only approx. 30% of the ^{14}C incorporated into trichloroacetic acid-precipitable protein was removed by performic acid oxidation. This suggested that the formyl group attached to lipoate may undergo some time-dependent intramolecular rearrangement into non-thioester linkage. This would be consistent with the observation that pyruvate dehydrogenase, after incubation with TPP and glyoxylate (1mM) for 4h under conditions of equilibrium microdialysis, was over 70% inactive (assay system I; see the Experimental section). Under the conditions of the experiment described in Table 7, enzyme which had been incubated with $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ for up to 15min showed full pyruvate dehydrogenase activity.

Fig. 13 presents the time-course of incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ into pyruvate dehydrogenase and pyruvate dehydrogenase phosphate. Incorporation of ^{14}C into pyruvate dehydrogenase was essentially complete after 5min, whereas even after 15min, incorporation into pyruvate dehydrogenase phosphate was less than 10% of that into the non-phosphorylated enzyme. The maximum extent of incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ into pyruvate dehydrogenase was approx. 540pmol/unit, in close agreement with the value obtained for acetylation from $[\text{3-}^{14}\text{C}]\text{pyruvate}$ and from $[\text{1-}^{14}\text{C}]\text{acetyl-CoA}$.

Estimation of enzyme bound 2- α -hydroxyethyl-TPP carbanion in pyruvate dehydrogenase: effect of phosphorylation of pyruvate dehydrogenase

Pyruvate dehydrogenase and pyruvate dehydrogenase $[\text{32P}]\text{phosphate}$ are completely retained by

Fig. 13. Time-course of the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ into acyl-bound groups in pyruvate dehydrogenase and pyruvate dehydrogenase phosphate

Pyruvate dehydrogenase (400munits, ○) and pyruvate dehydrogenase phosphate (366munits, ●), were preincubated with 20mM-potassium phosphate, pH 7.0, containing 1mM- MgCl_2 and 0.1mM-TPP for 30s at 19°C . The reaction was initiated by addition of $[\text{U-}^{14}\text{C}]\text{glyoxylate}$ ($50\mu\text{M}$ final concn., 7.5mCi/mmol) and at the times indicated $30\mu\text{l}$ samples were analysed for acyl-bound radioactivity (see the Experimental section). Results are the mean of observations in duplicate. Under these conditions there was no net incorporation of radioactivity either in the presence of 0.25mM-sodium arsenite and 1mM-NADH, or with $[\text{1-}^{14}\text{C}]\text{glyoxylate}$.

Millipore filtration (see the Experimental section). Evidence summarized below indicates that hydroxy- $[\text{1-}^{14}\text{C}]\text{ethyl-TPP}$ carbanion and $[\text{14C}]\text{acetylhydro-lipoate}$ bound to the enzyme were also retained by the membrane, and that enzyme-bound 2- α -hydroxyethyl-TPP carbanion can be selectively eluted with 10% trichloroacetic acid, permitting estimations of enzyme-bound 2- α -hydroxyethyl-TPP carbanion and of enzyme-bound acetylhydro-lipoate.

Pyruvate dehydrogenase was incubated for 1min with $[\text{3-}^{14}\text{C}]\text{pyruvate}$, TPP and MgCl_2 (conditions which lead to maximum incorporation of ^{14}C into enzyme-bound lipoate, Fig. 11), and subjected to Millipore filtration. Washing with 2-4ml of buffer (20mM-potassium phosphate, pH 7.0, plus 5mM-2-mercaptoethanol) removed virtually all the $[\text{3-}^{14}\text{C}]\text{pyruvate}$, as shown by omission of pyruvate dehydrogenase; more prolonged washing with buffer (up to 10ml) removed no further radioactivity. Subsequent washing of the Millipore filter with 10% trichloroacetic acid removed approx. 20% of the residual radioactivity (Table 9); that this represents non-covalently bound 2- α -hydroxyethyl-TPP carbanion is supported by the following. Washing with 10% trichloroacetic acid (unlike performic acid oxidation)

Table 8. *Characteristics of the formation of enzyme-bound 2- α -hydroxy[14 C]ethyl-TPP from [3- 14 C]pyruvate by pyruvate dehydrogenase or pyruvate dehydrogenase phosphate in the presence of NADH and arsenite*

Pyruvate dehydrogenase (250 munits) or pyruvate dehydrogenase phosphate (250 munits) was incubated for 1 min with 20 mM-potassium phosphate, pH 7.0, containing 5 mM-2-mercaptoethanol, 1 mM-MgCl₂, 0.25 mM-sodium arsenite, 1 mM-NADH and the concentrations of TPP indicated, for 1 min at 20°C. [3- 14 C]Pyruvate (0.125 mM; 7.5 mCi/mmol) was added, and after incubation for a further 1 min, enzyme-bound hydroxyethyl-TPP and acetylhydrolipoate were determined as described in the Experimental section. In expt. 1, pyruvate dehydrogenase was treated with Norit A to remove TPP (see the Experimental section). Results are the means of closely agreeing triplicate determinations. In all cases the incorporation of substrate into acetylhydrolipoate was less than 8 pmol/unit of enzyme.

Expt. no.	Enzyme	TPP (μ M)	Substrate	Time with substrate (min)	Incorporation of substrate into enzyme-bound hydroxy-ethyl-TPP (pmol/unit of enzyme)
1	Pyruvate dehydrogenase	0	[3- 14 C]Pyruvate	1	8
		10	[3- 14 C]Pyruvate	1	191
		10	[1- 14 C]Pyruvate	1	0
2	Pyruvate dehydrogenase	100	[3- 14 C]Pyruvate	0.5	170
		100	[3- 14 C]Pyruvate	1.0	197
		100	[3- 14 C]Pyruvate	5.0	143
3	Pyruvate dehydrogenase phosphate	100	[3- 14 C]Pyruvate	0.5	22
		100	[3- 14 C]Pyruvate	1.0	20
		100	[3- 14 C]Pyruvate	5.0	34

does not remove [14 C]-labelled-enzyme-bound acetylhydrolipoate. With enzyme that had been treated with arsenite and NADH (to block formation of [14 C]acetylhydrolipoate), the radioactivity incorporated from [3- 14 C]pyruvate was retained on the Millipore filter after prolonged washing with buffer, but at least 80% of the radioactivity was eluted with 50% (v/v) methanol (known to remove 2- α -hydroxyethyl-TPP), or with buffer plus 5 mM-acetaldehyde (known to stimulate conversion of enzyme-bound hydroxyethyl-TPP into acetoin), or with 10% trichloroacetic acid. The radioactivity eluted with 50% methanol after freeze-drying and suspension in water, could be absorbed by Norit A (50 mg/ml) in 0.1 M-HCl. Virtually no radioactivity was retained on the filter after incubation of pyruvate dehydrogenase with [1- 14 C]pyruvate plus TPP, or with [3- 14 C]pyruvate in the absence of TPP. When samples of pyruvate dehydrogenase which had been incubated with NADH, arsenite, TPP, MgCl₂ and then with [3- 14 C]pyruvate for 1 min (in an experiment analogous to that in Table 8) were filtered, and the filters washed with 2, 3, 4, 5, 7 or 9 ml of buffer, the respective amounts of enzyme-bound 2- α -hydroxyethyl-TPP were 273, 295, 255, 256, 246 and 246 pmol/unit (means of triplicate determinations). The assumption was therefore made that radioactivity determined by this Millipore procedure represented incorporation into enzyme-bound 2- α -hydroxyethyl-TPP.

The number of 2- α -hydroxyethyl-TPP groups associated with pyruvate dehydrogenase after incubation with pyruvate, TPP and Mg²⁺ was in the range of 150–250 pmol/unit of enzyme (Tables 8 and 9). This

value was attained within 30 s of the addition of pyruvate (Table 8), and was not greatly altered by prior incubation of the enzyme with arsenite and NADH, or with acetyl-CoA and NADH, either condition which markedly decreased the incorporation of 14 C from [3- 14 C]pyruvate into acetylhydrolipoyl groups (Table 9). Similar amounts of enzyme-bound 2- α -hydroxyethyl-TPP were detected after incubation of pyruvate dehydrogenase with [1- 14 C]acetyl-CoA and NADH for 1 min (Table 9), and for 0.5 and 5 min (results not shown). These results provide strong evidence of the presence of high-affinity binding sites for 2- α -hydroxyethyl-TPP in pyruvate dehydrogenase (approx. 250 pmol/unit of enzyme). All of these sites may not be identical, and the existence of low-affinity binding sites from which 2- α -hydroxyethyl-TPP is eluted during the washing procedure, is a possibility.

Phosphorylation markedly decreased the number of binding sites for 2- α -hydroxyethyl-TPP to less than 20% of the number in the non-phosphorylated enzyme (Tables 8 and 9). Similar values were obtained for pyruvate dehydrogenase phosphate incubated with or without arsenite and NADH (Table 9). The rate of acetylation from [3- 14 C]pyruvate of pyruvate dehydrogenase phosphate used in the present studies was markedly higher than with the preparation used in Fig. 11, although both preparations contained less than 2% of non-phosphorylated enzyme. A possible explanation of this difference is that the enzyme used in the previous studies had been stored for less than 1 week at -20°C, whereas the enzyme in study had been stored for approx. 4 weeks at -20°C;

Table 9. *Effect of phosphorylation of pyruvate dehydrogenase on the formation of enzyme bound 2- α -hydroxy[14 C]ethyl-TPP from [3- 14 C]pyruvate or from [1- 14 C]acetyl-CoA*

Pyruvate dehydrogenase (250munits) or pyruvate dehydrogenase phosphate (250munits) was incubated for 1 min at 20°C with 20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol, 1 mM-MgCl₂, 0.1 mM-TPP and other additions as indicated (total vol. 35 μ l). [3- 14 C]Pyruvate (0.125 mM; 7.5 mCi/mmol) or [1- 14 C]acetyl-CoA (0.125 mM; approx. 4 mCi/mmol) was added, and after incubation for a further 1 min at 20°C, enzyme-bound hydroxyethyl-TPP and acetylhydroliipoate were determined as described in the Experimental section. Results are means $\pm$ s.e.m. for the numbers of observations given in parentheses.

Enzyme	Additions	Substrate	Incorporation of substrate (as pmol/unit of enzyme) into		
			Hydroxyethyl-TPP plus acetylhydroliipoate	Acetylhydroliipoate	Hydroxyethyl-TPP
Pyruvate dehydrogenase	None	[3- 14 C]Pyruvate	1055 $\pm$ 47	815 $\pm$ 38	252 $\pm$ 27 (8)
	Arsenite (0.25 mM), NADH (1 mM)	[3- 14 C]Pyruvate	183 $\pm$ 10	5.3 $\pm$ 1.2	178 $\pm$ 10 (8)
	Acetyl-CoA (0.125 mM), NADH (1 mM)	[3- 14 C]Pyruvate	308 $\pm$ 36	89 $\pm$ 6.4	219 $\pm$ 39 (3)
	NADH (1 mM)	[1- 14 C]Acetyl-CoA	798 $\pm$ 51	542 $\pm$ 28	252 $\pm$ 36 (3)
Pyruvate dehydrogenase phosphate	None	[3- 14 C]Pyruvate	657 $\pm$ 64	601 $\pm$ 84	55 $\pm$ 50 (3)
	Arsenite (0.25 mM), NADH (1 mM)	[3- 14 C]Pyruvate	24 $\pm$ 5.2	2.0 $\pm$ 1.5	21.7 $\pm$ 5.5 (3)
	Acetyl-CoA (0.125 mM), NADH (1 mM)	[3- 14 C]Pyruvate	44 $\pm$ 6.0	23.0 $\pm$ 1.5	21 $\pm$ 1.5 (3)

such storage modifies the oxidation/reduction state of the lipoate. Addition of sodium pyrophosphate caused some decrease in the apparent number of sites (by approx. 10% and 40% at 0.25 and 1 mM-sodium pyrophosphate respectively) with enzyme incubated with arsenite, NADH, TPP (100 μ M), MgCl₂ and [3- 14 C]pyruvate as in Table 8, for periods of 0.5, 1 and 5 min (results not shown).

Discussion

Mechanism of the pyruvate dehydrogenase reaction and regulation by end-product inhibition

The reactions catalysed by the pyruvate dehydrogenase complex have been shown to involve three different enzymes acting in sequence (Gunsalus, 1954; Koike *et al.*, 1963; Massey, 1963; Hayakawa *et al.*, 1969; Barrera *et al.*, 1972; Roche & Reed, 1972). The initial-velocity patterns obtained in the present study at a constant concentration of pyruvate, with varying concentrations of TPP at either fixed concentrations of NAD⁺ and at a saturating concentration of CoA (Fig. 2a), or fixed concentration of CoA and a saturating concentration of NAD⁺ (Fig. 2b), are consistent with the three-site Ping Pong mechanism deduced for the bovine kidney complex by Tsai *et al.* (1973). Kinetic data and physicochemical characterization of the mammalian pyruvate dehydrogenase complex, performed primarily in the laboratories of Reed

(Linn *et al.*, 1969a; Barrera *et al.*, 1972; Roche & Reed, 1972) and Koike (Hayakawa & Koike, 1967; Hayakawa *et al.*, 1969), are the basis for the sequence of reactions presented in Scheme 1. Results obtained in the present study give a basis for a molecular stoichiometry for some of these reactions. These are summarized in Table 10. The maximum number of acetylation sites corresponds to one site per dihydroliipoate acetyltransferase subunit. There are approximately half this number of 2- α -hydroxyethyl-TPP binding sites, a value commensurate with no more than one site per $\alpha_2\beta_2$ pyruvate decarboxylase subunit. The average number of TPP binding sites determined was between 1 and 2 sites per $\alpha_2\beta_2$ pyruvate decarboxylase subunit; the results obtained do not allow a more definite conclusion.

In the present study several experiments have permitted an assessment of the interaction of TPP with the enzyme, but not an unequivocal assignment. The data include: (a) the initial-velocity patterns observed for the overall reaction (Scheme 1; reactions 1f, 2f, 3f, 4f and 5f) with varying concentrations of TPP and pyruvate in the presence of saturating concentrations of CoA and NAD⁺ (Fig. 3); (b) the dependency of the NAD⁺ and CoA-independent decarboxylation of pyruvate on TPP concentration (reactions 1 and 6) (Fig. 7); (c) the dependency of the partial back reaction on TPP concentration (Scheme 1; reactions 5r, 4r, 3r, 2r and 6r) (Fig. 9); and (d) the direct determination of the binding of TPP to the enzyme (Fig. 4).

A comparison of the evaluated constants is presented in Table 11. The initial-velocity pattern observed with varying concentrations of TPP at fixed concentrations of pyruvate is consistent with a mechanism involving formation of the ternary complex (pyruvate-Enz₁-TPP). If the binding of TPP is the first obligatory step, or if the mechanism of the pyruvate decarboxylase reaction is rapid-equilibrium random, then the point of intersection of reciprocal plots (Fig. 3) is a direct measure of the affinity of the enzyme for TPP. This value, K_{1a} , is in close agreement with the value of K_d (obtained by binding studies using equilibrium dialysis of TPP); possibly the small difference observed between the two methods of evaluation is a function of the 2000-fold difference in enzyme concentration. It is of interest that the K_m^{TPP} of the partial back reaction, which was performed at an intermediate enzyme concentration and which is also a measure of an interaction of TPP with Enz₁, is close to the K_d^{TPP} . Similarly, one of the interaction constants of TPP as an uncompetitive inhibitor of the pyruvate dehydrogenase kinase reaction (Cooper *et al.*, 1974) to which a specific mechanism has not been assigned, is of similar magnitude (7.2 μ M). It is thus possible that K_{1a} (overall reaction), K_d (binding), K_m (partial back reaction) and K_i (kinase inhibition) each describes the same interaction of TPP with Enz₁. Non-competitive inhibition of the overall reaction by glyoxylate against TPP (Fig. 5) would be compatible with an assignment of a rapid-equilibrium random mechanism for the decarboxylase reaction (Segel, 1975). However, glyoxylate did not modify the binding of TPP (Table 1) as would be anticipated if this assignment of mechanism were correct, but the observed formylation of the enzyme (Fig. 8) may complicate a simple interpretation. The K_m^{TPP} values determined for the overall pyruvate dehydrogenase

reaction at saturating concentrations of pyruvate is another measure of the interaction of TPP. This value (approx. 50 nM) is much lower than K_d or K_{1a} or K_m^{TPP} (partial back reaction) by some two orders of magnitude. This might suggest that the rate of the overall reaction is limited by the step: pyruvate-Enz₁-TPP $\rightarrow$ Enz₁-hydroxyethyl-TPP + CO₂ (i.e., that the rate is determined by the concentration of Pyruvate-Enz₁-TPP). In that case, pyruvate by a mass-action effect might be expected to lower the apparent K_m for TPP. The K_m^{TPP} value determined for the NAD⁺ and CoA-independent decarboxylation is an alternative measure of the interaction of TPP in the presence of pyruvate.

Experiments presented here have also provided insight into various aspects of the rate-limiting step(s) in the reaction sequence catalysed by the pyruvate dehydrogenase complex and of the position of equilibrium of some of the individual steps. Included in this are the following:

Table 10. *Maximum number of binding sites for TPP, 2- α -hydroxyethyl-TPP and acetylhydrolipate on pyruvate dehydrogenase and pyruvate dehydrogenase phosphate*

Parameter	Numbers of observations are given in parentheses No. of sites (pmol/unit) on:	
	Pyruvate dehydrogenase phosphate	
	Pyruvate dehydrogenase	Pyruvate dehydrogenase phosphate
TPP binding	490 $\pm$ 74*	543 $\pm$ 36*
2- α -Hydroxyethyl-TPP binding	245 $\pm$ 18 (14)	34.0 $\pm$ 17.6 (8)
Phosphorylation	—	710 $\pm$ 80 (2)
Acetylation	569 $\pm$ 33 (10)	784 $\pm$ 159 (3)

* Data from Fig. 4 analysed by linear regression.

Table 11. *Kinetic constants of the pyruvate dehydrogenase complex for TPP and other cofactors*

Observation	Parameter	Value (μ M)	Enzyme concn. (units/ml)
Overall pyruvate dehydrogenase reaction	K_m^{TPP}	0.05 $\pm$ 0.01	0.016
	K_{1a}^{TPP}	1.6 $\pm$ 0.3	0.016
NAD ⁺ - and CoA-independent pyruvate decarboxylation	K_m^{TPP} at [Pyruvate] = 100 μ M	0.11 $\pm$ 0.03	3.0
Partial back reaction	K_m^{TPP}	4.1 $\pm$ 0.5	0.103
Binding of TPP	K_d	8.2 $\pm$ 0.3	36.4
Inhibition of pyruvate dehydrogenase kinase (Cooper <i>et al.</i> , 1974)	K_{i1}^{TPP}	7.2 $\pm$ 1.3	3.0
	K_{i2}^{TPP}	20.8 $\pm$ 1.4	—
Overall pyruvate dehydrogenase reaction	K_m^{CoA}	1.9 $\pm$ 0.2	—
	K_m^{NAD}	53 $\pm$ 5	—
	$K_m^{Pyruvate}$	25 $\pm$ 4	—
NAD ⁺ - and CoA-independent pyruvate decarboxylation	$K_m^{Pyruvate}$	25	—

(a) The formation of acetoin from pyruvate does not involve enzyme-bound acetylhydrolipoate (Table 2).

(b) The rate-limiting step of the NAD^+ - and CoA-independent pyruvate decarboxylation (Scheme 1; reactions 1 and 6) is considerably slower (<0.5%) than the complete pyruvate dehydrogenase reaction. The rate-limiting step of the former must therefore involve some aspect of the formation of side products such as acetoin from the carbanion form of Enz_1 -2- α -hydroxyethyl-TPP (Reaction 6).

(c) The rate of the partial back reaction (Scheme 1; reactions 5r, 4r, 3r, 2r and 6r) is essentially identical with that of the NAD^+ - and CoA-independent decarboxylation, as would be expected if the rate-limiting step in both cases was some aspect of reaction 6r (Table 3).

(d) As mentioned above, the difference between the values of K_m and K_d for TPP (Fig. 3 and Table 11) indicates that pyruvate, by its interaction and further metabolism, displaces the equilibrium of the TPP-binding reaction, suggesting that the rate-limiting step of the overall reaction is subsequent to the binding of TPP or pyruvate.

(e) The equilibrium of reaction 2 in Scheme 1 (Enz_1 -hydroxyethyl-TPP + Enz_2 -lipoate $\rightleftharpoons$ Enz_1 -TPP + Enz_2 -acetylhydrolipoate) lies very much to the right (data of Table 6). When the reaction is studied in the reverse direction (i.e. with acetyl-CoA and NADH as substrates) the concentration of Enz_1 -hydroxyethyl-TPP formed by the interaction of Enz_1 -TPP with Enz_2 -acetylhydrolipoate is readily increased by addition of NADH, which removes Enz_2 -lipoate as Enz_2 -dihydrolipoate. This presumably reflects the mechanism by which NADH acts as a product inhibitor. In the presence of pyruvate, the ratio $[\text{NADH}]/[\text{NAD}^+]$ has little effect on the concentration of enzyme-bound acetylhydrolipoate (Table 5). This possibly results from the largely irreversible nature of reaction 1, facilitating removal of Enz_1 -TPP with re-formation of Enz_1 -hydroxyethyl-TPP. The observation that removal of acetyl-CoA by the citrate synthase reaction lowered the K_m for CoA by an order of magnitude in the complete pyruvate dehydrogenase reaction, would also be consistent with this interpretation. This would suggest that the ratio $[\text{acetyl-CoA}]/[\text{CoA}]$ may be of particular importance in end-product inhibition *in vivo*, when pyruvate is invariably present.

Mechanism of inhibition of pyruvate dehydrogenase by pyrophosphate and analogues of pyrophosphate

Pyrophosphate and the pyrophosphate analogues GTP, ADP and adenylyl imidodiphosphate were competitive inhibitors of TPP in the overall pyruvate dehydrogenase reaction over a range of pyruvate con-

centrations (Cooper *et al.*, 1974; K_i of pyrophosphate, 0.22 mM). Pyrophosphate was also a predominantly competitive inhibitor of the NAD^+ and CoA-independent decarboxylation of pyruvate (K_i 0.12 mM). These observations might suggest that pyrophosphate and its analogues interfere with TPP binding. However, direct measurements with equilibrium dialysis failed to show any effect of these compounds on K_d or on maximum binding of TPP. The observation that pyrophosphate did not modify K_{in}^{TPP} obtained from initial-velocity measurements supports this conclusion (data of Figs. 3 and 6). Pyrophosphate decreased the number of 2- α -hydroxyethyl-TPP-binding sites and was an inhibitor of the partial back reaction [K_i (non-competitive) 0.16 mM]. The close correspondence of the K_i values in each of these reactions might suggest that a single site of interaction is involved in the inhibition of each. This is apparently not the site at which TPP interacts under the conditions of the binding studies (absence of pyruvate, acetyl-CoA, NADH). Pyrophosphate is also an inhibitor of the pyruvate dehydrogenase kinase reaction, and the K_i (0.15 mM) corresponds closely to the K_i values for the complete and partial reactions of pyruvate dehydrogenase.

These actions of pyrophosphate must therefore stem from a binding site (or sites) which is different from the binding site for TPP in the absence of pyruvate or of acetyl-CoA plus NADH. The question thus arises as to why inhibition by pyrophosphate is apparently competitive with TPP when pyruvate decarboxylation is proceeding. One explanation could be that if the reaction is ordered, the binding of pyrophosphate displaces to the left the equilibrium of the reaction (Enz_1 -TPP + pyruvate $\rightleftharpoons$ pyruvate- Enz_1 -TPP). Another possibility is that reactions of the decarboxylase (eqns. 1 and 2) may involve an intramolecular transfer of hydroxyethyl-TPP to a second binding site on Enz_1 and that pyrophosphate interferes with this transfer.

Mechanism of regulation of the catalytic activity of pyruvate dehydrogenase by phosphorylation

Phosphorylation of pyruvate dehydrogenase inhibits the overall co-ordinated sequence of reactions, and this inhibition is not overcome by increasing the concentrations of the substrates TPP and pyruvate (Linn *et al.*, 1969a). The low rate of NAD^+ -dependent oxidation of acetoin catalysed by the pyruvate dehydrogenase complex is likewise inhibited. The number of TPP binding sites is not modified by phosphorylation (Fig. 4) and the minor alteration of the TPP affinity constant (less than twofold) is too small to be other than a secondary effect. By contrast, formation of enzyme-bound 2- α -hydroxyethyl-TPP is decreased substantially in the phosphorylated enzyme (Tables 8 and 9). The rate of formation of

Scheme 2. Summary of the effects of phosphorylation on the elementary reactions of pyruvate dehydrogenase

Permitted reaction sequences are shown in solid arrows, namely TPP binding and acetylation ($5r+4r+3r$). Blocked reaction sequences are shown in dotted arrows: these are overall reactions ($1f+2f+3f+4f+5f$), hydroxyethyl-TPP formation ($1f$), decarboxylation ($1f+6r$) and reversal ($5r+4r+3r+2r+6r$). Also blocked, but not shown, is acetoin oxidation ($6f+2f+3f+4f+5f$).

either enzyme-linked acetylhydrolipoate from pyruvate (Fig. 11) or enzyme-linked formylhydrolipoate from glyoxylate (Fig. 13) is markedly inhibited for pyruvate dehydrogenase phosphate. The maximum extent (as opposed to the rate) of enzyme-linked acetylhydrolipoate formation from pyruvate may not be significantly affected. As a corollary to these observations it has been demonstrated (Fig. 12) that phosphorylation of the enzyme inhibits the reaction ($\text{Enz}_2\text{-lipSH} \cdot \text{S} \cdot \text{acetyl} + \text{Enz}_1\text{-TPP} \rightleftharpoons \text{Enz}_2\text{-lipS}_2 + \text{Enz}_1\text{-hydroxyethyl-TPP}$ (Scheme 1; reaction $2r$). By contrast, the rate of formation of enzyme-linked acetylhydrolipoate from acetyl-CoA is not apparently modified in pyruvate dehydrogenase phosphate (Fig. 11). A summary of this information is presented in Scheme 2. All reactions in which $\text{Enz}_1\text{-2-}\alpha\text{-hydroxyethyl-TPP}$ is formed are inhibited on phosphorylation of the enzyme. No reaction in which $\text{Enz}_1\text{-2-}\alpha\text{-hydroxyethyl-TPP}$ is not involved is affected. The latter include: the binding of TPP, oxidation-reduction of the lipoyl moiety, and interaction of acetyl-CoA with dihydrolipoate acetyltransferase and resultant formation of enzyme-linked acetylhydrolipoate. In addition, although it has not been possible to measure pyruvate binding directly because of its decarboxylation (even in the absence of NAD^+ and CoA) it is unlikely that the pyruvate binding site is modified. Elevated pyruvate concentrations

in no way negate the inhibition of activity by phosphorylation. $\text{Enz}_1\text{-2-}\alpha\text{-hydroxyethyl-TPP}$ is the only common feature of the overall reaction, the NAD^+ - and CoA-independent decarboxylation, and of the partial back reaction. Thus the conclusion is drawn that phosphorylation modifies those reactions of enzyme-bound TPP which lead to the formation of 2- α -hydroxyethyl-TPP on the enzyme. In the parlance of descriptions of allosteric enzymes, the effect of phosphorylation of pyruvate dehydrogenase is of the 'V' type (Monod *et al.*, 1965), in which modification has affected the maximum velocity. By contrast, the phosphorylation of phosphorylase kinase (Krebs *et al.*, 1964), phosphorylase (Helmreich & Cori, 1964) and glycogen synthetase (Rosell-Perez & Lerner, 1964) each affect a parameter of the affinity of binding of a substrate or effector. It must be pointed out, however, that an exhaustive search for possible allosteric activators of pyruvate dehydrogenase phosphate has not, to our knowledge, been undertaken.

Pyruvate decarboxylase contains two subunits, one of which designated ' α ', undergoes phosphorylation (Barrera *et al.*, 1972). Roche & Reed (1972) have suggested that the α subunit of pyruvate decarboxylase specifically catalyses the decarboxylation of pyruvate (Scheme 1, reaction $1f$), whereas the β subunit catalyses the transfer of the C_2 moiety from TPP

to lipoate (reaction 2f), and that phosphorylation in consequence regulates the decarboxylation step. Evidence was presented that, whereas pyruvate oxidation was inhibited by 89% and pyruvate decarboxylation by 70% by phosphorylation, no effect was observed on the rate of NADH-dependent oxidation of hydroxyethyl-TPP or on the formation of Enz₂-acetylhydrolipoate from hydroxy[¹⁴C]ethyl-TPP. However, the rates of these two reactions were only 0.2 and 0.04% respectively of the rate of pyruvate oxidation, presumably because the formation of the carbanion of hydroxyethyl-TPP from hydroxyethyl-TPP is slow and rate-limiting; thus the capacity of the phosphorylated enzyme to catalyse the formation of Enz₂-acetylhydrolipoate from Enz₁-2- α -hydroxyethyl-TPP (reaction 2f) was in considerable excess of the observed rate of utilization of free hydroxyethyl-TPP, even if phosphorylation had decreased activity by 89%. The results of our own studies indicate that phosphorylation of the α subunit inhibits all reactions leading to the formation of enzyme-bound 2- α -hydroxyethyl-TPP carbanion from enzyme-bound TPP, including specifically reactions 1f and 2r. The catalytic role of the α and β subunits may thus not be that suggested by Roche & Reed (1972).

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