Biochem. J. (1979) **181**, 427–433
Printed in Great Britain

Regulation of Kinase Reactions in Pig Heart Pyruvate Dehydrogenase Complex

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(*Received* 22 *December* 1978)

1. Pig heart pyruvate dehydrogenase complex is inactivated by phosphorylation ($MgATP^{2-}$) of an α-chain of the decarboxylase component. Three serine residues may be phosphorylated, one of which (site 1) is the major inactivating site. 2. The relative rates of phosphorylation are site 1>site 2>site 3. 3. The kinetics of the inactivating phosphorylation were investigated by measuring inactivation of the complex with $MgATP^{2-}$. The apparent K_m for the Mg complex of ATP was 25.5 μM; ADP was a competitive inhibitor (K_i 69.8 μM) and sodium pyruvate an uncompetitive inhibitor (K_i 2.8 mM). Inactivation was accelerated by increasing concentration ratios of NADH/NAD^+ and of acetyl-CoA/CoA. 4. The kinetics of additional phosphorylations (predominantly site 2 under these conditions) were investigated by measurement of ^{32}P incorporation into non-radioactive pyruvate dehydrogenase phosphate containing 3–6% of active complex, and assumed from parallel experiments with ^{32}P labelling to contain 91% of protein-bound phosphate in site 1 and 9% in site 2. 5. The apparent K_m for the Mg complex of ATP was 10.1 μM; ADP was a competitive inhibitor (K_i 31.5 μM) and sodium pyruvate an uncompetitive inhibitor (K_i 1.1 mM). 6. Incorporation was accelerated by increasing concentration ratios of NADH/NAD^+ and of acetyl-CoA/CoA, although it was less marked at the highest ratios.

The pyruvate dehydrogenase complex of animal tissues (EC 1.2.4.1+EC 2.3.1.12+EC 1.6.4.3.) is phosphorylated and inactivated by an intrinsic kinase (Linn *et al.*, 1969*a*,*b*.). In complexes from bovine kidney or heart, or pig heart, sequence analyses of tryptic phosphopeptides have shown that inactivation is correlated with phosphorylation of one specific serine residue of the α-chain of the decarboxylase (EC 1.2.4.1) (Yeaman *et al.*, 1978; the preceding paper, Sugden *et al.*, 1979). The decarboxylase is a tetramer ($\alpha_2\beta_2$) (Barrera *et al.*, 1972), and, in the pig heart complex, inactivation may be represented by the equation (Sugden & Randle, 1978):

$$(\alpha_2\beta_2)_n + n\text{MgATP} \rightarrow (\alpha P \cdot \alpha\beta_2)_n + n\text{MgADP} \quad (1)$$

There is evidence for a similar stoicheiometry of inactivation by phosphorylation in bovine kidney and heart complexes (Yeaman *et al.*, 1978). Phosphorylation continues after inactivation is complete and results in phosphorylation of two other serine residues (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). These further phosphorylations may be represented by the equation:

$$(\alpha P \cdot \alpha\beta_2)_n + 2n\text{MgATP} \rightarrow (\alpha_2 P_3 \beta_2)_n + 2n\text{MgADP} \quad (2)$$

The inactivating phosphorylation (eqn. 1) can be monitored by measuring the disappearance of the active form of pyruvate dehydrogenase complex. The additional phosphorylations (eqn. 2) can be monitored by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into pyruvate dehydrogenase phosphate prepared by titration to inactivation with non-radioactive ATP (Sugden *et al.*, 1978). These methods have been used to investigate the influence of metabolite effectors on inactivating and additional phosphorylations in the pig heart complex. The effectors include ADP, pyruvate, acetyl-CoA, CoA, NADH and NAD^+ (Linn *et al.*, 1969*b*; Cooper *et al.*, 1974; Pettit *et al.*, 1975; Cooper *et al.*, 1975). These methods have also been used to investigate the relative rates of the inactivating and additional phosphorylations.

Experimental

Materials

The sources of biochemicals and radiochemicals were as given by Kerbey *et al.* (1976, 1977), Cooper *et al.* (1974) or Sugden *et al.* (1979).

Pyruvate dehydrogenase complex was purified from pig hearts. The poly(ethylene glycol)-fractionation procedure (Linn *et al.*, 1972) as modified for pig hearts by Cooper *et al.* (1974) was further modified as follows to increase the yield of complex. Dithiothreitol (2 mM) was used in place of 2-mercaptoethanol. After centrifugation of the heart-muscle homogenate (at 2075*g*), the supernatant was discarded. A total of eight re-extractions of this pellet in the Waring blender was found necessary to obtain maximum

yield of mitochondrial fraction. Dithiothreitol (0.1mM) was added to water used to wash the mitochondrial pellets, and to the 20mM-potassium phosphate (pH7) in which mitochondria were frozen and thawed. In later stages of the preparation, contaminating material was removed at pH6.25 instead of pH6.1. The frozen mitochondrial fraction could safely be stored at −60°C for up to 3 days. These modifications increased the yield of purified complex to 50–75 units per heart (approx. 0.5–0.75unit/g fresh wt. of heart muscle) (1 unit is 1μmol NADH formed/min at 30°C). Before the complex was used for kinase assays or for preparation of pyruvate dehydrogenase phosphate, 1ml samples were layered on to 6ml of 20mM-potassium phosphate/2mM-dithiothreitol/2% (w/v) sucrose, pH7, and centrifuged for 90min at 150000*g*. The pellets were taken up in phosphate/dithiothreitol and stored at −20°C. The ATPase activity of the preparations under the conditions of the pyruvate dehydrogenase kinase assays hydrolysed <0.15% of the ATP per min (method of Cooper *et al.*, 1974).

Pyruvate dehydrogenase phosphate ($\alpha P \cdot \alpha\beta_2$) was prepared with non-radioactive ATP by the method of Sugden *et al.* (1978). The preparations contained 3–6% of active complex. The preparations of pyruvate dehydrogenase phosphate were free of pyruvate dehydrogenase phosphate phosphatase [no formation of active complex on incubation for 10min at 30°C with $MgCl_2$ (added to 20mM) and $CaCl_2$ (added to 23μM)].

Methods

Assay of phosphorylations in pyruvate dehydrogenase complex. The rate of the inactivating phosphorylation (eqn. 1) was assayed at 30°C in 20mM-potassium phosphate/2mM-dithiothreitol/5mM-$MgCl_2$, pH7. The concentrations of ATP and of other additions are given in the text, the Tables or the Figures. The reaction was initiated by addition of pyruvate dehydrogenase complex to 2.5units/ml. The concentration of active complex was measured before, and 0.75min after, initiating the reaction, and the rate of the inactivating phosphorylation calculated from this difference (for progress curve, see the Results section).

The rate of additional phosphorylations (eq. 2) was assayed under the same general conditions with [γ-^{32}P]ATP (45μCi/μmol). The reaction was initiated by addition of pyruvate dehydrogenase phosphate ($\alpha P \cdot \alpha\beta_2$) (equivalent to 2.5 units of active complex/ml). The concentration of protein-bound ^{32}P was measured 5min after initiation of the reaction by the method of Corbin & Reimann (1974) (for progress curve, see the Results section).

Other assays. Pyruvate dehydrogenase complex was assayed spectrophotometrically by NADH formation (Cooper *et al.*, 1974). Pyruvate dehydrogenase phosphate ($\alpha P \cdot \alpha\beta_2$) was assayed spectrophotometrically after conversion into active complex by incubation with pig heart pyruvate dehydrogenase phosphate phosphatase (Severson *et al.*, 1974), by coupling to arylamine acetyltransferase (the preparations of phosphatase contain some lactate dehydrogenase, which interferes with the assay based on NADH formation).

The following solutions were standardized spectrophotometrically: ATP and ADP at 257nm in 1M-HCl; NADH at 340nm; NAD^+ as NADH after reduction with ethanol dehydrogenase (Estabrook & Maitra, 1962); CoA with 5,5'-dithiobis-(2-nitrobenzoate) (Srere *et al.*, 1963); acetyl-CoA with arylamine acetyltransferase, as described by Coore *et al.* (1971).

Paper electrophoresis of tryptic phosphopeptides. The three phosphorylation sites in fully phosphorylated pig heart pyruvate dehydrogenase complex ($\alpha_2P_3\beta_2$) are recovered, after tryptic digestion, in two phosphopeptides (Sugden *et al.*, 1979), as in bovine complexes (Davis *et al.*, 1977). Peptide A is Tyr-His-Gly-His-Ser(*P*)-Met-Ser-Asp-Pro-Gly-Val-Ser(*P*)-Tyr-Arg, and the inactivating site (site 1) is Ser-5 and the additional site (site 2) is Ser-12. Peptide B contains the site-3 phosphorylation at Ser-6 and is Tyr-Gly-Met-Gly-Thr-Ser-Val-Glu-Arg. In pyruvate dehydrogenase phosphate ($\alpha P \cdot \alpha\beta_2$), the major phosphopeptide (peptide A′) contains only the inactivating phosphoserine residue (Sugden *et al.*, 1979). The three peptides may be separated by electrophoresis on Whatman 3MM paper (57cm×23cm) in pH1.9 buffer [8% (v/v) acetic acid/2% (v/v) formic acid)] for 1h at 4kV in a Shandon Southern L24 high-voltage electrophoresis apparatus. The velocities relative to N^5-dinitrophenyl-lysine marker were 0.99 (peptide A), 1.40 (peptide A′), 0.61 (peptide B). The phosphopeptides were located by radioautography (Kodak Blue Brand BB5 X-ray film). The strips from the origin to 4cm beyond the fastest migrating phosphopeptide were excised, cut at 1cm intervals and assayed for ^{32}P by liquid-scintillation spectrometry. The profile of radioactivity was plotted on graph paper and the peaks excised and weighed. The distribution of ^{32}P between the three phosphoserine residues could then be calculated (site 1, peptide A′+0.5 peptide A; site 2, 0.5 peptide A; site 3, peptide B).

For tryptic digestion, phosphorylation was terminated and the complex precipitated by addition of trichloroacetic acid to 10% (w/v). Non-protein-bound ^{32}P was removed by repeated centrifugation and resuspension in 10% trichloroacetic acid (shown by assay of radioactivity in supernatant). The pellets were taken up in 8M-urea/2% (w/v) NH_4HCO_3, adjusted to pH7–8 with NH_3, and dialysed overnight against 2M-urea/2% NH_4HCO_3, pH8.3 (this dialysis

is not essential, provided that the pH is adjusted with NH_3). The protein solution was then incubated at 30°C with trypsin (added to 1mg/ml) for up to 6h (reaction was complete in 30min as judged by solubility of ^{32}P in 10% trichloroacetic acid). When samples required concentration before tryptic digestion, dialysis was against NH_4HCO_3 and the dialysis residue was freeze-dried and redissolved in 2M-urea/2% NH_4HCO_3, pH8.3.

Expression of results. Apparent K_m values were computed by the method of Jones (1970), and concentrations of $MgATP^{2-}$ were computed by using a dissociation constant of the Mg^{2+} complex of 5.24×10^{-5}M. The type of inhibition and K_i values were computed by using methods of analysis given by Cleland (1963*a*,*b*).

Results and Discussion

Progress curves of the phosphorylations

The time course of inactivation of the preparation of active complex used in the present study is shown in Fig. 1. Inactivation was first-order until approx. 80–86% of the complex had been inactivated (full curve not shown; see also Cooper *et al.*, 1974). The time for 50% inactivation was 0.96min, and at 0.75min (the time routinely used for rate measurements in studying activation and inhibition), inactivation was 39% complete. Incorporation of ^{32}P into active complex showed similar characteristics, being first-order until approx. 80–86% of the complex was inactivated. Fig. 2 shows this incorporation (in nmol) as a function of the units of active complex inactivated. The ratio (0.68 ± 0.03nmol/unit; mean±S.E.M.) was constant to 80–86% inactivation. The actual value for this ratio varied between different preparations over the range 0.40–0.68nmol/unit (not shown). The time course of incorporation of ^{32}P into pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$) is also shown in Fig. 1. The rate was much slower than the rate of inactivation (shown in the same Figure). The time for 50% incorporation was 12.7 min. The progress curve was approximately linear for 10min, and at 5min (routinely used for rate measurements), incorporation was 31% complete. Complete phosphorylation took approx. 60min (results not shown). Rates of

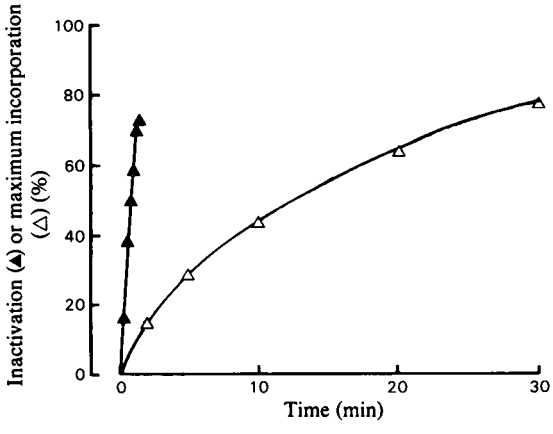

Fig. 1. *Rate of phosphorylations in pig heart pyruvate dehydrogenase complex*: (▲) *inactivating phosphorylation*; (△) *additional phosphorylations*

For details of enzyme preparations, assays and incubation medium, see the Experimental section. Concentrations: ATP (200μM); $MgCl_2$ (1mM); pyruvate dehydrogenase complex (▲) 2.5units/ml; pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$) (△) 2.5units/ml as active complex. Specific radioactivity of [γ-^{32}P]ATP, 49μCi/μmol. (▲) Samples taken for assay of active complex at times shown; maximum inactivation was 100%. (△) Samples taken for assay of protein-bound ^{32}P at times shown; maximum incorporation (at 60min) was 0.8nmol of *P*/unit. Each point represents the mean result for two experiments.

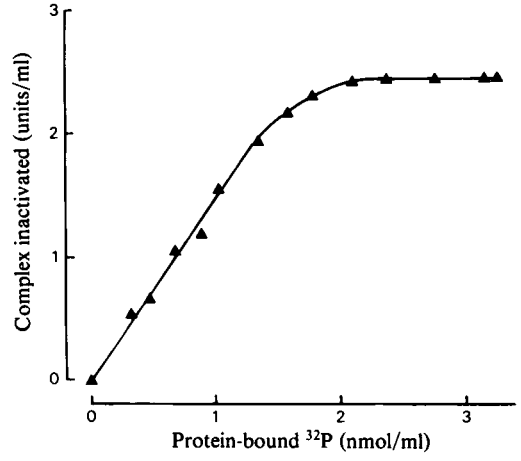

Fig. 2. *Incorporation of ^{32}P from [γ-^{32}P]ATP into pig heart pyruvate dehydrogenase complex during inactivation by phosphorylation*

For details of enzyme preparation, assays and incubation medium, see the Experimental section. Concentrations and specific radioactivity were as in Fig. 1. Samples were taken for assay of active complex and protein-bound ^{32}P at 20s, 40s and 1, 1.5, 2, 3, 4, 6, 10, 15, 30, 45 and 60min. Each point is the mean result for six experiments. The maximum incorporation of ^{32}P was 1.32±0.16nmol/unit of complex inactivated (mean±S.E.M.). The slope (up to 3min) was 0.684±0.03nmol of P/unit of complex inactivated (mean±S.E.M.) and the correlation coefficient (*r*) was 0.995.

inactivation and phosphorylation depended on the concentration of complex, which was fixed at 2.5 units/ml in all studies described.

The incorporation of phosphate into each of the three sites of phosphorylation was determined by high-voltage electrophoresis of the tryptic [^{32}P]phosphopeptides. Under the conditions used to study inactivation, incorporation of ^{32}P at 87% inactivation was 84±1.1%, site 1 (inactivating site); 15±0.64%, site 2 (same tryptic phosphopeptide as site 1); and 1±0.48%, site 3 (mean±S.E.M. for 12 observations on a single preparation). When active complex was titrated to inactivation with incremental additions of [γ-^{32}P]ATP (i.e. conditions used to prepare $\alpha P\cdot\alpha\beta_2$), 91±7.3% of protein-bound ^{32}P was in site 1 and 9±0.62% in site 2. In fully phosphorylated complex, protein-bound ^{32}P was equally distributed between the three sites. Fig. 3 shows the time course of incorporation of ^{32}P into sites 2 and 3 of pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$). This experiment was performed with a preparation of pyruvate dehydrogenase complex different from the one used in Figs. 1, 2, 4, 5, 6 and 7. In the first 5 min, the rate of phosphorylation of site 2 was three times that of site 3. Site 2 is also phosphorylated more rapidly than site 3 when active complex is phosphorylated with [γ-^{32}P]ATP (see above). Under the conditions used to study activation and inhibition of additional phosphorylations in $\alpha P\cdot\alpha\beta_2$, the major site of phosphorylation is therefore site 2 (see also Sugden *et al.*, 1979). The regulation of site-3 phosphorylation in the absence of site-2 phosphorylation has yet to be reliably measured.

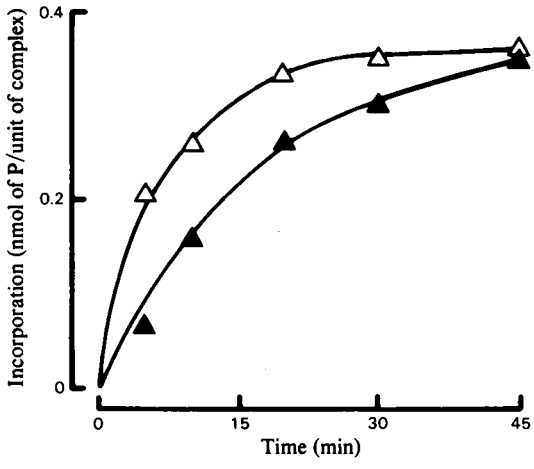

Fig. 3. *Time course of phosphorylation of sites 2 and 3 in pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$)*

Pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$), prepared with non-radioactive ATP as described in the Experimental section, was incubated at 30°C for the time shown with [γ-^{32}P]ATP (0.5 mM; 35 μCi/μmol) and 1 mM-$MgCl_2$. Duplicate samples (25 μl) were taken for assay of protein-bound ^{32}P (see the Experimental section), and the reaction was terminated by addition of trichloroacetic acid to 10% (w/v). Each time-period sample was a single tube containing, in 1.05 ml, 2.63 units of pyruvate dehydrogenase phosphate (expressed as active complex). The trichloroacetic acid-precipitate pellet was washed with 10% (w/v) trichloroacetic acid to remove acid-soluble radioactivity, dissolved in 8 M-urea/2%(w/v) NH_4HCO_3 and adjusted to pH 8.0–8.5 with NH_3. It was then diluted wih 3 vol. of 2% (w/v) NH_4HCO_3 and digested for 6 h with trypsin (1 mg/ml); the relative incorporations into sites 2 and 3 were then determined by high-voltage electrophoresis (see the Experimental section). In calculating relative incorporations, corrections were applied for the presence of 9% of active complex and for phosphorylation of 9% of site 2 with non-radioactive phosphate during the preparation of pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$). (△) Site-2 phosphorylation; (▲) site-3 phosphorylation.

Activation and inhibition

Apparent K_m^{ATP} and K_i^{ADP}. The apparent K_m for the Mg complexes of ATP for inactivation (25.5 μM) was significantly greater than the K_m for additional

Table 1. *Kinetic constants for inactivating and additional phosphorylations*

Details of enzyme preparations, incubation medium and assays are given in the Experimental section. The rate of the inactivating phosphorylation was determined by measuring the concentration of active pyruvate dehydrogenase complex at zero time (2.5 units/ml) and after 0.75 min. The rate of additional phosphorylations was determined by measuring incorporation of ^{32}P from [γ-^{32}P]ATP (49 μCi/μmol) into pyruvate dehydrogenase phosphate ($\alpha P\cdot\alpha\beta_2$) 5 min after initiating the reaction with pyruvate dehydrogenase phosphate. Concentrations were: ATP, 20, 25, 33.3, 50, 100 and 200 μM; ADP, 200 and 500 μM; sodium pyruvate, 1, 2, 5 and 10 mM (inactivating phosphorylation) and 2.5 and 5 mM (additional phosphorylations); $MgCl_2$, 5 mM. At least three measurements were made at each concentration of ATP. Results are means± S.E.M. K_1 is competitive inhibition. K_2 is uncompetitive inhibition. *$P<0.05$, †$P<0.001$ against inactivating phosphorylation.

	Phosphorylation	
	Inactivating	Additional
Mg complex of ATP, apparent K_m (μM)	25.5± 4.67	10.1±2.64*
ADP, K_i (μM)	K_1 69.8±11.63	K_1 31.5±6.7†
Pyruvate, K_i (mM)	K_2 2.8± 0.67	K_2 1.1±0.08*

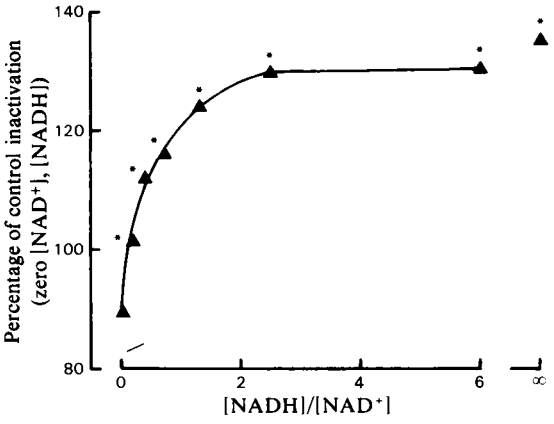

Fig. 4. *Effect of concentration ratio of NADH/NAD⁺ on the rate of the inactivating phosphorylation in pig heart pyruvate dehydrogenase complex*

For details of enzyme preparation, assays and incubation medium see the Experimental section. Concentrations: 200μM-ATP; 35μM-ADP; 5mM-$MgCl_2$; 7mM-(NAD^++NADH), ratios as shown; 2.5 units of pyruvate dehydrogenase complex/ml. The concentration of active complex was assayed before, and 0.75 min after, initiating phosphorylation; the final concentration in the control (zero [NAD^+], [NADH]) was 1.4 units/ml. Each point represents the mean result for at least nine observations. *$P<0.01$ for difference from zero [NADH]; other differences, $P<0.05$.

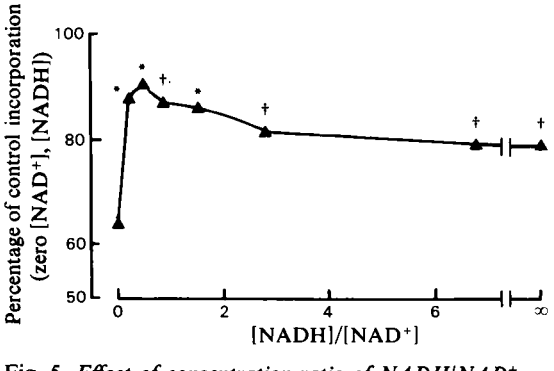

Fig. 5. *Effect of concentration ratio of NADH/NAD⁺ on rate of phosphorylation of additional sites in pyruvate dehydrogenase phosphate* ($\alpha P \cdot \alpha\beta_2$)

General conditions and concentrations were as given in Fig. 4, except that pyruvate dehydrogenase phosphate was used (2.5 units/ml). The specific activity of ATP was 49μCi/μmol and the concentration of protein-bound ^{32}P was measured after 5 min; the incorporation in the control (zero [NAD^+], [NADH]) was 0.17 nmol of *P*/unit of complex. Each point is the mean for at least seven observations. *$P<0.01$, †$P<0.05$ for difference from zero [NADH], 7mM-NAD^+.

phosphorylations (10.1μM). The K_i value for ADP (a competitive inhibitor) was 69.8μM for inactivation and 31.5μM for additional phosphorylations. Full details of the analyses are shown in Table 1. The K_m value for the Mg complex of ATP for inactivation is comparable with that seen in a previous study in which short-time incorporation of ^{32}P was measured (Cooper *et al.*, 1974); the K_i for ADP is somewhat higher.

Inhibition by pyruvate. Sodium pyruvate was an uncompetitive inhibitor both of inactivation by ATP and of additional phosphorylations. The K_i for inactivation was 2.8mM and the K_i for additional phosphorylations was 1.14mM (for details, see Table 1). These values are higher than those obtained by Cooper *et al.* (1974) (0.64mM). The cause of this variation in K_i for pyruvate is not known. It is known that inhibition of the pyruvate dehydrogenase kinase reaction(s) by pyruvate is influenced by nutritional factors, being impaired by starvation in the rat (Hutson & Randle, 1978; Baxter & Coore, 1978). This effect of starvation is sufficiently stable to persist through isolation, incubation and extraction of rat heart mitochondria. Our studies with the pig heart complex have necessarily involved hearts obtained from local abattoirs. It is possible that the extent of starvation of animals before slaughter could influence the kinetics of phosphorylation and inactivation of the complex and of inhibition by pyruvate.

The effect of pyruvate on the time course of inactivation is to diminish the fraction of complex inactivated by the first-order reaction (not shown). The initial rate of the first-order reaction is probably not altered, but this is difficult to investigate because of the short time period involved. Inactivation cannot be measured continuously, because at the high dilution of complex necessary, phosphorylation with $MgATP^2$ does not occur. This feature of the effect of pyruvate is also shown by dichloroacetate, another uncompetitive inhibitor of kinase reactions (Whitehouse *et al.*, 1974). Pyruvate dehydrogenase kinase is tightly bound to the pyruvate dehydrogenase complex, and current evidence suggests that a single kinase molecule may phosphorylate as many as six decarboxylase tetramers (Barrera *et al.*, 1972). One explanation of the effect of pyruvate on phosphorylation could be that it restricts mobility of the kinase, or its access to decarboxylase tetramers.

Effect of concentration ratio NADH/NAD⁺. Fig. 4 shows the effect of the concentration ratio NADH/NAD^+ on the rate of inactivation of pyruvate dehydrogenase complex by phosphorylation. The inactivating phosphorylation was stimulated (relative to NAD^+ alone) by increasing concentration ratios of NADH/NAD^+. The maximum stimulation (at ratios of 2.5 or above) was approx. 50%; the ratio for half-maximal stimulation was approx. 0.3. As Fig. 5 shows, the additional phosphorylations were also

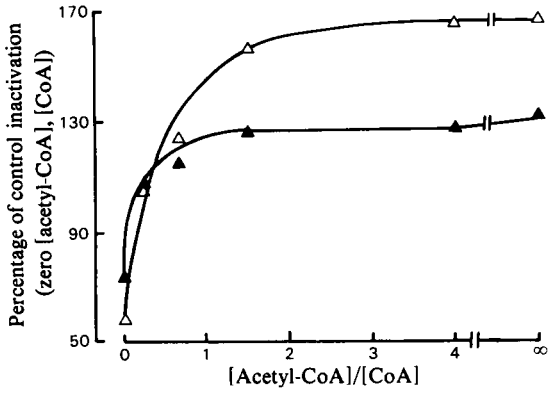

Fig. 6. *Effect of concentration ratio of acetyl-CoA/CoA on the rate of the inactivating phosphorylation in pig heart pyruvate dehydrogenase complex*

The concentration of (acetyl-CoA+CoA) was 1.15mM and the ratios were as shown, in the absence (▲) or presence (△) of 6mM-NAD^+ plus 1mM-NADH. Other concentrations were as given in Fig. 4. Each point represents the mean result for at least five observations. $P<0.001$ for all differences from zero [acetyl-CoA], 1.15mM-CoA.

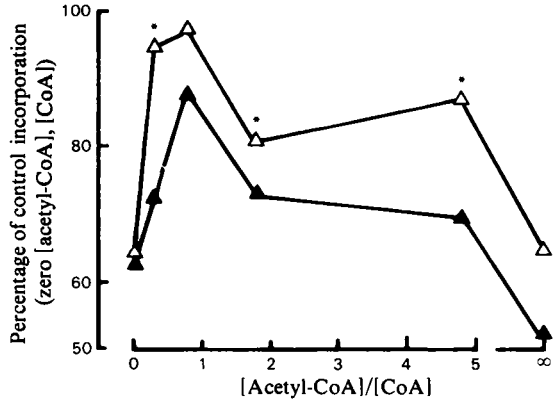

Fig. 7. *Effect of concentration ratio of acetyl-CoA/CoA on rate of phosphorylation of additional sites in pyruvate dehydrogenase phosphate* ($\alpha P \cdot \alpha\beta_2$)

The concentration of (acetyl-CoA+CoA) was 1.15mM and the ratios were as shown, in the absence (▲) or presence (△) of 6mM-NAD^+ plus 1mM-NADH. Other concentrations and specific radioactivity and time of incubation were as described in Fig. 5. Incorporation in control (zero [NAD^+], [NADH], [acetyl-CoA], CoA]) was 0.15nmol of P/unit of complex. Each point represents the mean result for at least three observations. $*P<0.01$ for difference from zero [acetyl-CoA]. Other differences from zero [acetyl-CoA], $P>0.05$.

stimulated (relative to NAD^+ alone) by increasing the ratio of NADH/NAD^+. The maximum stimulation (at ratios between 0.2 and 1.5) was approx. 40%, and declined at ratios above 1.5. The ratio for half-maximal stimulation was less than 0.2. In each of these experiments the total concentration of (NAD^++NADH) was 7mM.

Effect of concentration ratio acetyl-CoA/CoA. Fig. 6 shows the effect of the concentration ratio acetyl-CoA/CoA on the rate of inactivation of pyruvate dehydrogenase complex by phosphorylation. This was studied in the absence and in the presence of 6mM-NAD^+ and 1mM-NADH (the latter is optimal for stimulation by acetyl-CoA; Cooper *et al.*, 1975). Increasing ratios of acetyl-CoA/CoA stimulated the inactivating kinase reaction approx. 2.85-fold (with NADH+NAD^+) or 1.7-fold (absence of NAD^++NADH) at ratios of 1.5 or above. The ratio for half-maximal stimulation was approx. 0.4. As Fig. 7 shows, additional phosphorylation reactions were also stimulated by increasing ratios of acetyl-CoA/CoA (relative to CoA alone). The maximum stimulation was approx. 50% at a concentration ratio of approx. 0.8. The degree of stimulation declined at concentration ratios greater than 0.8. In each of these experments the total concentration of (CoA+acetyl-CoA) was 1.15mM and that, where added, of (NADH+NAD^+) was 7mM.

General conclusions

The present studies have shown that the rate of the inactivating phosphorylation (site 1) is faster than that of site-2 phosphorylation, which in turn is more rapid than site-3 phosphorylation (see also Sugden & Randle, 1978). These conclusions are in agreement with studies in the bovine kidney and heart complexes by Davis *et al.* (1977) and Yeaman *et al.* (1978). It is generally assumed that the inactivating (site 1) and additional (sites 2 and 3) phosphorylations are sequential, although this has not been shown unequivocally so far as we are aware. The crucial point is the absence of a tryptic phosphopeptide in which site 2 is phosphorylated but not site 1. Because site-1 phosphorylation is at least five times as rapid as site-2 phosphorylation, the absence of this phosphopeptide is not easy to prove.

It seemed important to attempt to show that metabolite effectors of the kinase reactions influence both inactivating (site 1) and additional (sites 2 and 3) phosphorylations. In order to do this we have used inactivation as an index of site-1 phosphorylation. This appears justifiable, because inactivation can be accomplished (>85%) with only 16% phosphorylation of site 2 and zero phosphorylation of site 3. Additional phosphorylations have been measured by using a preparation of complex in which site 1 is approx. 98% phosphorylated and site 2 only 9%

phosphorylated. Under the conditions used the additional phosphorylation was mainly site 2.

Subject to these limitations, the present study has shown that pyruvate inhibits, and increasing concentration ratios of ATP/ADP, NADH/NAD^+ and acetyl-CoA/CoA stimulate, both inactivating and additional phosphorylations. Current evidence suggests that it is the concentration ratios of ATP/ADP, NADH/NAD^+ and acetyl-CoA/CoA that may regulate the proportion of inactive phosphorylated complex *in vivo* (cf. Garland & Randle, 1964; Hansford, 1976; Kerbey *et al.*, 1976; Paetzke-Brunner *et al.*, 1978). The present study suggests that this regulation may extend to additional phosphorylations which may influence the rate of reactivation by pyruvate dehydrogenase phosphate phosphatase (Sugden *et al.*, 1978).

This work was supported by grants from the Medical Research Council and the British Diabetic Association.

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