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Regulation of Heart Muscle Pyruvate Dehydrogenase Kinase

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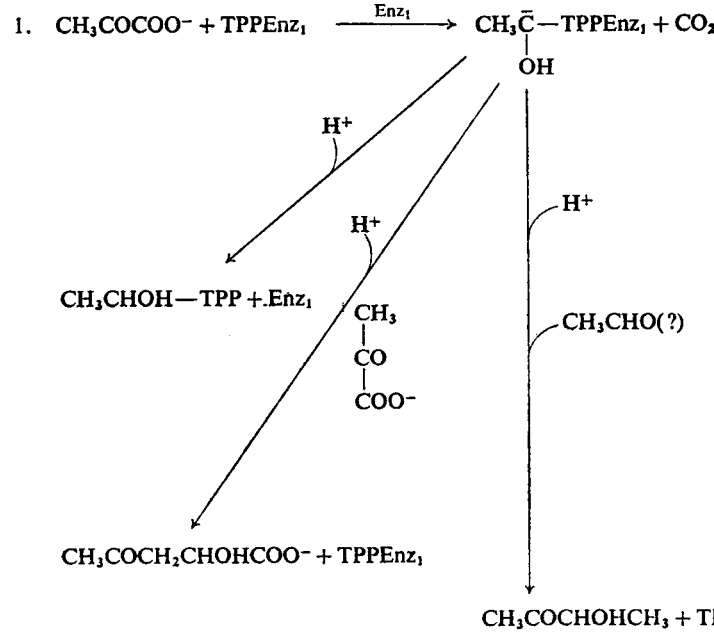
1. The activity of pig heart pyruvate dehydrogenase kinase was assayed by the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into the dehydrogenase complex. There was a very close correlation between this incorporation and the loss of pyruvate dehydrogenase activity with all preparations studied. 2. Nucleoside triphosphates other than ATP (at 100μM) and cyclic 3′:5′-nucleotides (at 10μM) had no significant effect on kinase activity. 3. The K_m for thiamin pyrophosphate in the pyruvate dehydrogenase reaction was 0.76μM. Sodium pyrophosphate, adenylyl imidodiphosphate, ADP and GTP were competitive inhibitors against thiamin pyrophosphate in the dehydrogenase reaction. 4. The K_m for ATP of the intrinsic kinase assayed in three preparations of pig heart pyruvate dehydrogenase was in the range 13.9–25.4μM. Inhibition by ADP and adenylyl imidodiphosphate was predominantly competitive, but there was nevertheless a definite non-competitive element. Thiamin pyrophosphate and sodium pyrophosphate were uncompetitive inhibitors against ATP. It is suggested that ADP and adenylyl imidodiphosphate inhibit the kinase mainly by binding to the ATP site and that the adenosine moiety may be involved in this binding. It is suggested that thiamin pyrophosphate, sodium pyrophosphate, adenylyl imidodiphosphate and ADP may inhibit the kinase by binding through pyrophosphate or imidodiphosphate moieties at some site other than the ATP site. It is not known whether this is the coenzyme-binding site in the pyruvate dehydrogenase reaction. 5. The K_m for pyruvate in the pyruvate dehydrogenase reaction was 35.5μM. 2-Oxobutyrate and 3-hydroxypyruvate but not glyoxylate were also substrates; all three compounds inhibited pyruvate oxidation. 6. In preparations of pig heart pyruvate dehydrogenase free of thiamin pyrophosphate, pyruvate inhibited the kinase reaction at all concentrations in the range 25–500μM. The inhibition was uncompetitive. In the presence of thiamin pyrophosphate (endogenous or added at 2 or 10μM) the kinase activity was enhanced by low concentrations of pyruvate (25–100μM) and inhibited by a high concentration (500μM). Activation of the kinase reaction was not seen when sodium pyrophosphate was substituted for thiamin pyrophosphate. 7. Under the conditions of the kinase assay, pig heart pyruvate dehydrogenase forms $^{14}CO_2$ from [1-^{14}C]pyruvate in the presence of thiamin pyrophosphate. Previous work suggests that the products may include acetoin. Acetoin activated the kinase reaction in the presence of thiamin pyrophosphate but not with sodium pyrophosphate. It is suggested that acetoin formation may contribute to activation of the kinase reaction by low pyruvate concentrations in the presence of thiamin pyrophosphate. 8. Pyruvate effected the conversion of pyruvate dehydrogenase phosphate into pyruvate dehydrogenase in rat heart mitochondria incubated with 5mM-2-oxoglutarate and 0.5mM-L-malate as respiratory substrates. It is suggested that this effect of pyruvate is due to inhibition of the pyruvate dehydrogenase kinase reaction in the mitochondrion. 9. Pyruvate dehydrogenase kinase activity was inhibited by high concentrations of Mg^{2+} (15mM) and by Ca^{2+} (10nM–10μM) at low Mg^{2+} (0.15mM) but not at high Mg^{2+} (15mM).

Mammalian pyruvate dehydrogenases (EC 1.2.4.1) catalyse the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2 in the presence of thiamin pyrophosphate and Mg^{2+} by reactions shown in Scheme 1 (Gunsalus, 1954; Reed, 1960; Ullrich & Mannschreck, 1967). The reaction sequence involves three enzymes in the complex which we will refer to as pyruvate decarboxylase, dihydrolipoate acetyltransferase and dihydrolipoate dehydrogenase. In the absence of CoA and NAD^+ pyruvate is decarboxylated and the end products (other than CO_2) may include carbanion



2. $CH_3\bar{C}(OH)-TPPEnz_1 + H^+ + \text{lipoate-}Enz_2 \xrightleftharpoons{Enz_1} TPPEnz_1 + \text{acetylhydrolipoate-}Enz_2$

3. $\text{Acetylhydrolipoate-}Enz_2 + CoA \xrightleftharpoons{Enz_2} \text{acetyl CoA} + \text{dihydrolipoate-}Enz_2$

4. $\text{Dihydrolipoate-}Enz_2 + NAD \xrightleftharpoons{Enz_3} \text{lipoate-}Enz_2 + NADH_2$

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

In this scheme, the abbreviations are: TPP, thiamin pyrophosphate; Enz_1, pyruvate decarboxylase; Enz_2, dihydrolipoate acetyltransferase; Enz_3, dihydrolipoate dehydrogenase.

and protonated forms of 2-α-hydroxyethylthiamin-pyrophosphate, acetoin and acetolactate (Scheme 1; Jagannathan & Schweet, 1952; Juni & Heym, 1956; Downes & Sykes, 1957; Breslow, 1958; Ullrich & Mannschreck, 1967). A fourth enzyme in the complex (pyruvate dehydrogenase kinase) catalyses the phosphorylation and inactivation of pyruvate decarboxylase by reaction with $ATPMg^{2-}$ (Linn *et al.*, 1969*a*; Hucho *et al.*, 1972; Roche & Reed, 1972). These enzymes are tightly bound in the complex and are co-purified during fractional precipitation and ultracentrifugation. A fifth enzyme, pyruvate dehydrogenase phosphate phosphatase, catalyses the dephosphorylation and reactivation of pyruvate decarboxylase (Linn *et al.*, 1969*a*,*b*). It is more loosely bound in the complex and may be separated from the other components in the ultracentrifuge (mol.wt. of complex approx. 10^7; mol.wt. of phosphatase approx. 10^5).

Regulation of pyruvate dehydrogenase kinase was suggested by the observation that pyruvate or ADP can inhibit phosphorylation of pyruvate dehydrogenase (Linn *et al.*, 1969*b*). Pyruvate dehydrogenase kinase is also inhibited by thiamin pyrophosphate and by PP_i (Roche & Reed, 1972; Randle & Denton, 1973; Wieland *et al.*, 1973). Preparations of pyruvate dehydrogenase can contain variable amounts of thiamin pyrophosphate, and when the coenzyme is present pyruvate can be decarboxylated during kinase assays. Therefore it was important to investigate interactions between pyruvate and thiamin pyrophosphate in kinase regulation. Wieland *et al.* (1973) on the basis of kinetic studies suggested that PP_i may be a competitive inhibitor against ATP of pyruvate dehydrogenase kinase. Our own preliminary studies suggested that the inhibition was uncompetitive and this has led us to investigate and compare effects of PP_i, thiamin pyrophosphate and of other pyrophos-

phates. Pyruvate dehydrogenase phosphate phosphatase requires Mg^{2+} for activity and is substantially activated by Ca^{2+} in the presence of Mg^{2+} (Denton *et al.*, 1972; Siess & Wieland, 1972; Pettit *et al.*, 1972). The effects of Ca^{2+} and Mg^{2+} on the activity of pyruvate dehydrogenase kinase have therefore been investigated. The results of these and other studies are reported here.

Experimental

Materials

Chemicals. Nucleotides, coenzymes, sodium pyruvate and adenylyl imidodiphosphate were from Boehringer (London) Corp., London W.5, U.K. Thiamin pyrophosphate, thiamin, and the sodium salts of glyoxylate, 2-oxobutyrate, 2-oxo-3-methylbutyrate, 2-oxo-3-methylpentanoate, 2-oxo-4-methylpentanoate, L-lactate, 3-fluoropyruvate, phenylpyruvate and *p*-hydroxyphenylpyruvate were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; 3-bromopyruvate was from Kodak Ltd., Kirby, Liverpool, U.K.; 2-(*N*-morpholino)propanesulphonic acid (Mops) was from Calbiochem, San Diego, Calif. U.S.A. Dithiothreitol and L-carnitine chloride were from Koch–Light Ltd., Colnbrook, Bucks., U.K. Acetyl-CoA was synthesized by the method of Simon & Shemin (1953). Acetyl-L-carnitine was synthesized by the method of Fraenkel & Friedman (1957). Spectrograde $MgSO_4$ and $CaCl_2$ were from Johnson Matthey Chemicals Ltd., London E.C.4, U.K. [γ-^{32}P]ATP (sodium salt, 0.5–3mCi/μmol), $^{45}CaCl_2$ and [1-^{14}C]pyruvate were from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were Analytical Reagent Grade or the highest grade available.

Pig heart pyruvate dehydrogenase. Preparations of pyruvate dehydrogenase for kinase assay must be low in ATPase (adenosine triphosphatase) and pyruvate dehydrogenase phosphate phosphatase activities. None of the existing methods of purification proved entirely satisfactory. A modification of the polyethylene glycol fractionation procedure of Linn *et al.* (1972) has consistently given satisfactory preparations and is described below. Unless otherwise stated, operations were at 4°C; pH was measured with a glass electrode.

Pig hearts were obtained fresh from the carcass and immediately packed in ice (important). The muscle was cut into 2.5cm (1in) cubes and fat removed, and dispersed in a Waring blender into 30mM-potassium phosphate–1mM-EDTA, pH7.6 (400ml per heart, added gradually during dispersion). The pH was raised to between 6.5–7.0 with 5M-KOH if necessary. The homogenate was centrifuged at 2075*g* for 40min in an MSE Mistral 6L centrifuge. The supernatant was filtered through muslin and retained; the pellet was redispersed as described above in phosphate–EDTA (300ml per heart) and the supernatant retained. The combined supernatants were adjusted to pH5.4 with 10% (v/v) acetic acid and precipitated mitochondria were separated at 15000*g* for 20min, washed once by resuspending in water and collected by centrifuging.

The mitochondrial pellets were suspended in water (approx. 40–60ml per heart), 0.02vol. of 1M-potassium phosphate, pH7.0, was added and the pH adjusted to 6.8 with 5M-KOH. The mitochondrial suspension was shell-frozen and thawed (three times) by using alternately liquid N_2 and a water bath at 45°C. The slurry was then centrifuged for 1h at 53000*g* in the 6×300ml head of the MSE 65 centrifuge. The clear supernatant was aspirated carefully (important), 0.01vol. of 1M-$MgCl_2$ was added and the pH readjusted to 6.8. The solution was incubated at 30°C for 20min to effect conversion of pyruvate dehydrogenase phosphate into pyruvate dehydrogenase. Pyruvate dehydrogenase activity was then assayed by coupling to arylamine transacetylase as described by Coore *et al.* (1971).

The solution was adjusted to pH6.5 with 10% (v/v) acetic acid and 0.01vol. of 5M-NaCl added. Polyethylene glycol (mol.wt. 6000; 0.03vol. of 50% w/v) was added at room temperature with stirring and after 10min the clear supernatant was recovered by centrifuging at 18°C for 20min at 15000*g*. Pyruvate dehydrogenase was then precipitated, by addition of a further 0.07 vol. of 50% (w/v) polyethylene glycol at room temperature, and collected by centrifuging at 18°C for 20min at 15000*g*. The pyruvate dehydrogenase was dispersed at 0°C into 50mM-2-(*N*-morpholino)propanesulphonate–1mM-dithiothreitol, pH7.0, in a Teflon–glass Potter–Elvehjem homogenizer (15–25 units/ml of buffer, approx.). These operations were completed in 1 day.

After standing overnight at 0°C the suspension was cleared by centrifuging at 4°C for 30min at 40000*g* and the supernatant assayed for pyruvate dehydrogenase by coupling to arylamine transacetylase. It was then warmed to 20°C, 0.02vol. of 0.5M-potassium EDTA, pH7.0, added, and the pH lowered to 6.5 with 10% (v/v) acetic acid. Polyethylene glycol (0.03vol. of 50% w/v) was added with stirring and after 10min the supernatant recovered by centrifuging at 18°C for 20min at 32000*g*. Pyruvate dehydrogenase was then precipitated by lowering the pH to 5.4 (by addition of 0.5M-$MgCl_2$ which displaces protons from EDTA) and recovered after 10min by centrifuging at 18°C for 20min at 32000*g*. The pellets were redissolved in 20mM-potassium phosphate–5mM-2-mercaptoethanol (phosphate–mercaptoethanol) to give approx. 40 units of pyruvate dehydrogenase/ml and the pH was adjusted to 7.0 with 1M-KOH. The pyruvate dehydrogenase was left for 1h at 0°C and then the pH was lowered to 6.1 with 10%

(v/v) acetic acid and precipitated material removed by centrifuging at 4°C for 20min at 32000*g*. The supernatant was adjusted back to pH7.0 with 5M-KOH and assayed for pyruvate dehydrogenase activity. After dilution with phosphate–mercaptoethanol to approx. 15 units/ml, and addition of 0.004 vol. of 0.5M-potassium EDTA, pH7.0, the solution was centrifuged at 2°C for 90min at 150000*g* and the supernatant discarded.

The pellets were redissolved in phosphate–mercaptoethanol (1ml per 40–50 units of pyruvate dehydrogenase) and further contaminating material was removed by precipitation at pH6.1 (see preceding paragraph). Pyruvate dehydrogenase was then assayed spectrophotometrically by NAD^+ reduction (see below), as the preparation was then substantially free of lactate dehydrogenase. Centrifugation at 150000*g* was repeated but without addition of EDTA and the pellets were redissolved in phosphate–mercaptoethanol (to 20–40 units/ml). Subsequent treatment depended on the appearance of the 150000*g* pellet. If this was small and pale yellow in colour with only a small brown spot in the centre the preparation was usually low in ATPase activity and further purification was not necessary. If the pellets were bulky and contained appreciable brown material or if the subsequent solution was turbid, precipitation at pH6.1 was repeated. If the activity of pyruvate dehydrogenase phosphate phosphatase was too high (see below) centrifugation at 150000*g* was repeated. The purified dehydrogenase (20–40 units/ml) was stored at −10°C in suitable batches (0.2, 0.5 or 1ml). Loss of activity on storage was about 10–20% per week, and individual preparations lasted approx. 3 weeks. The approximate yields were: 100–180 units per heart after extraction from the mitochondrial fraction and incubation with $MgCl_2$; 60–70 units per heart after the first precipitation with polyethylene glycol; 40–50 units per heart after the second polyethylene glycol precipitation at pH5.4; 30–40 units per heart after the first pH6.1 fractionation; 20–35 units per heart, final yield.

Rat heart pyruvate dehydrogenase. This was partially purified from frozen rat hearts by the procedure for partial purification of adipose-tissue pyruvate dehydrogenase phosphate phosphatase described by Severson *et al.* (1974). In the final step of that procedure pyruvate dehydrogenase is separated as a pellet by centrifugation at 150000*g*. The pellet was taken up in phosphate–mercaptoethanol and the pH lowered to 6.1 with acetic acid. After centrifuging the supernatant was adjusted to pH7.0 with KOH and the preparation stored at −10°C. The initial activity was approx. 4 units/ml.

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 1.5ml of medium containing 100mM-Tris–HCl, 0.5mM-EDTA, 1mM-$MgCl_2$, 5mM-2-mercaptoethanol 1mM-thiamin pyrophosphate, 0.5mM-NAD^+, 0.1mM-CoA, 1mM-pyruvate, pH7.8.

ATPase activity in pyruvate dehydrogenase preparations. This was assayed by the release of [^{32}P]phosphate from [γ-^{32}P]ATP. Pyruvate dehydrogenase (0.25–0.35 unit) and [γ-^{32}P]ATP (10nmol; specific radioactivity 100μCi/μmol) were incubated in phosphate–mercaptoethanol, pH7.0 (total vol. 100μl) at 30°C in Beckman Microfuge tubes. Samples (20μl) were removed at 2, 5 and 10min into 0.5ml of acid charcoal (5mg of Norit GSX in 1M-HCl), mixed, and centrifuged for 2min in an Eppendorf 3200 centrifuge. The supernatant was assayed for radioactivity in methoxyethanol–toluene-based scintillator (Severson *et al.*, 1974).

Lactate dehydrogenase in pyruvate dehydrogenase preparations. This was assayed spectrophotometrically by the rate of ΔE_{340} at 30°C on addition of pyruvate dehydrogenase to phosphate–mercaptoethanol containing 1mM-pyruvate and 0.1mM-NADH.

2-Oxoglutarate dehydrogenase in pyruvate dehydrogenase preparations. This was assayed as for pyruvate dehydrogenase except that 1.3mM-2-oxoglutarate was used in place of 1mM-pyruvate.

Pyruvate dehydrogenase kinase; radioassay. This involved measurement of the initial rate of transfer of [^{32}P]phosphate from [γ-^{32}P]ATP into pyruvate dehydrogenase. Separation of protein-bound radioactivity was achieved by trichloroacetic acid precipitation on paper by the method given by Wastila *et al.* (1971). Incubations were made in phosphate–mercaptoethanol. Compounds to be tested were dissolved in the same buffer and the pH was adjusted to 7.0 where necessary. The concentrations of various additions are shown in the text, Figures or Tables. Stock solutions of [γ-^{32}P]ATP were prepared by drying a suitable sample of [γ-^{32}P]ATP *in vacuo* and redissolving in 1mM-ATP containing an appropriate concentration of $MgCl_2$ (usually 20mM) in phosphate–mercaptoethanol at pH7.0. The final specific radioactivity was approx. 100μCi/μmol. Whatman 3MM paper was dipped into 2.5mM-ATP–5mM-$MgCl_2$, pH7.0, and air-dried at room temperature (to minimize adsorption of radioactive ATP). It was cut into 1.5cm squares for use.

Incubations were done at 30°C in Beckman 152 Microfuge tubes. Pyruvate dehydrogenase (50–200 munits) and other additions (except $ATPMg^{2-}$) as given in the text, Figures or Tables, were equilibrated for 10min at 30°C (unless otherwise stated) before initiation of the kinase reaction by addition of $ATPMg^{2-}$. The total volume of incubation mixture was 50μl. After incubation (usually for 2min), the reaction was terminated by applying 30μl to a square

of Whatman 3MM paper which was immediately placed in 10% (w/v) trichloroacetic acid at 0°C. The subsequent steps were as given by Wastila *et al.* (1971). Radioactivity was assayed by liquid-scintillation spectrometry in toluene-based scintillator (for composition see Severson *et al.*, 1974). Appropriate blanks (omitting pyruvate dehydrogenase) were included. Control experiments with a number of preparations of pyruvate dehydrogenase showed that incorporation was linear for up to 3min and hence 2min of incubation was used as a routine.

Pyruvate dehydrogenase kinase; spectrophotometric assay. This involved measurement of the initial rate of inactivation of pyruvate dehydrogenase by phosphorylation. The general conditions of incubation were as described for the radioassay except that non-radioactive ATP was used (unless both assays were being made simultaneously). The reaction was terminated by transfer of a sample to a cuvette for assay of pyruvate dehydrogenase as described above.

Experiments with calcium-depleted reagents. Phosphate–mercaptoethanol and pyruvate dehydrogenase were depleted of calcium with Chelex-100 resin and dialysis as described by Randle *et al.* (1974). Spectrograde $MgSO_4$ and $CaCl_2$ were used in experiments with calcium-depleted reagents.

Assay of pyruvate dehydrogenase phosphate phosphatase in pyruvate dehydrogenase preparations. This was assayed by the release of [^{32}P]phosphate from pig heart pyruvate dehydrogenase [^{32}P]phosphate as described by Randle *et al.* (1974). The assay medium was phosphate–mercaptoethanol. Concentrations were: pyruvate dehydrogenase phosphate, 1.5μM-protein-bound phosphate (equivalent to 2.5 units of pyruvate dehydrogenase/ml), 10mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], 9.75mM-$CaCl_2$ and 32mM-$MgCl_2$.

Assay of pyruvate decarboxylase activity. In the absence of CoA and NAD^+, pyruvate is decarboxylated by pyruvate dehydrogenase to 2-α-hydroxyethylthiamin pyrophosphate and acetoin or acetolactate (see the introduction). This decarboxylation was assayed by the release of $^{14}CO_2$ from [1-^{14}C]-pyruvate. Incubations (final volume 40μl) were made in small glass tubes inserted into scintillation-counting bottles containing 0.5ml of 2-phenethylamine–methanol (1:1, v/v) to absorb CO_2 and sealed with skirted rubber caps. The assay medium was phosphate–mercaptoethanol; other additions are given in the text or Tables. The reaction was initiated by injection of [1-^{14}C]pyruvate, and terminated and CO_2 liberated into the phenethylamine by addition of 100μl of 0.8M-citric acid–0.04M-Na_2HPO_4. After shaking for 60min to complete absorption of CO_2 the tubes were removed and radioactivity assayed by liquid-scintillation spectrometry with methoxyethanol–toluene-based scintillator (Severson *et al.*, 1974).

Experiments with rat heart mitochondria. Mitochondria were prepared from rat hearts and ATP was assayed by methods given by Whitehouse *et al.* (1974). The conditions of incubation for assays of mitochondrial pyruvate dehydrogenase are given in the text, Tables and Figures. Pyruvate dehydrogenase and pyruvate dehydrogenase phosphate were extracted and assayed as described by Severson *et al.* (1974).

Calculations. Concentrations of Ca^{2+} (free calcium) and Mg^{2+} (free magnesium) and of bound forms of these metals were calculated by using a computer program (Feldman *et al.*, 1972). The dissociation constants for EGTA, phosphate and sulphate were as given by Severson *et al.* (1974). Other dissociation constants calculated from molar concentrations were $MgATP^{2-}$ 5.24×10^{-5}M; $CaATP^{2-}$ 1.32×10^{-4}M; $MgP_2O_7^{2-}$ 2.78×10^{-4}M; $MgADP^-$ 5.80×10^{-4}M; magnesium adenylylimidodiphosphate 7.85×10^{-5}M.

In studies of enzyme kinetics K_m, $V_{max.}$ and K_i were computed by the method of Jones (1970) by using a program provided by Dr. P. J. England of this Department.

ATP, $ATPMg^{2-}$ and pyruvate dehydrogenase kinase activity. In describing the results of assays of pyruvate dehydrogenase kinase activities the concentration of ATP is given as the total concentration of ATP. Since rates of phosphorylation of pyruvate dehydrogenase by ATP are much slower in the absence of added $MgCl_2$ or $MgSO_4$, the substrate for phosphorylation is presumably $ATPMg^{2-}$. Under the conditions that we have used as a routine for kinase assays (20–200μM-ATP; 2mM-$MgCl_2$) the concentration of $ATPMg^{2-}$ was calculated to be 80–85% of that of total ATP. Because we have found that higher concentrations of $MgCl_2$ are inhibitory, it was impracticable to add sufficient $MgCl_2$ to ensure that all of the ATP was present as $ATPMg^{2-}$. For convenience K_m values are expressed as a concentration of total ATP. If ATP is not inhibitory towards $ATPMg^{2-}$ then the K_m for $ATPMg^{2-}$ will be approx. 80–85% of the values quoted. We have not been able to ascertain whether or not ATP is inhibitory towards $ATPMg^{2-}$.

Results and Discussion

Purity of pyruvate dehydrogenase preparations

Seven preparations of pig heart pyruvate dehydrogenase (PDH Z5–31) have been used in these studies. The specific activity was approx. 9 units/mg. Electrophoresis of preparations PDH 29, 30 and 31 on sodium dodecyl sulphate gels (by Dr. Donal A. Walsh and Helen T. Pask) (Weber & Osborn 1969) revealed four major protein bands corresponding to the α and β subunits of pyruvate decarboxylase, dihydrolipoyl acetyltransferase and dihydrolipoyl dehydrogenase described for the bovine kidney and heart enzymes (Barrera *et al.*, 1972). These protein bands accounted for at least 70% of the protein present.

Table 1 shows kinase and phosphatase activities of

Table 1. *Enzyme activities of pig heart pyruvate dehydrogenase preparations*

Kinase activities were measured by radioassay and by the spectrophotometric assay (see the Experimental section). The initial concentration of pyruvate dehydrogenase was 1–4 units/ml. Phosphatase activity was assayed on pyruvate dehydrogenase [^{32}P]phosphate prepared from each dehydrogenase preparation (see the Experimental section). The units of activity were as follows: kinase: % of pyruvate dehydrogenase phosphorylated after 1 min; phosphatase: % of pyruvate dehydrogenase phosphate hydrolysed/min. Thiamin pyrophosphate dependence: 100× velocity without added thiamin pyrophosphate/velocity with added thiamin pyrophosphate.

Pyruvate dehydrogenase preparation	Activities (units)		Thiamin pyrophosphate dependence
	Kinase	Phosphatase	
25	18	2.5	—
26	14	0.6	—
27	24.5	0.4	—
28	32	0.04	30
29	35	0.4	1.25
30	33	1.8	9.0
31	40	0.1	9.0

the preparations of pyruvate dehydrogenase used in these studies. The phosphatase activity was low relative to the kinase activity and calculation showed that no more than 1% of the pyruvate dehydrogenase phosphate formed would be hydrolysed during kinase assays. The phosphatase assays shown in Table 1 were performed at saturating concentrations of Mg^{2+} and Ca^{2+} and the initial concentration of substrate was equal to the K_m (1.5 μM-protein-bound phosphate; 32mM-$MgCl_2$, 10mM-EGTA, 9.75mM-$CaCl_2$; Randle *et al.*, 1974). Except for experiments shown in Tables 7 and 8 the concentration of Mg^{2+} in kinase assays (0.25mM) was well below the K_m of the phosphatase for Mg^{2+} (1mM; Randle *et al.*, 1974). The concentration of pyruvate dehydrogenase phosphate at the end of the kinase assay was approx. 0.5–1.9 μM-protein-bound phosphate.

The ATPase activity of the dehydrogenase preparations was low and would have hydrolysed no more than 3% of the ATP present/min under the conditions of the kinase assay. Because, for each preparation, reaction velocity in the kinase assay was constant for the duration of the assay (2min) this ATPase activity was considered acceptable. The activities of lactate dehydrogenase and 2-oxoglutarate dehydrogenase were negligible (<0.1% of that of pyruvate dehydrogenase).

Time-course of phosphorylation; pH dependence

Fig. 1 shows the incorporation of [^{32}P]phosphate into pyruvate dehydrogenase and disappearance of dehydrogenase activity during 12min of incubation of preparation PDH 26 with [γ-^{32}P]ATP. There was very close correlation between the incorporation of ^{32}P and the disappearance of pyruvate dehydrogenase activity. Each of the preparations used in this study showed a similar, close correlation. Fig. 1 also shows that the rate of phosphorylation was essentially constant for up to 6min for preparation PDH 26 when approx. 70% of the enzyme was phosphorylated. Even in other preparations, in which the kinase was more active (see Table 1) constant rates of phosphorylation were maintained for at least 2min, during which time 50–80% of the enzyme was phosphorylated. As a routine assays of kinase activity were terminated after 2min.

The pyruvate dehydrogenase complex is unusual in that the kinase molecules are tightly bound to the complex containing the substrate (pyruvate decarboxylase). Analyses of bovine kidney and heart pyruvate dehydrogenase by Barrera *et al.* (1972) have indicated that there may be only one molecule of kinase per 12 decarboxylase units ($\alpha\beta$), that one phosphate residue is incorporated per decarboxylase α chain, and that kinase and decarboxylase are attached to a core of dihydrolipoate acetyltransferase.

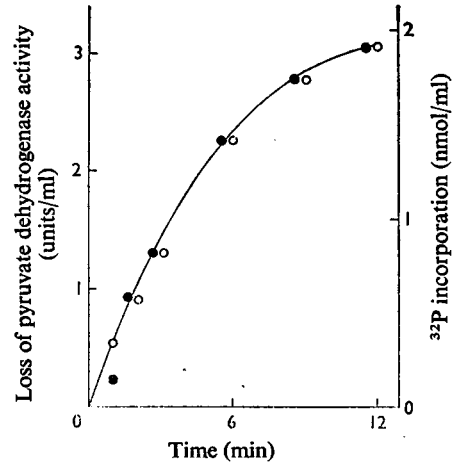


Fig. 1. *Time-course of phosphorylation and inactivation of pig heart pyruvate dehydrogenase (PDH 26)*

Pyruvate dehydrogenase (3.34 units/ml) was incubated at 30°C in phosphate–mercaptoethanol, pH 7.0, containing 0.2mM-$MgCl_2$ and 0.1mM-[γ-^{32}P]ATP (45 μCi/μmol). Samples were taken at the times shown and assayed for protein-bound [^{32}P]phosphate and pyruvate dehydrogenase activity (see the Experimental section). Each point is the mean of three determinations. Loss of pyruvate dehydrogenase activity (●) is (pyruvate dehydrogenase activity at zero time−activity at time shown). ^{32}P incorporation (○) (nmol/ml) is (protein-bound radioactivity in d.p.m./ml of incubation mixture)/(specific radioactivity of ATP in d.p.m./nmol).

Thus each molecule of kinase may catalyse multiple sequential phosphorylations while remaining apparently bound to the complex. The time-course of phosphorylation for preparations PDH 26–29 was analysed by the integrated Michaelis equation (Dixon & Webb, 1958). There was no correlation between $y \cdot t^{-1}$ and $2.303 \cdot t^{-1} \log S_0 \cdot (S_0 - y)^{-1}$ ($r = 0.23$ for 250 observations on four preparations) where S_0 = initial pyruvate dehydrogenase concn., y = pyruvate dehydrogenase phosphate concn. at time t. A negative correlation ($r = -1$) would be expected if Michaelis–Menten kinetics are obeyed. The near-constant rate of phosphorylation until 50–80% of sites are phosphorylated would suggest that phosphorylation of up to half the pyruvate decarboxylase units has little or no effect on the rate of phosphorylation of other pyruvate decarboxylase units.

There was a linear increase ($r = 1.0$) in the initial rate of phosphorylation with increasing concentrations of pyruvate dehydrogenase (preparation PDH 28) over the range 1–6 units/ml (at constant ATP concentration). The range of pyruvate dehydrogenase concentrations in all other experiments was between 1 and 4 units/ml. In any one experiment the concentration of pyruvate dehydrogenase was constant. Fig. 2 shows the effect of pH on the initial rate of phosphorylation of preparation PDH 27. Between pH6.0 and 7.0 the rate of phosphorylation was virtually independent of pH. Below pH6.0 or above pH7.0 the rate fell. Kinase assays were performed as a routine at pH7.0 and care was taken to adjust the pH of any additions to 7.0.

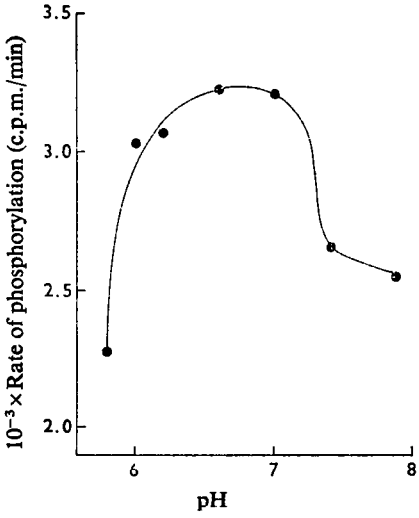


Fig. 2. *Effect of pH on rate of phosphorylation of pig heart pyruvate dehydrogenase (PDH 27)*

Pyruvate dehydrogenase (2.2 units/ml) was preincubated at 30°C in phosphate–mercaptoethanol for 10min at the pH shown. Reaction was initiated by addition of 1mM-$MgCl_2$ and 0.1mM-[γ-^{32}P]ATP (150μCi/μmol). Reaction was terminated and protein-bound radioactivity assayed after 2min. Assays were made in quadruplicate.

Table 2. *Effect of nucleoside triphosphates and cyclic 3′:5′ nucleotides on pyruvate dehydrogenase kinase activity*

Kinase activity was assayed on pig heart pyruvate dehydrogenase (preparation PDH 25) by radioassay (see the Experimental section). Concentrations were: [γ-^{32}P]ATP, 100μM; $MgCl_2$, 2μmol/μmol of nucleoside triphosphate; pyruvate dehydrogenase, 1.5 units/ml; other additions as shown (additional ATP was non-radioactive). There were three observations in each group. The specific radioactivities of ATP were constant within any one experiment. Kinase activities are given as c.p.m. in protein-bound phosphate incorporated/2min per 30μl of incubation mixture.

Experiment	Addition	Kinase activity (Mean±S.E.M.)
1.	None	1579±217
	ATP (100μM)	971± 39
	GTP (100μM)	1565± 23
	CTP (100μM)	1555± 13
	UTP (100μM)	1388± 63
2.	None	2321± 26
	3′:5′-Cyclic AMP (10μM)	2278± 68
	3′:5′-Cyclic GMP (10μM)	2222± 98
	3′:5′-Cyclic CMP (10μM)	2288±128
	3′:5′-Cyclic UMP (10μM)	2270± 28
	3′:5′-Cyclic deoxythymidine monophosphate (10μM)	2320±231

Effects of nucleoside triphosphates and cyclic 3′:5′ nucleotides on pyruvate dehydrogenase kinase activity

These data are shown in Table 2. Addition of unlabelled ATP produced the expected decrease in ^{32}P incorporation. None of the other nucleoside triphosphates tested at 100μM diminished the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (100μM) into pyruvate dehydrogenase significantly. These findings are in agreement with those obtained with bovine kidney enzyme by Linn *et al.* (1969*a*). Our data show no evidence for phosphorylation of pyruvate dehydrogenase by GTP, CTP or UTP or for competitive inhibition by these nucleoside triphosphates of phosphorylation with ATP at the concentrations used. Mixed inhibition by adenylyl imidodiphosphate and ADP is described in the next section.

At a concentration of 10μM the five cyclic 3′:5′ nucleotides shown had no effect on pyruvate dehydrogenase kinase activity.

Table 3. *Inhibition of pig heart pyruvate dehydrogenase by sodium pyrophosphate, adenylyl imidodiphosphate, GTP and ADP*

Pig heart pyruvate dehydrogenase activity (preparation PDH 29; 0.015 unit) was assayed spectrophotometrically by measurement of NAD^+ reduction (see the Experimental section). Measurements were made in duplicate at the following concentrations of thiamin pyrophosphate (0, 0.9, 1.37, 2.73, 5.25 and 10.5 μM) with and without 0.5 mM concentrations of sodium pyrophosphate, adenylyl imidodiphosphate, GTP or ADP. Corrections were made for the rate in the absence of added thiamin pyrophosphate (1.25 % of $V_{max.}$). K_m and $V_{max.}$ were computed (see the Experimental section). K_i was calculated by substitution in the equation for competitive inhibition. Results are means ± S.E.M.

Inhibitor	K_m for thiamin pyrophosphate (μM)	$10^3 \times V_{max.}$ (ΔE_{340}/min)	K_i (competitive) (μM)
None	0.76 ± 0.09	44 ± 1.2	—
Sodium pyrophosphate	4.55 ± 0.61	43.6 ± 2.7	100
Adenylyl imidodiphosphate	1.83 ± 0.40	40.7 ± 3.1	355
GTP	1.43 ± 0.12	40 ± 1.2	507
ADP	1.1 ± 0.07	47.3 ± 0.94	1118

Table 4. *Inhibition of activity of pig heart pyruvate dehydrogenase kinase by thiamin pyrophosphate, sodium pyrophosphate, ADP and adenylyl imidodiphosphate*

Pyruvate dehydrogenase kinase activity was assayed by radioassay (see the Experimental section) with triplicate determinations. Concentrations were: ATP, 20, 25, 50, 100, 200 μM; $MgCl_2$, 2 mM; pyruvate dehydrogenase, preparation PDH 25, 3 units/ml; preparation PDH 28, 1 unit/ml; preparation PDH 29, 3 units/ml; preparation PDH 31, 2 units/ml; thiamin pyrophosphate, 10 μM; sodium pyrophosphate, 100 μM; adenylyl imidodiphosphate, 0.2 mM; ADP, 0.5, 1.0, and 2.0 mM. In experiments with fixed ATP* (100 μM-ATP, 2 mM-$MgCl_2$) thiamin pyrophosphate concentrations were 0, 1, 2, 5 and 10 μM. K_1, competitive K_i; K_2, non-competitive K_i; K_3, uncompetitive K_i. Results are means ± S.E.M.

Preparation	Inhibitor	K_m for ATP (μM)	$V_{max.}$ (c.p.m./2min per 30 μl of incubation mixture)	K_i (μM)
PDH 29	None	17.4 ± 1.9	1969 ± 65	—
	Thiamin pyrophosphate	13.5 ± 1.0	1373 ± 26	K_3 20.8 ± 1.4
				K_3 7.2 ± 1.3*
PDH 25	None	25.4 ± 2.5	8656 ± 261	—
	Sodium pyrophosphate	15.8 ± 1.4	5190 ± 113	K_3 146 ± 9.3
PDH 28	None	13.9 ± 4.5	1573 ± 117	
	Adenylyl imidodiphosphate	59.5 ± 8.6	1017 ± 63	K_1 39.5 ± 13
				K_2 351 ± 151
PDH 31	None	13.5 ± 1.1	3338 ± 67	K_1 28.4 ± 3.1
	ADP (0.5 mM)	144 ± 20	2055 ± 161	K_2 779 ± 218
	ADP (1.0 mM)	311 ± 49	1774 ± 195	
	ADP (2.0 mM)	592 ± 15	1227 ± 25	

Effects of thiamin pyrophosphate, sodium pyrophosphate, adenylyl imidodiphosphate, GTP and ADP on the activities of pyruvate dehydrogenase and its intrinsic kinase

As shown in Table 3, the K_m of pig heart pyruvate dehydrogenase for thiamin pyrophosphate was 0.76 μM. Sodium pyrophosphate, adenylyl imidodiphosphate, GTP and ADP (all at 0.5 mM) were competitive inhibitors of the dehydrogenase with respect to thiamin pyrophosphate. The calculated K_i values were as shown. In further experiments (not shown) with preparation PDH 28, which exhibited significant activity in the absence of added thiamin pyrophosphate (see Table 1), it was found that ATP (1 mM) added during the course of the reaction produced immediate inhibition of the dehydrogenase, which was partially reversed by adding thiamin pyrophosphate. This suggested the possibility that ATP may inhibit with respect to thiamin pyrophosphate, but this was difficult to test because ATP can phosphorylate and inactivate the dehydrogenase. This led to the use of adenylyl imidodiphosphate, an analogue of ATP that does not phosphorylate the dehydrogenase (experiments not shown). These findings are consistent with the possibility that the pyrophosphate moiety is involved in the binding of the thiamin pyrophosphate to the dehydrogenase (Morey & Juni, 1970).

Table 4 shows that the K_m for ATP of the intrinsic kinase of three pig heart pyruvate dehydrogenase preparations varied from 13.9 to 25.4 μM, very similar

to the value of approx. 20μM for bovine heart and kidney enzymes (Hucho *et al.*, 1972). Thiamin pyrophosphate and sodium pyrophosphate were uncompetitive inhibitors with respect to ATP. The K_i for inhibition of intrinsic kinase activity by thiamin pyrophosphate was at least one order of magnitude higher than its K_m with pyruvate dehydrogenase. Thiamin itself, at concentrations as high as 0.5mM, had no effect on kinase activity. Inhibition by ADP and adenylyl imidodiphosphate was mainly competitive with ATP, but each compound significantly decreased $V_{max.}$. The competitive K_i for ADP is somewhat lower than the range of values (100–300μM) for bovine heart and kidney enzymes (Hucho *et al.*, 1972). These findings suggest that thiamin pyrophosphate and sodium pyrophosphate inhibit pyruvate dehydrogenase kinase at site(s) other than the ATP-binding site, and that ADP and adenylyl imidodiphosphate may inhibit by binding both at the ATP site and at another site.

In experiments with partially purified rat heart pyruvate dehydrogenase the K_m for ATP was 27.4±4.2μM. Sodium pyrophosphate was an uncompetitive inhibitor (K_i 210±26μM).

Effects of pyruvate, glyoxylate, 3-hydroxypyruvate, 2-oxobutyrate and dichloroacetate on the activity of pyruvate dehydrogenase kinase in the presence or absence of thiamin pyrophosphate or sodium pyrophosphate

The K_m of pig heart pyruvate dehydrogenase (PDH 31) for pyruvate was 35.8±6.8μM (with 1mM-thiamin pyrophosphate, 0.5mM-NAD^+, 0.1mM-CoA). The enzyme also oxidized 3-hydroxypyruvate (the rate with 1mM-hydroxypyruvate was 5.4% of that with 1mM- pyruvate)and 2-oxobutyrate (the rate with 1mM-2-oxobutyrate was 47% of that with 1mM-pyruvate). The initial rate of oxidation of 1mM-pyruvate was decreased to 36% of the control by 1mM-3-hydroxypyruvate. Glyoxylate (1mM) was

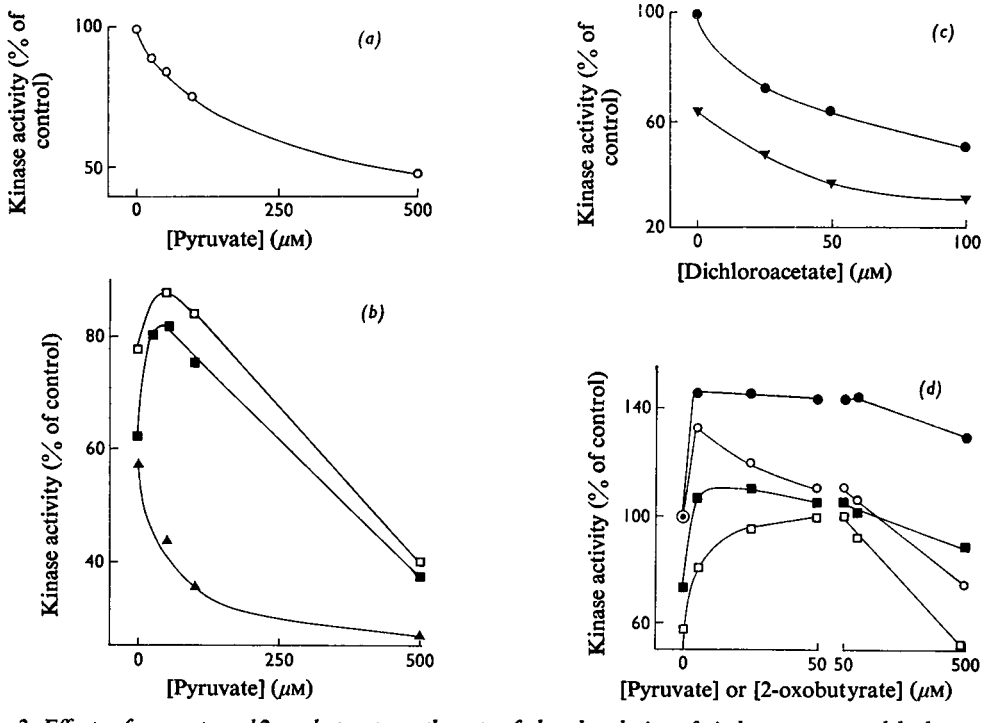


Fig. 3. *Effects of pyruvate and 2-oxobutyrate on the rate of phosphorylation of pig heart pyruvate dehydrogenase*

(*a*), (*b*), (*c*), Preparation PDH 29; (*d*) preparation PDH 30. Pyruvate dehydrogenase was preincubated for 10min at 30°C in phosphate–mercaptoethanol with additions specified below. The kinase reaction was initiated by addition of 0.1mM-[γ-^{32}P]ATP and 2mM-$MgCl_2$, terminated and protein-bound radioactivity was assayed after 2min. (*a*) No further addition other than pyruvate at the concn. shown; (*b*) □, 2μM-thiamin pyrophosphate; ■, 10μM-thiamin pyrophosphate; ▲, 50μM-sodium pyrophosphate. (*c*) ●, No further addition other than dichloroacetate at the concentration shown; ▼, 10μM-thiamin pyrophosphate. (*d*) ○, Varying pyruvate at concn. shown; □, 10μM-thiamin pyrophosphate, varying pyruvate concn.; ●, varying 2-oxobutyrate at concn. shown; ■, 10μM-thiamin pyrophosphate, varying 2-oxobutyrate concn. Each point is the mean of three observations. Ordinates show kinase activity as percentage of control, which is the activity with ATP and $MgCl_2$ only.

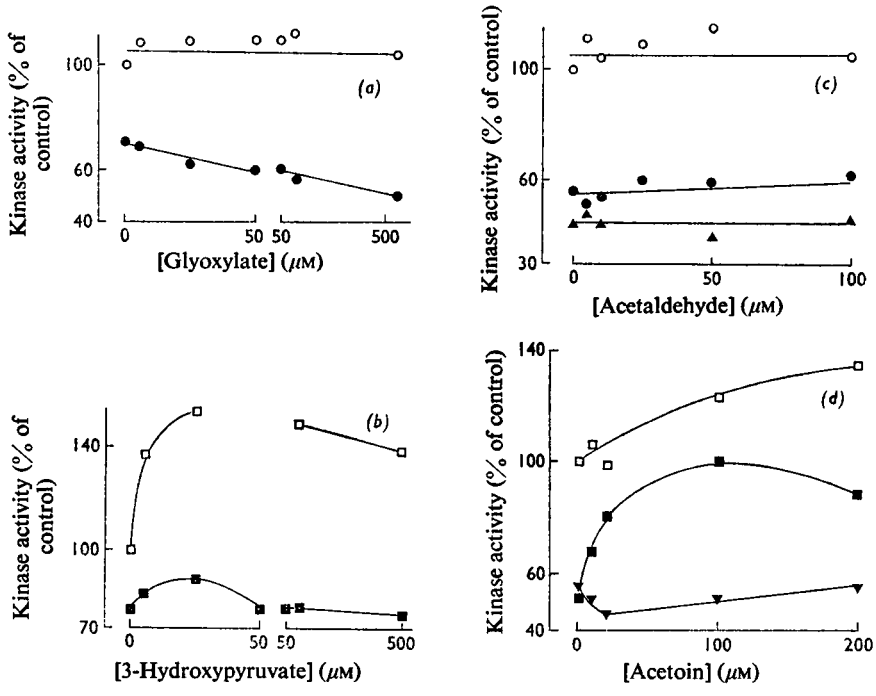


Fig. 4. *Effects of glyoxylate, 3-hydroxypyruvate, acetaldehyde and acetoin on the rate of phosphorylation of pig heart pyruvate dehydrogenase*

(*a*) and (*b*), Preparation PDH 30; (*c*) and (*d*), preparation PDH 31. The conditions of incubation were as given in Fig. 3. (*a*) ○, No further addition other than glyoxylate at concn. shown; ●, 10 μM-thiamin pyrophosphate and glyoxylate at concn. shown. (*b*) □, No further addition other than hydroxypyruvate at concn. shown; ■, 10 μM-thiamin pyrophosphate and hydroxypyruvate at concn. shown. (*c*) ○, No further addition other than acetaldehyde at concn. shown; ●, 10 μM-thiamin pyrophosphate and acetaldehyde at concn. shown; ▲, 25 μM-sodium pyrophosphate and acetaldehyde at concn. shown. (*d*) □, No further addition other than acetoin at concn. shown; ■, 10 μM-thiamin pyrophosphate and acetoin at concn. shown; ▼, 25 μM-sodium pyrophosphate and acetoin at concn. shown. Each point is the mean of three observations. Ordinates show kinase activity as percentage of control, which is the activity with ATP and $MgCl_2$ only.

not oxidized by pyruvate dehydrogenase and it decreased the rate of oxidation of 1 mM-pyruvate to 57% of the control.

Fig. 3 shows the effects of pyruvate on the activity of the intrinsic kinase of pyruvate dehydrogenase (PDH 29), which contained little if any endogenous thiamin pyrophosphate (see Table 1). In the absence of added thiamin pyrophosphate (Fig. 3*a*) all concentrations of pyruvate (ranging from 25 to 500 μM) were inhibitory. The inhibition was uncompetitive (not shown). The K_i with 0.25 mM-pyruvate and various [ATP] (for concentrations see legend to Table 4) was 0.85±0.29 mM. In another experiment with fixed ATP (100 μM) and varying [pyruvate] the K_i was 0.42±0.07 mM. These may be compared with values of 0.08–0.3 mM for bovine heart and of 0.9–2 mM for bovine kidney pyruvate dehydrogenase (Hucho *et al.*, 1972). In the presence of thiamin pyrophosphate (2 or 10 μM) low concentrations of pyruvate (25–100 μM) activated the pyruvate dehydrogenase kinase reaction, whereas a higher concentration (500 μM) was inhibitory (Fig. 3*b*). With preparation PDH 30, which contained more endogenous thiamin pyrophosphate, activation of the pyruvate dehydrogenase kinase reaction by low concentrations of pyruvate was seen in the absence and in the presence of added thiamin pyrophosphate (Fig. 3*d*). Activation of the kinase reaction by pyruvate in the presence of thiamine pyrophosphate was also seen with preparation PDH 31 (Fig. 6). Fig. 3(*b*) shows also that no activation of the kinase reaction was seen with pyruvate when sodium pyrophosphate was substituted for thiamin pyrophosphate in experiments with preparation PDH 29. Sodium dichloroacetate (an uncompetitive inhibitor of pyruvate dehydrogenase kinase, which unlike pyruvate is not metabolized) was inhibitory in the presence or absence of added thiamin pyrophosphate (Fig. 3*c*). Fig. (3*d*) also shows that 2-oxobutyrate activates the kinase reaction in preparation PDH 30 at concentrations from 5 to

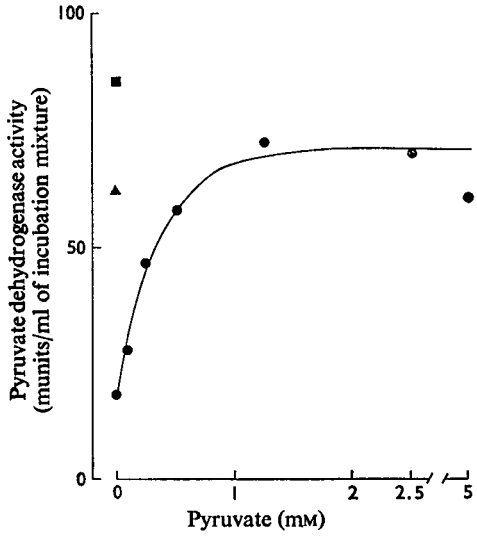


Fig. 5. *Effect of pyruvate concentration on the activity of pyruvate dehydrogenase in rat heart mitochondria*

Mitochondria were prepared from rat heart (see the Experimental section) and incubated for 5min at 30°C in 0.12M-KCl, 2mM-Tris–HCl, 1mM-EGTA, 5mM-potassium phosphate, pH7.4, with additions given below. Mitochondria were then separated by centrifugation, the supernatant was discarded and the mitochondria were frozen. Pyruvate dehydrogenase was then extracted and assayed as described in the Experimental section. ●, Mitochondria incubated with 0.5mM-L-malate and 5mM-2-oxoglutarate and pyruvate at concn. shown; ▲, mitochondria incubated without substrate; ■, total pyruvate dehydrogenase activity measured after incubation with pyruvate dehydrogenase phosphate phosphatase.

100μM in the presence or absence of thiamin pyrophosphate. As shown in Fig. 4(*b*), 3-hydroxypyruvate activated the kinase reaction with preparation PDH 30 in the absence of thiamin pyrophosphate at all concentrations tested (range 5–500μM). In the presence of 10μM-thiamin pyrophosphate, 3-hydroxypyruvate had little effect on kinase activity. With the same preparation glyoxylate (5–500μM) had little effect on kinase activity in the absence of thiamin pyrophosphate and was weakly inhibitory in the presence of the coenzyme (Fig. 4*a*).

Phosphorylation of partially purified rat heart pyruvate dehydrogenase was inhibited uncompetitively by pyruvate; the K_i (with 0.5mM-pyruvate and ATP concentrations as given at the head of Table 4) was 0.95±0.07mM.

As shown in Fig. 5 pyruvate may promote dephosphorylation and activation of pyruvate dehydrogenase in rat heart mitochondria. In rat heart mitochondria incubated without substrates, approx. 70% of pyruvate dehydrogenase is in the active form. Incubation with 5mM-2-oxoglutarate+0.5mM-L-malate increased the concentration of ATP from 0.7 to 4.6nmol/mg of mitochondrial protein and the activity of pyruvate dehydrogenase was decreased to approx. 30% of the no-substrate control. When pyruvate was added in addition to oxoglutarate and malate complete reactivation of the dehydrogenase to the value in the no-substrate control was seen when the initial pyruvate concentration in the medium was above 1mM (Fig. 5). The initial concentration of pyruvate in the medium required for half-maximum reactivation was approx. 0.25mM. If pyruvate is concentrated by rat heart mitochondria, as in liver mitochondria (Halestrap & Denton, 1974), then these data might suggest that the concentration of pyruvate in mitochondria required to inhibit the kinase reaction is similar to that required with isolated pyruvate dehydrogenase. There was no evidence in these experiments for activation of pyruvate dehydrogenase kinase in mitochondria by pyruvate.

Mechanism of activation of pyruvate dehydrogenase kinase by pyruvate in the presence of thiamin pyrophosphate

As described above, low concentrations of pyruvate activate pig heart pyruvate dehydrogenase kinase in the presence of thiamin pyrophosphate, but not in its absence or when sodium pyrophosphate is substituted for thiamin pyrophosphate. This suggests that the products of pyruvate decarboxylation might be responsible for activation of the kinase reaction by pyruvate in the presence of thiamin pyrophosphate. As shown in Scheme 1 (see the introduction) these products may include carbanion and protonated forms of 2-α-hydroxyethylthiamin pyrophosphate, acetoin and acetolactate. It is known that glyoxylate (which did not activate the kinase reaction)

Table 5. *Decarboxylation of pyruvate by pig heart pyruvate dehydrogenase in the absence of CoA and NAD$^+$*

Decarboxylation was measured by the incorporation of ^{14}C from [1-^{14}C]pyruvate into CO_2 (see the Experimental section). Concentrations were: pyruvate, 100μM; $MgCl_2$, 0.25mM; pyruvate dehydrogenase and thiamin pyrophosphate as indicated.

Preparation	Thiamin pyrophosphate	Pyruvate decarboxylation (nmol/ml) after: 2min	5min
PDH 30 (2.5 units/ml)	—	14	7
	10μM	44	68
PDH 31 (6 units/ml)	—	—	17
	10μM	51	83
PDH 31 (1.5 units/ml)	—	—	2.5
	10μM	—	24.7

can give rise to 2-α-hydroxymethylthiamin pyrophosphate (Kohlaw *et al.*, 1965), which is not further metabolized in the presence of CoA and NAD^+. It is known also that 3-hydroxypyruvate and 2-oxobutyrate, which showed some activation of the kinase, are decarboxylated to 2-α-β-dihydroxyethylthiamine pyrophosphate (Holzer *et al.*, 1962) and 2-α-hydroxypropionylthiamine pyrophosphate respectively; these products are further metabolized in the presence of CoA and NAD^+.

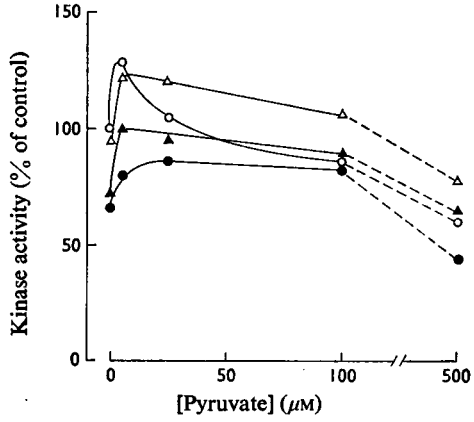


Fig. 6. *Effect of pyruvate on the rate of phosphorylation of pig heart pyruvate dehydrogenase (PDH 31)*

Pyruvate dehydrogenase was incubated for 2min at 30°C in phosphate–mercaptoethanol containing 0.1mM-ATP and 2mM-$MgCl_2$, and the reaction was terminated and protein-bound phosphate assayed (see the Experimental section). ○, Preincubation for 10min with pyruvate at concn. shown before initiation of kinase reaction with ATP and $MgCl_2$. ●, Preincubation for 10min with 10μM-thiamin pyrophosphate and pyruvate at concn. shown. △, Incubation with pyruvate at concn. shown without preincubation. ▲, Incubation with 10μM-thiamin pyrophosphate and pyruvate at concn. shown without preincubation. There were three observations at each point. Ordinate shows kinase activity as percentage of control, which is the activity with ATP and $MgCl_2$ only.

As shown in Table 5 pig heart pyruvate dehydrogenase preparations PDH 30 and 31 catalysed substantial decarboxylation of pyruvate in the presence of thiamin pyrophosphate. Decarboxylation was substantially less in the absence of added thiamin pyrophosphate. In the standard kinase assay pyruvate dehydrogenase was preincubated for 10min at 30°C with additions such as pyruvate and thiamin pyrophosphate before initiation of the kinase reaction with ATP. Hence activation of the kinase by low concentrations of pyruvate in the presence of thiamin pyrophosphate could be caused by a product of pyruvate decarboxylation and not by pyruvate itself. Fig. 6 shows the effect of preincubation on the extent of activation of the kinase reaction by pyruvate in the presence or absence of thiamin pyrophosphate (preparation PDH 31). Preincubation had little effect on the activation of the kinase reaction by low concentrations of pyruvate. The extent of decarboxylation of pyruvate under the conditions used in Fig. 6 was measured with [1-^{14}C]pyruvate. The results are shown in Table 6. Substantially greater decarboxylation of pyruvate was found with preincubation, whereas preincubation had little effect on the extent of activation of the kinase by pyruvate.

The products of decarboxylation of pyruvate by pyruvate dehydrogenase in the absence of NAD^+ and CoA have been listed above and are shown in Scheme 1. Acetoin and acetaldehyde (which has been suggested as an intermediate in acetoin formation) were the only products available. Fig. 4(*d*) shows that acetoin activated the pyruvate dehydrogenase kinase reaction (preparation PDH 31). The activation was more pronounced with 10μM-thiamin pyrophosphate than in the absence of added coenzyme. The minimum concentration required for activation was between

Table 6. *Decarboxylation of pyruvate by pig heart pyruvate dehydrogenase (PDH 31) under conditions of kinase assay in Fig. 6*

Decarboxylation was measured by the incorporation of ^{14}C from [1-^{14}C]pyruvate into CO_2 (see the Experimental section). Concentrations were: pyruvate and thiamin pyrophosphate as indicated; $MgCl_2$, 2mM; pyruvate dehydrogenase (PDH 31), 2.9 units/ml; during incubation, ATP, 100μM. Incubation was for 2min.

Preincubation (min)	Pyruvate (nmol/ml)	Thiamin pyrophosphate (μM)	Pyruvate decarboxylation (nmol/ml)	Maximum acetoin concn. (nmol/ml)
—	100	—	7	3.5
—	100	10	54	27
—	25	—	5	2.5
—	25	10	17	8.5
10	100	—	20	10
10	100	10	84	42
10	25	—	15	7.5
10	25	10	20	10

2 and 10μM and the concentration required for half-maximum activation was approx. 15μM. With 25μM-sodium pyrophosphate in place of thiamin pyrophosphate acetoin did not activate the pyruvate dehydrogenase kinase reaction (Fig. 4*d*). Acetaldehyde (which has been suggested as an intermediate in acetoin formation) had no significant effect on kinase activity with or without addition of thiamin pyrophosphate or sodium pyrophosphate (Fig. 4*c*). Table 6 shows the maximum acetoin concentration likely to be present during the kinase assays shown in Fig. 6, assuming that all of the pyruvate decarboxylated is converted into acetoin. These data suggest that acetoin formed by decarboxylation may contribute to the activation of the kinase reaction by low concentrations of pyruvate in the presence of thiamin pyrophosphate. However, it is difficult to ascribe the activation by pyruvate solely to acetoin.

Acetoin and acetaldehyde did not reduce NAD^+ in the presence of CoA (0.1mM), NAD^+ (0.5mM), thiamin pyrophosphate (1mM) and PDH 31 (15munits per cuvette). Acetoin (2mM) was a competitive inhibitor of pyruvate oxidation and the K_i was 71.2μM. Acetaldehyde (2mM) was an uncompetitive inhibitor of pyruvate oxidation and the K_i was 1.3mM. These observations may suggest that acetoin either cannot give rise to the carbanion of 2-α-hydroxyethylthiamine pyrophosphate or prevents further metabolism of the carbanion, and that acetoin either binds in the vicinity of the catalytic site for pyruvate or prevents the formation of 2-α-hydroxyethylthiamine pyrophosphate from pyruvate or its further metabolism. Acetaldehyde would appear to inhibit pyruvate dehydrogenase by another mechanism. This might involve stimulation of acetoin formation (see Scheme 1). No production of acetaldehyde by pyruvate dehydrogenase was detected on incubation of preparation PDH 32 (1.3 units/ml) with pyruvate (1mM), thiamin pyrophosphate (32μM), $MgCl_2$ (0.16mM), NADH (50–80μM) and ethanol dehydrogenase (13 units/ml). If acetaldehyde is an intermediate in acetoin formation it is not released in detectable amount during pyruvate decarboxylation.

The oxygen consumption of rat heart mitochondria incubated with 0.5mM-L-malate and 1μmol of ADP was not supported by 5mM-acetoin or 5mM-acetaldehyde. With 0.5mM-pyruvate, 0.5mM-L-malate and 1μmol of ADP, the rate of oxygen consumption was decreased to 52% of the control by 5mM-acetoin and to 15% of the control with 5mM-acetaldehyde.

Effect of EGTA, CaEGTA buffers, calcium depletion, $CaCl_2$ and $MgCl_2$ on the activity of pyruvate dehydrogenase kinase

The activity of pig heart pyruvate dehydrogenase kinase was depressed by CaEGTA buffers calculated to give a free Ca^{2+} concentration of 7.5μM (Table 7, Expts. 2 and 3). Kinase activity was increased by EGTA in some experiments (e.g. Expt. 3, Table 7) but not in others. It is suggested that some reagents may contain sufficient Ca^{2+} to inhibit the kinase and when this is so addition of EGTA leads to activation of the kinase. Fig. 7 shows the effect of various concentrations of Ca^{2+} (achieved by use of CaEGTA buffers) on the activity of pig heart pyruvate dehydrogenase kinase. Significant inhibition was seen over the range of Ca^{2+} concentration from 10nM to

Table 7. *Effects of EGTA, CaEGTA buffers, calcium depletion and $CaCl_2$ on activity of pyruvate dehydrogenase kinase*

Pyruvate dehydrogenase kinase was assayed by radioassay (see the Experimental section). Concentrations were: [γ-^{32}P]ATP, 100μM; pig heart pyruvate dehydrogenase: Expts. 1 and 2, preparation PDH 26, 1.3 units/ml; Expt. 3, preparation PDH 25, 4 units/ml; other concentrations were as shown. The concentrations of Ca^{2+}, Mg^{2+}, $ATPMg^{2-}$ and $ATPCa^{2-}$ were calculated from the dissociation constants of calcium and magnesium salts of EGTA, ATP, orthophosphate and sulphate (see the Experimental section). In Expt. 1 with calcium-depleted reagents Spectrograde $MgSO_4$ and $CaCl_2$ were used (see the Experimental section). Spectrograde $MgSO_4$ was used in Expt. 2. Analytical-reagent grade $MgCl_2$ was used instead of $MgSO_4$ in Expt. 3. Results are means±S.E.M. for the numbers of observations given in parentheses.

	Additions			Calculated concentrations				
Expt. no.	EGTA (mM)	$CaCl_2$ (mM)	$MgSO_4$ (mM)	Ca^{2+} (μM)	Mg^{2+} (mM)	$ATPMg^{2-}$ (μM)	$ATPCa^{2-}$ (μM)	Pyruvate dehydrogenase kinase activity (c.p.m./2min per 30μl of incubation mixture)
1.	—	—	5	—	0.72	93	—	946± 70 (3)
		0.03	5	6.6	0.72	93	0.3	896± 73 (3)
		0.30	5	66	0.73	90	3.2	762± 85 (3)
		0.60	5	134	0.74	88	6.3	698± 18 (3)
2.	—	—	10	Not known	1.5	97	—	653± 40 (4)
	10	—	10	—	1.4	96	—	654± 8 (4)
	10	9.75	10	7.5	1.5	96	0.19	383± 11 (4)
3.	—	—	2	Not known	0.29	85	—	3122±104 (4)
	10	—	2	—	0.27	84	—	4069±203 (4)
	10	9.75	2	7.3	0.29	84	0.83	2746± 51 (4)

10μM. Table 8 shows effects of Mg^{2+} concentration (0.12–0.14 and 12.6–15.3mM) and of Ca^{2+} (7 or 12μM; achieved by CaEGTA buffers) at low and high Mg^{2+} concentrations. High Mg^{2+} inhibited kinase activity by lowering $V_{max.}$; the K_m for ATP was not changed significantly. The value of $V_{max.}$ was approximately halved by Ca^{2+} at low [Mg^{2+}], but was only decreased by about 20% at high [Mg^{2+}]. The K_m for ATP was not changed significantly by Ca^{2+}. When the kinase was inhibited by the presence of Ca^{2+} (in Expts. 2 and 3 in Table 7, and in Table 8) [$ATPCa^{2-}$] was calculated to be no more than 2% of [$ATPMg^{2-}$]. These findings would suggest that Mg^{2+} and Ca^{2+} can inhibit kinase activity, and that the effects of Ca^{2+} are unlikely to be due to $ATPCa^{2-}$ being a poor substrate for the kinase by comparison with ATP Mg^{2-}.

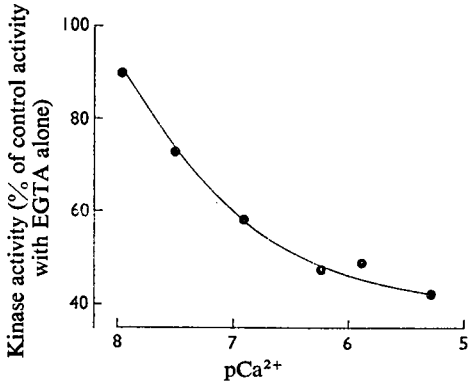


Fig. 7. *Effect of Ca^{2+} concentration in CaEGTA buffers on the rate of phosphorylation of pig heart pyruvate dehydrogenase (preparation PDH 26)*

Pyruvate dehydrogenase was preincubated for 10min at 30°C in phosphate–mercaptoethanol containing 10mM-EGTA and $CaCl_2$ at concentrations calculated to give Ca^{2+} concentrations as shown (see the Experimental section). [γ-^{32}P]ATP was then added to 0.1mM and $MgCl_2$ to 2mM, and the reaction was terminated and protein bound radioactivity assayed after 2min of incubation. There were three observations at each point. $pCa^{2+} = -\log [Ca^{2+}]$ where [Ca^{2+}] is in mol/l.

Expt. 1 in Table 7 shows that the kinase was inhibited by $CaCl_2$ in the absence of EGTA when reagents were depleted of calcium by use of Chelex 100 resin (see the Experimental section). The concentrations of Ca^{2+} required for inhibition were much higher than with CaEGTA buffers. A similar discrepancy has been noted in the activation of pyruvate dehydrogenase phosphate phosphatase by $CaCl_2$ and CaEGTA (Randle *et al.*, 1974). The reason for this discrepancy is not yet known. In kinase assays with $CaCl_2$ (as opposed to CaEGTA) the concentration of $ATPCa^{2-}$ was 3.6 and 7.2% of that of $ATPMg^{2-}$ at 0.3 and 0.6mM-$CaCl_2$.

Effects of various carboxylic acids on the activity of pig heart pyruvate dehydrogenase kinase

Pig heart pyruvate dehydrogenase kinase activity in preparation PDH 27 was inhibited by acetate, propionate, *n*-butyrate, isobutyrate and *n*-valerate (Fig. 8) and by 3-fluoropyruvate, 2-oxo-3-methylpentanoate and 2-oxo-4-methylpentanoate (Fig. 9). Kinase activity was increased by 3-bromopyruvate and to a lesser extent by 2-oxo-3-methylbutyrate. The dehydrogenase preparation used in the studies in Fig. 9 (PDH 28) contained a significant amount of endogenous thiamin pyrophosphate (see Table 1). As described elsewhere (Whitehouse *et al.*, 1974),

Table 8. *Effects of EGTA, CaEGTA and $MgCl_2$ on activity of pyruvate dehydrogenase kinase and K_m for ATP*

Pyruvate dehydrogenase kinase was assayed by radioassay (see the Experimental section). Concentrations were: EGTA, 10mM; [γ-^{32}P]ATP, 20, 30, 50, 100, 200μM; pig heart pyruvate dehydrogenase, preparation PDH 26, 1.7 units/ml. The concentrations of Ca^{2+}, Mg^{2+}, $ATPMg^{2-}$ and $ATPCa^{2-}$ were calculated as described in Table 7. There were four observations at each ATP concentration; results are means±S.E.M. Calculated concentrations are given for highest and lowest ATP concentrations used.

Concentrations of additions			Calculated concentrations				Kinetic constants	
Total ATP (μM)	$CaCl_2$ (mM)	$MgCl_2$ (mM)	Ca^{2+} (μM)	Mg^{2+} (mM)	$ATPMg^{2-}$ (μM)	$ATPCa^{2-}$ (μM)	K_m of ATP (μM)	$V_{max.}$ (c.p.m./2min per 30μl of incubation mixture)
20	—	1	—	0.14	14.4	—		
200	—	1	—	0.12	139	—	34.8±7.7	3330±251
20	—	32	—	12.7	19.9	—		
200	—	32	—	12.6	199	—	22.2±4.9	1752±111
20	9.75	1	7	0.15	15	0.29		
200	9.75	1	7	0.13	139	3.14	28.4±5.5	1557± 96
20	9.75	32	12	15.4	19.9	0.006		
200	9.75	32	12	15.3	199	0.06	17.9±6.2	1360±119

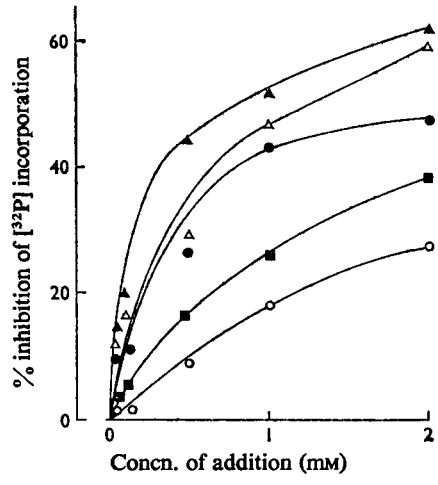


Fig. 8. *Effects of acetate, propionate, n-butyrate, isobutyrate and n-valerate on rate of phosphorylation of pig heart pyruvate dehydrogenase (preparation PDH 27)*

Pyruvate dehydrogenase was preincubated for 10min, at 10°C in phosphate–mercaptoethanol with the additions at the concn. shown. [γ-^{32}P]ATP (to 0.1mM) and $MgCl_2$ (to 2mM) were then added, and the reaction was terminated and protein-bound radioactivity assayed after 2min of incubation. There were three observations at each point. ○, Potassium acetate; ▲, potassium propionate; ●, potassium *n*-butyrate; △, potassium isobutyrate; ■, potassium *n*-valerate.

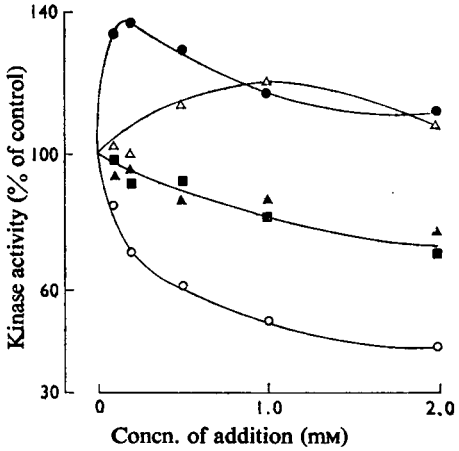


Fig. 9. *Effects of 3-fluoropyruvate, 3-bromopyruvate, 2-oxo-3-methylbutyrate, 2-oxo-3-methylpentanoate and 2-oxo-4-methylpentanoate on the rate of phosphorylation of pig heart pyruvate dehydrogenase (preparation PDH 28)*

The conditions of preincubation and incubation were as in Fig. 8. ○, 3-Fluoropyruvate; ●, 3-bromopyruvate; △, 2-oxo-3-methylbutyrate; ▲, 2-oxo-3-methylpentanoate; ■, 2-oxo-4-methylpentanoate. Control was incubated with ATP and $MgCl_2$ only. There were three observations at each point.

pyruvate dehydrogenase kinase is also inhibited by monochloroacetate, dichloroacetate, difluoroacetate, trichloroacetate, 2-chloropropionate, 2,2′-dichloropropionate and 3-chloropropionate.

Effects of miscellaneous compounds on the activity of pig heart pyruvate dehydrogenase kinase

There was no significant activation or inhibition of kinase activity by the following compounds at the concentrations shown: phenylpyruvate, *p*-hydroxyphenylpyruvate, α-cyano-4-hydroxycinnamate, L-lactate, glycellate, citrate, palmitoyl-L-carnitine (all at 0.5mM), malonate, succinate, L-malate, itaconate, L-carnitine and acetyl-L-carnitine (all at 1mM), and L-alanine (2mM). Kinase activity was inhibited by approx. 20% by pent-4-enoate (0.5mM).

Conclusions

Ligand-binding sites

Although binding studies are required to show the number and subunit location of binding sites involved in the regulation of pyruvate dehydrogenase kinase, the results of the present study suggest some general conclusions. There was a substantial competitive element (against ATP) in the inhibition of the kinase reaction by ADP and adenylyl imidodiphosphate, and these compounds may bind to the substrate site for ATP. Binding at this site may involve the adenosine moiety, as other pyrophosphates were uncompetitive inhibitors of the kinase reaction and there was an additional non-competitive element to the inhibition by ADP and adenylyl imidodiphosphate.

Sodium pyrophosphate, adenylyl imidodiphosphate and ADP were competitive inhibitors (against thiamin pyrophosphate) of the pyruvate dehydrogenase reaction; these compounds may bind to the coenzyme site through pyrophosphate or imidodiphosphate moieties. Thiamin pyrophosphate and sodium pyrophosphate were uncompetitive inhibitors of the kinase reaction and there was a non-competitive element in the inhibition by adenylyl imidodiphosphate and ADP. These effects on the kinase reaction could involve binding to a common site through pyrophosphate (or imidodiphosphate) moieties. Whether this is the coenzyme binding site or some additional regulator site is not known.

There are multiple effects of Mg^{2+} and of Ca^{2+} in the reactions of the pyruvate dehydrogenase complex. Mg^{2+} is required for the pyruvate dehydrogenase reaction and may be involved in the binding of thiamin pyrophosphate. Addition of Ca^{2+} or its removal with EGTA had no detectable effect on the activity of pyruvate dehydrogenase (not shown). The kinase reaction was inhibited by either Ca^{2+} or Mg^{2+} and the inhibition was non-competitive (or possibly uncompetitive) against ATP. The phosphatase reaction, on the other hand, requires Mg^{2+}

and when Mg^{2+} is present Ca^{2+} induces further activation by lowering the K_m for Mg^{2+} and for pyruvate dehydrogenase phosphate (Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974). These results may suggest that there are separate binding sites for Mg^{2+} and Ca^{2+}. One possibility is that Mg^{2+} may inhibit the kinase reaction by inducing a conformational charge in the complex and that Ca^{2+} may facilitate the action of Mg^{2+}. In the phosphatase reaction Ca^{2+} may facilitate binding of the phosphatase, as suggested by Pettit *et al.* (1972), and the action of Mg^{2+}.

Pyruvate may bind and act at separate substrate and regulator sites. The K_i for pyruvate in the kinase reaction is high and some 20 or 30 times greater than its K_m in the dehydrogenase reaction. Hucho *et al.* (1972) found that pyruvate can inhibit phosphorylation of casein by purified pyruvate dehydrogenase kinase and suggested that there may be a binding site for pyruvate on the kinase. The kinase reaction is also inhibited by chloroacetates and chloropropionates (Whitehouse & Randle, 1973; Whitehouse *et al.*, 1974) and by a number of straight and branched short-chain fatty acids and some other oxo acids (present study). Whether these may act by binding to a regulator site for pyruvate is not known.

Regulation of pyruvate dehydrogenase kinase in vivo

Pyruvate increased the proportion of active (dephosphorylated) pyruvate dehydrogenase in experiments *in vitro* with perfused rat liver (Patzelt *et al.*, 1973) with epididymal fat-pads (Martin *et al.*, 1972) and with mitochondria from rat liver, heart or epididymal fat-cells (Portenhauser & Wieland, 1972; present study; Martin *et al.*, 1972). In fat-cell, rat heart or rat kidney mitochondria there was an inverse correlation between the concentration of ATP and the proportion of active pyruvate dehydrogenase (Martin *et al.*, 1972; Whitehouse *et al.*, 1974; present study). These studies have been interpreted as showing that pyruvate and a high ratio of [ADP]/[ATP] inhibit pyruvate dehydrogenase kinase *in vivo* and thus facilitate conversion of pyruvate dehydrogenase phosphate into active pyruvate dehydrogenase. The potential regulation of pyruvate dehydrogenase kinase and of pyruvate dehydrogenase phosphate phosphatase by intramitochondrial [Mg^{2+}] and [Ca^{2+}] has yet to be shown to operate *in vivo*.

Activation of pyruvate dehydrogenase kinase has not been described previously. It has been shown in the present study that low concentrations of pyruvate or acetoin can activate the kinase in the presence of thiamin pyrophosphate. The mechanism is not known but the possibilities may include reversal of thiamin pyrophosphate inhibition when pyruvate or acetoin is bound to the substrate site for pyruvate, or activation of the kinase reaction when acetoin is bound, or activation of the kinase reaction when thiamin pyrophosphate is converted into the carbanion of 2-α-hydroxyethylthiamin pyrophosphate. Activation of the kinase reaction by pyruvate in the presence of thiamin pyrophosphate has only been shown in the absence of CoA and NAD^+ (it has not been practicable to study the kinase reaction with the dehydrogenase reaction proceeding). Under these conditions metabolism of pyruvate by the normal dehydrogenase sequence forms only acetylhydrolipoate and the carbanion of 2-α-hydroxyethylthiamin pyrophosphate. Side reactions may lead to the formation of acetoin and acetolactate and the protonated form of 2-α-hydroxyethylthiamin pyrophosphate (see Scheme 1). Pyruvate dehydrogenase is subject to end-product inhibition by high ratios of [acetyl-CoA]/[CoA] and of [NADH]/[NAD^+]. The mechanism may involve accumulation of acetylhydrolipoate (see Scheme 1) (Garland & Randle, 1964*a*; Randle *et al.*, 1966; Tsai *et al.*, 1973). With conditions of end-product inhibition metabolism of pyruvate may be restricted to the side reactions mentioned above and it is possible that pyruvate might activate the kinase reaction under these conditions. The possibility is of relevance *in vivo* because the respiration of acetate, ketone bodies and long-chain fatty acids in the perfused rat heart increases the proportion of inactive pyruvate dehydrogenase phosphate (Wieland *et al.*, 1971; Whitehouse & Randle, 1973). These substrates may also favour end-product inhibition of the dehydrogenase because the tissue ratio of [acetyl-CoA]/[CoA] is markedly increased (Garland & Randle, 1964*a*,*b*). We have no definitive evidence on this point, as we have not been able to show with the purified dehydrogenase activation of the kinase reaction by pyruvate under conditions of end-product inhibition. The problem has been variable effects of acetyl-CoA, CoA, NADH and NAD^+ on the activity of the kinase. There are other possible mechanisms for the effects of respiration of fatty acids or ketone bodies on the phosphorylation of pyruvate dehydrogenase *in vivo*. For example, products of the respiration of fatty acids and ketone bodies could alter intramitochondrial concentrations of effectors of the kinase or phosphatase (i.e. pyruvate, ADP, ATP, Ca^{2+}, Mg^{2+}).

Two mechanisms may be involved in the regulation of pyruvate dehydrogenase, namely end-product inhibition and the phosphorylation–dephosphorylation cycle. It seems possible that one function of the inactivation of pyruvate dehydrogenase by phosphorylation is to prevent decarboxylation of pyruvate by the side reactions shown in Scheme 1 under conditions where substrate flow through the complete sequence is prevented by end-product inhibition. Pyruvate dehydrogenase phosphate is much less active in the decarboxylation reaction than pyruvate dehydrogenase (results not shown), in conformity with the observations that the kinase phosphorylates

the α-chain of the decarboxylase and specifically inhibits the decarboxylation of pyruvate (Roche & Reed, 1972).

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