Conversion of Inactive (Phosphorylated) Pyruvate Dehydrogenase Complex into Active Complex by the Phosphate Reaction in Heart Mitochondria is Inhibited by Alloxan-Diabetes or Starvation in the Rat

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1. The conversion of inactive (phosphorylated) pyruvate dehydrogenase complex into active (dephosphorylated) complex by pyruvate dehydrogenase phosphate phosphatase is inhibited in heart mitochondria prepared from alloxan-diabetic or 48 h-starved rats, in mitochondria prepared from acetate-perfused rat hearts and in mitochondria prepared from normal rat hearts incubated with respiratory substrates for 6 min (as compared with 1 min). 2. This conclusion is based on experiments with isolated intact mitochondria in which the pyruvate dehydrogenase kinase reaction was inhibited by pyruvate or ATP depletion (by using oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone), and in experiments in which the rate of conversion of inactive complex into active complex by the phosphatase was measured in extracts of mitochondria. The inhibition of the phosphatase reaction was seen with constant concentrations of Ca^{2+} and Mg^{2+} (activators of the phosphatase). The phosphatase reaction in these mitochondrial extracts was not inhibited when an excess of exogenous pig heart pyruvate dehydrogenase phosphate was used as substrate. It is concluded that this inhibition is due to some factor(s) associated with the substrate (pyruvate dehydrogenase phosphate complex) and not to inhibition of the phosphatase as such. 3. This conclusion was verified by isolating pyruvate dehydrogenase phosphate complex, free of phosphatase, from hearts of control and diabetic rats and from heart mitochondria incubated for 1 min (control) or 6 min with respiratory substrates. The rates of re-activation of the inactive complexes were then measured with preparations of ox heart or rat heart phosphatase. The rates were lower (relative to controls) with inactive complex from hearts of diabetic rats or from heart mitochondria incubated for 6 min with respiratory substrates. 4. The incorporation of $^{32}\text{P}_i$ into inactive complex took 6 min to complete in rat heart mitochondria. The extent of incorporation was consistent with three or four sites of phosphorylation in rat heart pyruvate dehydrogenase complex. 5. It is suggested that phosphorylation of sites additional to an inactivating site may inhibit the conversion of inactive complex into active complex by the phosphatase in heart mitochondria from alloxan-diabetic or 48 h-starved rats or in mitochondria incubated for 6 min with respiratory substrates.

The pyruvate dehydrogenase complex of animal tissues (EC 1.2.4.1+EC 2.3.1.12+EC 1.6.4.3) is inactivated by phosphorylation with MgATP^{2-} , catalysed by an intrinsic kinase. Re-activation is catalysed by a more loosely associated phosphatase (Linn *et al.*, 1969). There is evidence, based on experiments with isolated rat heart mitochondria, that the kinase and phosphatase reactions constitute a cycle that regulates the proportions of active (dephosphorylated) and inactive (phosphorylated) complex. Compounds such as pyruvate or dichloroacetate, which inhibit the kinase reaction in purified bovine heart and kidney, and rat and pig heart complexes (Linn *et al.*, 1969; Cooper *et al.*, 1974), increase the propor-

tion of active complex in isolated rat heart mitochondria (Whitehouse *et al.*, 1974; Kerbey *et al.*, 1976). The kinase reaction in bovine kidney and pig heart complexes is accelerated by high concentration ratios of ATP/ADP , NADH/NAD^+ and acetyl-CoA/CoA (Pettit *et al.*, 1975; Cooper *et al.*, 1975). The proportion of active complex in rat heart mitochondria is decreased by high concentration ratios of these metabolite pairs (Hansford, 1976; Kerbey *et al.*, 1977).

In rat heart, the proportion of active complex is decreased by alloxan-diabetes, by starvation and (in perfused heart) by oxidation of fatty acids and ketone bodies (Wieland *et al.*, 1971*a,b*; Kerbey *et al.*, 1976). It has been suggested that oxidation of lipid fuels increases the concentration ratio of acetyl-CoA/CoA

Abbreviation used: Tos-Lys- CH_2Cl , 7-amino-1-chloro-3-L-tosylamidoheptan-2-one.

thus leading to phosphorylation and inactivation of the complex (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976). However, there is also evidence suggesting inhibition of the phosphatase reaction. Inhibitors of the kinase reaction (pyruvate and dichloroacetate) are much less effective in bringing about conversion of inactive complex into active complex in heart mitochondria of diabetic rats, than in mitochondria from non-diabetic animals.

In this paper evidence is given that the conversion of inactive complex into active complex through the phosphatase reaction is inhibited in heart mitochondria by alloxan-diabetes in the rat, by starvation of the rat, by metabolism of acetate in perfused heart, or by incubation of heart mitochondria with respiratory substrates. Evidence is given that the inhibition of the conversion of inactive complex into active complex under these conditions is due to factors associated with pyruvate dehydrogenase phosphate and not to an inhibition of the phosphatase itself.

Experimental

Materials

Dithiothreitol, oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone and Tos-Lys-CH₂Cl were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. The sources of other biochemicals, chemicals and enzymes were as given by Kerbey *et al.* (1976, 1977). Pyruvate dehydrogenase was purified from pig heart by a modification of the method of Linn *et al.* (1972) as given by Cooper *et al.* (1974). Pyruvate dehydrogenase phosphate (0.4 nmol of P/unit of pyruvate dehydrogenase inactivated) was prepared by the method of Sugden *et al.* (1978). Pyruvate dehydrogenase phosphate phosphatase was partially purified from frozen ox or pig hearts by the method of Severson *et al.* (1974) and was completely free of pyruvate dehydrogenase complex (active and inactive forms).

Methods

Rats, heart perfusion, heart mitochondria. Details of feeding of rats, perfusion of hearts, induction of alloxan-diabetes, preparation and incubation of mitochondria and extraction and assay of mitochondrial ATP, ADP, NADH and NAD⁺ were as described by Kerbey *et al.* (1976, 1977). Extraction and assay of pyruvate dehydrogenase (active and inactive forms) was as given by Kerbey *et al.* (1976), except that the extraction medium was 100 mM-potassium phosphate/2 mM-EDTA/2 mM-dithiothreitol, pH 7.0.

Rat heart pyruvate dehydrogenase phosphate. Rat hearts were frozen with a tissue clamp at liquid-N₂ temperature, powdered with a pestle and mortar,

warmed to the temperature of solid CO₂ and extracted at 0°C (3 × 30 s bursts, Polytron PT20 homogenizer, position 6) with 30 mM-potassium phosphate/10 mM-EDTA/2 mM-dithiothreitol, pH 7.0 (4 ml/g of powder). After centrifuging at 2°C for 30 min at 38 000g, the supernatant was warmed to 20°C, 0.01 vol. of 5 M-NaCl added, and pH adjusted to 6.5 with 10% (v/v) acetic acid. After addition of 0.1 vol. of 50% (w/v) poly(ethylene glycol) the precipitate was collected (20 min, 38 000g, 18°C), rubbed into 1–2 ml of 30 mM-potassium phosphate/2 mM-EDTA/2 mM-dithiothreitol, pH 7.0, and the solution clarified (10 min, 38 000g, 2°C). The pyruvate dehydrogenase phosphate was freed of phosphatase by layering on to 6 ml of the same buffer containing 2% (w/v) sucrose and centrifuging twice at 2°C for 90 min at 150 000g. The pellet containing pyruvate dehydrogenase phosphate, but free of phosphatase and containing only 3–5% of active complex, was rubbed into 0.5–1 ml of 30 mM-potassium phosphate/2 mM-dithiothreitol, pH 7.0 (no EDTA), the solution cleared (2°C, 10 min, 55 000g) and where necessary stored at –10°C.

Rat heart pyruvate dehydrogenase phosphate phosphatase. Phosphatase was extracted (see the preceding section) and precipitated together with pyruvate dehydrogenase (all forms) by addition of solid (NH₄)₂SO₄ (0.21 g/ml). The pellet (20 min, 38 000g, 2°C) was taken up in 1 ml of phosphate/dithiothreitol and dialysed twice against 400 ml of the same buffer (2°C, total 18 h). The preparation of phosphatase was completely freed of pyruvate dehydrogenase (all forms) by twice centrifuging at 2°C for 90 min at 150 000g.

Pyruvate dehydrogenase phosphate from rat heart mitochondria. Frozen mitochondrial pellets (approx. 2 mg of mitochondrial protein) were extracted by ultrasonic disintegration (Kerbey *et al.*, 1976) into 0.15 ml of 30 mM-potassium phosphate/10 mM-EDTA/2 mM-dithiothreitol, pH 7.0. Mitochondria were prepared from hearts of 28 rats (total yield 0.5 g of mitochondrial protein) and incubated for 1 min or 6 min with 5 mM-2-oxoglutarate + 0.5 mM-L-malate by using methods given by Kerbey *et al.* (1976). The combined extracts in each group were cleared (2°C, 15 min, 38 000g) and pyruvate dehydrogenase phosphate was prepared as described for rat heart.

Assay of pyruvate dehydrogenase phosphate phosphatase. Phosphatase was assayed by measuring the rate of formation of active pyruvate dehydrogenase complex from inactive phosphorylated complex at 30°C (assayed spectrophotometrically by coupling to arylamine acetyltransferase; Coore *et al.*, 1971).

In experiments with pyruvate dehydrogenase phosphate purified from rat hearts, the reaction mixture contained 100 µl of (50 mM-Tris/HCl/2 mM-Tos-Lys-CH₂Cl/20 mM-EGTA/20 mM-CaCl₂, pH 7.5; Ca²⁺, 55 µM), 25 µl of ox heart phosphatase, 5–40 µl of

pyruvate dehydrogenase phosphate (60 munits of pyruvate dehydrogenase equivalent) and made up to 200 μ l with 50mM-Tris/HCl, pH7.5. A 30 μ l sample was taken for assay of zero-time pyruvate dehydrogenase (active forms). After 2min preincubation at 30°C, the phosphatase reaction was initiated by addition of MgCl_2 (1M) to 11.75mM and samples were taken at intervals (see the Figures) for assay of active complex. In experiments with pyruvate dehydrogenase phosphate prepared from mitochondria, the same procedure was followed but the volumes were 40 μ l of Tris/HCl/Tos-Lys- CH_2Cl /EGTA/ CaCl_2 , 15 μ l of ox heart phosphatase, 23 or 45 μ l of pyruvate dehydrogenase phosphate and 30mM-potassium phosphate, pH7.0, to 100 μ l. Reaction was initiated by addition of MgCl_2 (1M) to 14.3mM. The preparations of rat heart and mitochondrial pyruvate dehydrogenase phosphate were free of phosphatase. The preparations of rat heart pyruvate dehydrogenase phosphate contained 3–5% of active pyruvate dehydrogenase; those from mitochondria contained pyruvate dehydrogenase amounting to 35% of the total (sum of active+inactive forms from mitochondria incubated for 1min) and to 9% of the total (from mitochondria incubated for 6min). The total activity of pyruvate dehydrogenase phosphate was assayed by complete conversion at high phosphatase concentration (10–15min incubation required).

For assay of phosphatase in mitochondrial extracts, the frozen pellet (1.25mg of mitochondrial protein) was extracted by ultrasonic disintegration into 0.25ml of 50mM-Tris/HCl/5mM-2-mercaptoethanol, pH7.5. The extract was mixed with an equal volume of 50mM-Tris/HCl/20mM-EGTA/19.8mM- CaCl_2 /2mM-Tos-Lys- CH_2Cl , pH7.5 (Ca^{2+} approx. 23 μ M). A 30 μ l sample was taken for assay of initial pyruvate dehydrogenase activity and the remainder preincubated for 2min at 30°C. The phosphatase reaction was then started with MgCl_2 (1M) to 10mM (there was no conversion without addition of MgCl_2). Samples of incubation mixture (30 μ l) were taken for assay of active complex at times shown in the Figures. In some experiments phosphatase activity in extracts of mitochondria was assayed with pig heart pyruvate dehydrogenase phosphate (see under 'Materials'). The assay details were as described above, except that the reaction was started by adding pyruvate dehydrogenase phosphate (final concentration equivalent to 2.5 units of pyruvate dehydrogenase/ml) in a more than 10-fold excess, and that MgCl_2 (10mM) was present during preincubation.

Enzyme activity. One unit of pyruvate dehydrogenase forms 1 μ mol of acetyl-CoA or NADH per min at 30°C. One unit of pyruvate dehydrogenase phosphate phosphatase forms 1munit of pyruvate dehydrogenase per min at 30°C from pyruvate dehydrogenase phosphate.

Results and Discussion

Concentrations of active and inactive forms of the pyruvate dehydrogenase complex in rat heart mitochondria

The proportion of active pyruvate dehydrogenase in freshly prepared mitochondria from hearts of fed normal rats was $61.5 \pm 3.5\%$ (mean $\pm$ S.E.M. for ten observations). The corresponding values for heart mitochondria from starved or alloxan-diabetic rats were $27.8 \pm 9.5\%$ (four observations) and $31.4 \pm 5.6\%$ (five observations) respectively. The total activity of pyruvate dehydrogenase in heart mitochondria (sum of active and inactive forms) was not altered by alloxan-diabetes or starvation. The values in munits of pyruvate dehydrogenase activity/mg of mitochondrial protein (means $\pm$ S.E.M. with numbers of observations in parentheses) were: control 75.4 ± 5 (13), diabetic 73.3 ± 7.3 (6); control 59.1 ± 3.0 (5), starved 60.0 ± 6.7 (5). The total activity of pyruvate dehydrogenase showed no difference in mitochondria prepared from hearts perfused with glucose (5.5mM) + insulin (20munits/ml) or with the same medium plus sodium acetate (5mM). These values (in munits/mg of protein) were glucose, insulin, 81.5 ± 2.1 (4), glucose, insulin, acetate, 75 ± 4.7 (4), and are comparable with those obtained by Kerbey *et al.* (1976), Hansford (1976) and Portenhausser & Wieland (1977). The total activity of pyruvate dehydrogenase in batches of mitochondria prepared on different days varied for unknown reasons between 50 and 80 munits/mg of protein. All experiments comparing different batches of mitochondria were carried out side by side on the same day.

Effects of sodium pyruvate. The proportion of active complex in heart mitochondria of fed normal rats oxidizing 2-oxoglutarate+L-malate (Fig. 1) or succinate (Fig. 2) was increased by sodium pyruvate at concentrations between 0.25 and 10mM. Pyruvate was most effective when present from the beginning of incubation or added after 1min of incubation with succinate (Fig. 2) or 2-oxoglutarate+malate (not shown). Pyruvate had little or no effect when added after 6min of incubation with succinate (Fig. 2) or 2-oxoglutarate+malate (not shown). The loss of response to pyruvate after 6min of incubation was not associated with any difference in the proportion of active complex at the time of its addition; this did not change between 1min and 6min of incubation with succinate (Fig. 2).

The effect of pyruvate (present from the beginning of incubation) on the proportion of active complex was also decreased in mitochondria prepared from hearts of alloxan-diabetic rats or starved (48h) rats, or from hearts of fed rats perfused with medium containing acetate (Fig. 1). The proportion of active complex at the beginning of incubation was lower in mitochondria from hearts of diabetic or starved rats

Fig. 1. Proportion of active pyruvate dehydrogenase complex in rat heart mitochondria; effect of pyruvate concentration

Mitochondria were incubated for 6 min at 30°C in KCl medium (1 mg of protein in 0.55 ml) containing 5 mM-2-oxoglutarate, 0.5 mM-L-malate and pyruvate at concentration shown. Mitochondria were separated, frozen, extracted and assayed for pyruvate dehydrogenase (active form) as described in the Experimental section. Total pyruvate dehydrogenase was assayed after conversion of inactive complex into active complex. This was achieved either by incubation of extracts with phosphatase (▲) or by parallel incubations of mitochondria for 6 min without substrate (●, △, ○; see Kerbey *et al.*, 1976; Portenhauser & Wieland, 1977). The sources of mitochondria were as follows: ●, hearts of fed normal rats before or after 5 min of perfusion with medium containing glucose (5.5 mM), insulin (20 munits/ml); ○, hearts of fed alloxan-diabetic rats, not perfused; ▲, hearts of normal rats starved for 48 h, not perfused; △, hearts of fed normal rats perfused for 6 min with medium containing glucose (5.5 mM), insulin (20 munits/ml) and sodium acetate (5 mM). Because the control values (●) were very similar in these experiments, they were averaged and the experimental values (○, ▲, △) were each multiplied by the ratio of this new mean control value to the control value of that particular experiment. The *P* values calculated from the non-adjusted data for (control-experimental) were <0.001 , except for: (non-diabetic-diabetic) zero and 0.25 mM-pyruvate, $P<0.05>0.02$; (fed-starved), zero pyruvate, $P>0.05$, 0.5 mM-pyruvate, $P<0.05>0.02$. *P* values were not calculated with 10 mM-pyruvate as there were only two observations in each group. Other points are means for at least four observations.

Fig. 2. Effects of pyruvate on the proportion of active pyruvate dehydrogenase complex in heart mitochondria from fed normal rats

For experimental details see Fig. 1. In (a) the time of incubation was 11 min and sodium pyruvate was added to the concentration shown at zero time (●), or at 1 min (○) or at 6 min (△) of incubation with 5 mM-succinate. In (b) the time of incubation with 5 mM-succinate was as shown and sodium pyruvate was added to 5 mM at zero time (●) or after 1 min (○) or after 6 min (△). ▲, No pyruvate added. In (a) each point is the mean for two incubations. In (b) the combined means and standard errors (as % of total activity) for six observations were 1 (●) $87.1 \pm 2.46\%$; 2 (○) $70.7 \pm 1.20\%$; 3 (△) $20.3 \pm 0.5\%$; 4 (▲) $21.2 \pm 1.14\%$. For 1 versus 2, 3 or 4 and for 2 versus 3 or 4, $P<0.001$. For other difference (4 versus 3) $P>0.05$. The total activity of pyruvate dehydrogenase (76.5 munits/mg of protein) was unaffected by the conditions or duration of incubation.

or after perfusion with acetate (see preceding section).

Pyruvate has no detectable effect on the pyruvate dehydrogenase phosphate phosphatase reaction

(Denton *et al.*, 1975). It is assumed to increase the proportion of active complex in mitochondria by inhibiting the kinase reaction, thus facilitating con-

version of inactive complex into active complex by the phosphatase. A diminished effect of pyruvate could be due to either a lack of inhibition of the kinase reaction or to an inhibition of the phosphatase reaction. We therefore attempted to distinguish between these two possibilities by depleting mitochondria of ATP with oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone, and thereby arresting the kinase reaction.

Fig. 3. Proportion of active pyruvate dehydrogenase complex in rat heart mitochondria; effect of oligomycin (20 μ g/ml) and carbonyl cyanide *m*-chlorophenylhydrazone (1 μ M)

Heart mitochondria from fed normal rats were incubated in KCl medium containing 5 mM-succinate (all symbols) and oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone added shown by arrow either after 1 min of incubation (▲) or 6 min of incubation (△) or not added (●). (a) Mitochondria separated, frozen, extracted and assayed for pyruvate dehydrogenase. (b) Samples (50 μ l) of these same incubations removed into HClO₄ and assayed for ATP. For details see the Experimental section. In (a) each point is the mean for four observations on two different mitochondrial preparations; the overall values (means $\pm$ S.E.M. of % of active dehydrogenase, total numbers of observations in parentheses) were: 1 (●) 15.7 ± 0.94 (12); 2 (▲) 90.8 ± 2.67 (16); 3 (△) 29.4 ± 0.61 (12). $P < 0.001$ for 1 versus 2, 1 versus 3, 2 versus 3. In (b) each point is the mean for at least four observations; $P < 0.001$ for control versus oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone at all time points and for control at 1 min or 6 min versus control at 11 min. The total activity of pyruvate dehydrogenase (sum of active and inactive forms) was 43.9 munits/mg of protein and was unaffected by the conditions or duration of incubation.

Effects of oligomycin and carbonyl cyanide m-chlorophenylhydrazone. In these experiments, succinate was used as respiratory substrate because its oxidation (unlike that of 2-oxoglutarate) does not involve the formation of GTP (and thus ATP) at the level of succinyl-CoA synthetase. The results are shown in Figs. 3, 4 and 5. In each Figure (a) shows the proportion of active pyruvate dehydrogenase complex and (b) the ATP concentration.

When heart mitochondria from fed normal rats were incubated with 5 mM-succinate, the proportion of active complex fell to a minimum value within 1 min. This was maintained over 11 min of incubation in spite of a decline in ATP (Fig. 3). Addition of oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone after 1 min (Fig. 3) or 2 min (Figs. 4 and 5) of incubation with succinate led to almost complete conversion of inactive complex into active complex by the phosphatase (to over 85%). This conversion generally occurred within 1–2 min (Figs. 3 and 4), but in one experiment (Fig. 5) conversion took approx. 4 min. The rate of conversion was significantly lower in heart mitochondria from fed alloxan-diabetic rats (Fig. 4) or 48 h-starved non-diabetic animals (Fig. 5). Addition of oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone to heart mitochondria from fed animals after 6 min of incubation with succinate resulted in very little conversion of inactive complex by the phosphatase (Fig. 3). The total concentration of pyruvate dehydrogenase complex (sum of active and inactive forms) was not changed significantly by the duration or conditions of incubation, alloxan-diabetes, or starvation (see legends to Figs. 3, 4 and 5).

As shown in Figs. 3, 4 and 5 oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone led to a rapid fall in ATP concentration (within 1 min) regardless of the source of heart mitochondria or the time of addition of oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone during incubation with succinate. In each experiment the final concentration of ATP with oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone was in the range 0.2–1 mM. The ATP concentration in mitochondria incubated without substrate for 5–15 min is also in this range (not shown). Therefore heart mitochondria are not totally depleted of ATP by incubation with oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone or in the absence of respiratory substrates. Earlier studies with the purified bovine heart and kidney and pig heart complexes and with rat heart mitochondria showed that the kinase reaction is inhibited by low concentration ratios of ATP/ADP, NADH/NAD⁺ and acetyl-CoA/CoA (Linn *et al.*, 1969; Cooper *et al.*, 1974, 1975; Pettit *et al.*, 1975; Hansford, 1976; Kerbey *et al.*, 1977). The effects of oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone on concentration ratios of ATP/ADP and of NADH/NAD⁺ have been

Fig. 4. Effects of oligomycin (20 μ g/ml) and carbonyl cyanide *m*-chlorophenylhydrazone (1 μ M) on the activity of pyruvate dehydrogenase in heart mitochondria from control and alloxan diabetic rats

Experiments were carried out as described in Fig. 3, except that mitochondria were incubated for 2 min in the presence of 1 mM-succinate before the addition of oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone (shown by arrow). (a) Pyruvate dehydrogenase activity (% of active form) for control (●) and alloxan-diabetic (○) mitochondria. (b) Concentrations of ATP (nmol/mg of protein) measured in the same incubations; ●, control; ○, diabetic. Each point is mean $\pm$ S.E.M. of values from four different mitochondrial preparations. * $P < 0.001$ and ** $P < 0.01$ as compared with control values. All other points are not significantly different. Total pyruvate dehydrogenase activity was 56.8 ± 3.7 munits/mg of protein for control and 58.0 ± 3.7 munits/mg of protein for diabetic preparations.

compared in heart mitochondria from fed normal and fed alloxan-diabetic rats (Table 1). The concentration ratio of acetyl-CoA/CoA was not measured in this experiment because a previous study showed that this ratio is very low in mitochondria incubated with succinate (Kerbey *et al.*, 1977).

Fig. 5. Effect of oligomycin (20 μ g/ml)+carbonyl cyanide *m*-chlorophenylhydrazone (1 μ M) (indicated by arrows) on the activity of pyruvate dehydrogenase in heart mitochondria from control (fed) and 48 h-starved rats

For details of protocol see Fig. 4. (a) Pyruvate dehydrogenase activity (% of active form) for fed control (●) and 48 h-starved (▲) and (b) the corresponding values for ATP concentration in nmol/mg of protein. Points are means for four estimations on two different mitochondrial preparations from each group of rats. Total pyruvate dehydrogenase activities (sum of active and inactive forms) were (in munits/mg of protein; means $\pm$ S.E.M.) 63.5 ± 4.5 (fed) and 56.2 ± 9.3 (starved). For differences between fed and starved in (a) $P < 0.01$ at all times except 2 min and 7 min where $P > 0.05$; in (b) $P < 0.01$ at zero time; for all other times $P > 0.05$.

The results in Table 1 show that the concentration ratios of ATP/ADP and of NADH/NAD⁺ are much lower (relative to controls) in heart mitochondria from diabetic rats at zero time or 2 min of incubation with 1 mM-succinate. This has been noted previously (Kerbey *et al.*, 1977) and it is assumed to be due to uncoupling of oxidative phosphorylation by an increased concentration of free fatty acids in the diabetic muscle (Garland & Randle, 1964). Addition of oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone decreased the concentration ratio of ATP/ADP to

Table 1. *Effect of oligomycin (20 µg/ml)+carbonyl cyanide m-chlorophenylhydrazone (CCCP) (1 µM) on concentrations of ATP, ADP, NADH and NAD⁺ in rat heart mitochondria*

For details of experimental protocol see Fig. 4. For methods of analysis see the Experimental Section. Each value is the mean $\pm$ S.E.M. of two incubations, assayed in duplicate with one preparation of mitochondria from each type of rat (control or alloxan-diabetic).

Time after addition of oligomycin+CCCP (min)		Concentration (nmol/mg of protein)				Concentration ratio	
		ATP	ADP	NADH	NAD ⁺	ATP/ADP	NADH/NAD ⁺
-2	Control	3.14	0.78	0.46	4.29	4.03	0.11
	Diabetic	0.62	2.70	0.09	1.93	0.21	0.05
0	Control	4.32	0.11	4.24	1.02	39.3	4.16
	Diabetic	1.44	1.22	0.64	2.09	1.18	0.31
1	Control	1.21	2.05	0.06	3.74	0.59	0.02
	Diabetic	0.86	1.16	0.02	2.05	0.74	0.01
2	Control	1.19	2.90	0.44	3.20	0.41	0.14
	Diabetic	0.43	1.59	0	2.02	0.27	0
3	Control	0.78	3.07	0.46	4.17	0.25	0.11
	Diabetic	0.38	2.25	0	2.80	0.17	0
4	Control	0.81	3.05	0.51	3.35	0.27	0.15
	Diabetic	0.44	1.50	0	2.94	0.29	0

approximately one-sixteenth in the control and to approximately one-sixth in diabetic mitochondria. The absolute ratios with oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone were comparable in the two groups. The concentration ratios of NADH/NAD⁺ fell to zero in the diabetic mitochondria and to values which were not significantly different from zero in the control.

Because the concentration of ATP did not fall to zero it is not possible to conclude that oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone led to complete inhibition of the kinase reaction in these experiments. Nevertheless, the results show that the phosphatase reaction converts inactive complex into active complex in heart mitochondria when the kinase reaction is inhibited by lowering the concentration ratios of ATP/ADP and of NADH/NAD⁺. The results suggest therefore that the phosphatase reaction is inhibited in mitochondria from hearts of alloxan-diabetic or starved rats. The results also suggest that the phosphatase reaction is inhibited in mitochondria that are incubated for 6min with succinate. It is known that the phosphatase reaction requires Mg²⁺ (Linn *et al.*, 1969) and that it is activated by Ca²⁺ (Denton *et al.*, 1972). In order to ascertain whether the inhibition of the phosphatase reaction induced by diabetes or by starvation or by incubation of mitochondria with respiratory substrates is dependent on the concentration of these bivalent ions, the phosphatase reaction was studied in extracts of mitochondria.

Pyruvate dehydrogenase phosphate phosphatase activity in extracts of heart mitochondria

Defining assay conditions. In order to study the re-activation of pyruvate dehydrogenase phosphate by

phosphatase in extracts of mitochondria, it was essential to define conditions in which the reaction was linear with time and in which pyruvate dehydrogenase was stable. It was assumed that instability of pyruvate dehydrogenase in extracts is due to proteolysis (Wieland, 1975). We have found that use of a low Ca²⁺ concentration (5–23 µM; achieved with Ca/EGTA buffer) and inclusion of the proteinase inhibitor Tos-Lys-CH₂Cl stabilized the complex in extracts of mitochondria. Thus totally active pyruvate dehydrogenase was stable for at least 5min under these conditions (not shown; the exact details of the reaction mixture are given in the Experimental section). The rate of re-activation was constant over the first minute with extracts of heart mitochondria from fed normal rats (see Figs. 6 and 7). An alternative proteinase inhibitor phenylmethanesulphonyl fluoride was unsuitable because it was an inhibitor of phosphatase activity.

The formation of active complex in extracts of mitochondria under these conditions is assumed to be due to endogenous phosphatase and not to proteolysis. Firstly, 90–100% of the total pyruvate dehydrogenase activity (assayed with pig heart phosphatase) was regained in 15min of incubation (not shown). Secondly, the active pyruvate dehydrogenase formed in the mitochondrial extracts could be inactivated within 1–2min by addition of ATP (0.5mM), oligomycin (25 µg/ml) and EGTA (0.4mM) (not shown).

The zero-time concentrations of ATP present in the phosphatase reaction mixtures were 1.72 and 1.30 µM for extracts from control and alloxan-diabetic heart mitochondria respectively. These values are only 7–9% of the *K_m* of rat heart pyruvate dehydrogenase kinase for ATP (Cooper *et al.*, 1974). The Ca²⁺

Fig. 6. Conversion of inactive pyruvate dehydrogenase complex into active complex in extracts of heart mitochondria from normal and alloxan-diabetic rats

Mitochondria were incubated for 1 min with 5 mM-2-oxoglutarate+0.5 mM-L-malate (to convert active complex into inactive complex) and separated, frozen, extracted by ultrasonic disintegration and extracts incubated as described in Experimental section. Each point is the mean for five incubations of mitochondria from fed normal (●) and alloxan-diabetic (○) rats. * $P<0.001$; ** $P<0.01$ (normal versus diabetic). Zero-time activities of pyruvate dehydrogenase (active form) were (means $\pm$ S.E.M. in munits/mg of protein) 16.1 ± 1.3 (control) and 10.4 ± 0.6 (diabetic).

concentrations (5–23 μ M) present in these incubations are well above the K_i (1 μ M) of pig heart pyruvate dehydrogenase kinase for Ca^{2+} (Cooper *et al.*, 1974). These two factors should result in little, if any, pyruvate dehydrogenase kinase activity in these experiments.

Effects of alloxan-diabetes, starvation and respiratory substrates. Fig. 6 compares the initial rate of conversion of inactive complex into active complex in extracts of heart mitochondria from control (fed normal) and alloxan-diabetic rats. Before extraction, both preparations of mitochondria were incubated for 1 min with 2-oxoglutarate+L-malate to form inactive (phosphorylated) complex. The phosphatase reaction in extracts of diabetic mitochondria is clearly inhibited relative to controls. Nearly all (70–90%) of the inactive complex present was eventually converted into the active form in both control and diabetic extracts (after 15 min of incubation; not shown). Very similar results were obtained comparing extracts of heart mitochondria from fed and 48 h-starved animals (not shown). With excess of purified pig heart pyruvate dehydrogenase phosphate as substrate in the assay (see the Experimental section), there was no detectable difference between diabetic and control extracts in the initial rate of formation of

Fig. 7. Conversion of inactive pyruvate dehydrogenase complex into active complex in extracts of mitochondria incubated for 1 min (●) or 6 min (▲) with 5 mM-2-oxoglutarate+0.5 mM-L-malate

For other details see Fig. 6 and Experimental section. Each point is the mean for eight incubations with two different preparations of mitochondria. $P<0.001$ for (●) versus (▲) at 0.5 and 1 min of incubation. The zero-time pyruvate dehydrogenase activities (in munits/mg of protein, means $\pm$ S.E.M.) were 15.0 ± 2.9 (1 min) and 11.7 ± 1.3 (6 min).

active complex. The values (in munits of active complex formed/min per mg of mitochondrial protein) were 66.8 ± 8.1 (control) and 59.0 ± 8.9 (alloxan-diabetic) (means $\pm$ S.E.M. of 21 determinations from two separate preparations of mitochondria). This is in agreement with Kerbey *et al.* (1976), who measured release of $^{32}\text{P}_i$ from pig heart pyruvate dehydrogenase [^{32}P]phosphate.

As was seen in Figs. 2 and 3 there appeared to be an inhibition of active pyruvate dehydrogenase formation in intact mitochondria after 6 min of incubation with either 5 mM-succinate or 5 mM-2-oxoglutarate and 0.5 mM-L-malate. The following experiments were performed in order to see if this inhibition persisted into extracts prepared from mitochondria. Heart mitochondria from normal fed rats were pre-incubated for either 1 or 6 min with 5 mM-2-oxoglutarate and 0.5 mM-L-malate and pyruvate dehydrogenase phosphate phosphatase activity was subsequently assayed in extracts prepared from the mitochondria. The initial concentrations of the active and inactive forms of pyruvate dehydrogenase

in extracts of these two groups of mitochondria were not significantly different (see legend to Fig. 7). However, there was a large decrease in the initial rate of formation of active pyruvate dehydrogenase in extracts of mitochondria incubated with substrate for 6 min as compared with 1 min (Fig. 7). As was mentioned previously, there was no fall in total pyruvate dehydrogenase activity under these conditions. The initial rate of re-activation of purified pig heart pyruvate dehydrogenase phosphate with phosphatase in extracts of the two groups of mitochondria showed no significant difference (not shown).

These experiments show that the phosphatase reaction with endogenous substrate is inhibited (relative to controls) in extracts of heart mitochondria from alloxan-diabetic or 48 h-starved rats. The phosphatase reaction also becomes inhibited in extracts prepared from heart mitochondria incubated with respiratory substrates, between the second and sixth minute of incubation. These inhibitory effects are unlikely to be due to differences in Ca^{2+} or Mg^{2+} concentrations. The concentration of added MgCl_2 was fixed and control experiments showed that there was insufficient Mg^{2+} in the mitochondrial extracts to activate the phosphatase. The concentration of added Ca^{2+} was buffered by EGTA and the potential contribution of mitochondrial calcium to the total calcium present was negligible (25 ng-atoms of mitochondrial calcium as compared with 2.5 μg -atoms of added calcium). Inhibition of the phosphatase reaction was only seen with rat heart mitochondrial pyruvate dehydrogenase phosphate as substrate and not with the purified pig heart pyruvate dehydrogenase phosphate. It may be noted that the concentration of pig heart pyruvate dehydrogenase phosphate added in these experiments was very near to the K_m of the phosphatase for pyruvate dehydrogenase phosphate (Randle *et al.*, 1974). The conditions of assay would therefore have been sensitive to a competitive inhibitor of the phosphatase if one had been present. It therefore seemed likely that these inhibitory effects were due to some stable factor associated with the rat heart mitochondrial pyruvate dehydrogenase phosphate and not to inhibition of the phosphatase *per se*. We have therefore sought to confirm this conclusion by purifying rat heart pyruvate dehydrogenase phosphate and following its re-activation with added ox heart or rat heart phosphatase.

Re-activation of partially purified rat heart pyruvate dehydrogenase phosphate by ox heart or rat heart phosphatase

Effect of alloxan-diabetes. Hearts were perfused before extraction and purification of pyruvate dehydrogenase phosphate in order to minimize the effects of anaesthesia and excision of the heart. The

Fig. 8. Rate of re-activation by ox heart phosphatase of inactive pyruvate dehydrogenase complex purified from hearts of normal (●) or alloxan-diabetic (○) rats

Six hearts were perfused either with medium containing glucose (5.5 mM)+insulin (20 munits/ml) (control) or with medium containing 16.5 mM-glucose (diabetic). For details of heart perfusion and induction of alloxan-diabetes see Kerbey *et al.* (1976). At the end of perfusion hearts were frozen, powdered, and inactive pyruvate dehydrogenase complex was extracted, purified, freed of phosphatase and incubated with ox heart phosphatase as described in the Experimental section. $P < 0.01$ for control versus diabetic at all times after zero time. In incubations with phosphatase initial concentrations (in munits pyruvate dehydrogenase/ml) were: active form 15 (control) and 9 (diabetic); inactive form 300 (control and diabetic). Total pyruvate dehydrogenase activity was the same in both incubations as determined with excess ox heart phosphatase (see the Experimental section).

perfusion conditions used were designed to simulate conditions *in vivo* of insulinization and normal blood glucose in the control and insulin deficiency and hyperglycaemia in the diabetic. Fig. 8 compares the rates of formation of active complex from pyruvate dehydrogenase phosphate extracted and purified from alloxan-diabetic and fed normal hearts with ox heart pyruvate dehydrogenase phosphate phosphatase. The preparations of substrate were essentially free of phosphatase. The preparations of phosphatase were free of active or inactive complex. As shown in Fig. 8, the initial rate of conversion of inactive complex into active complex was greater with inactive complex from control hearts than with inactive complex from diabetic hearts. The ratio of initial rates (control/diabetic) was approx. 3:1. Total pyruvate dehydrogenase activity was measured with an excess of ox heart phosphatase (see the Experimental section) and adjusted to the same total activity in both sets of incubations (see legend to Fig. 8). Essentially the same results (not shown) were obtained with these same preparations and

Fig. 9. Rate of re-activation by ox heart phosphatase of inactive pyruvate dehydrogenase complex purified from rat heart mitochondria incubated for 1 min (●) or 6 min (▲) with 5 mM-2-oxoglutarate + 0.5 mM-L-malate

For details of separation of mitochondria and extraction, purification and incubation of inactive pyruvate dehydrogenase complex with ox heart phosphatase see the Experimental section. Each point is the mean for three incubations. $P < 0.01$ for (●) versus (▲) at 2 and 5 min of incubation. In the incubations with phosphatase the initial concentrations in munits of pyruvate dehydrogenase/ml were: active form 154 (●) and 21 (▲); inactive form 441 (●) and 235 (▲). The rates shown (▲) have been adjusted by multiplying by 441/235 (see the text for explanation).

utilizing rat heart phosphatase (free of active and inactive complexes). These experiments lead us to conclude that the conversion of inactive complex into active complex by the phosphatase is inhibited in alloxan-diabetes by a stable change in pyruvate dehydrogenase phosphate.

Effect of respiratory substrates in mitochondria. Fig. 9 compares the rate of formation of active complex from inactive complex extracted and purified from rat heart mitochondria incubated for 1 or 6 min with 2-oxoglutarate + malate. Conversion was effected with ox heart phosphatase free of active and inactive pyruvate dehydrogenase complexes. Because of shortage of material it was not possible to utilize the same concentration of pyruvate dehydrogenase phosphate in the two sets of incubations. The concentrations of pyruvate dehydrogenase phosphate (in units of pyruvate dehydrogenase equivalent/ml) were 0.44 (preparation from mitochondria incubated for 1 min) and 0.24 (preparation from mitochondria incubated for 6 min). On the assumption that the concentration of pyruvate dehydrogenase phosphate is below the K_m for the phosphatase, we have corrected the rates for the preparation from mitochondria incubated for 6 min by multiplying by 441/235. The K_m of pig heart

phosphatase for pig heart pyruvate dehydrogenase phosphate is 4 unit/ml (Randle *et al.*, 1974). The correction that we have applied would tend to over-estimate the rate with the preparation of inactive complex from mitochondria incubated for 6 min. The results of this experiment, given in Fig. 9, show that inactive complex prepared from heart mitochondria incubated with respiratory substrates for 1 min is re-activated more rapidly by the ox heart phosphatase than inactive complex prepared from mitochondria incubated for 6 min. Because measurements were only made at two time periods, it is not possible to give an accurate estimate of the ratio of initial rates. The data suggest that this ratio (1 min/6 min of incubation of mitochondria) is not less than 3:1. These results lead us to conclude that the pyruvate dehydrogenase phosphate phosphatase reaction in rat heart mitochondria incubated with 2-oxoglutarate + L-malate becomes inhibited between the first and sixth minutes of incubation as a result of a stable change associated with pyruvate dehydrogenase phosphate.

General Discussion and Conclusions

Recent studies have shown that the bovine kidney and pig heart complexes may incorporate up to three phosphates per tetramer ($\alpha_2\beta_2$) of pyruvate decarboxylase (EC 1.2.4.1) (Davis *et al.*, 1977; Sugden *et al.*, 1978; Sugden & Randle, 1978). These studies have shown that inactivation of the complex is largely achieved (>95%) by incorporation of one phosphate according to the equation:

Two further phosphates may then be incorporated according to the equation:

The fully phosphorylated complex (containing $\alpha_2\text{P}_3\beta_2$) is re-activated in the phosphatase reaction at approximately one-third of the initial rate of re-activation of the partially phosphorylated complex (containing $\alpha_2\text{P}\cdot\beta_2$) (Sugden *et al.*, 1978).

In the present study, comprehensive evidence is given for inhibition by alloxan-diabetes and by 48 h starvation in the rat of the conversion of inactive pyruvate dehydrogenase complex into active complex by the phosphatase reaction in heart mitochondria. Evidence is given that this is due to a stable change associated with the inactive complex (pyruvate dehydrogenase phosphate) and not to an inhibition of the phosphatase *per se*. We have also given evidence that a similar stable change associated with pyruvate dehydrogenase phosphate leads to inhibition of conversion by the phosphatase reaction in heart mito-

Fig. 10. $^{32}\text{P}_i$ incorporation into rat heart mitochondrial pyruvate dehydrogenase phosphate

Heart mitochondria from fed normal rats were incubated (1 mg of protein in 0.5 ml) at 30°C for 6–10 min in KCl medium (Kerbey *et al.*, 1976) modified to contain 2 mM- $^{32}\text{P}[\text{K}_2\text{HPO}_4]$ (40 $\mu\text{Ci}/\mu\text{mol}$), separated by centrifugation and frozen. The pellets were extracted by ultrasonic disintegration into 0.1 M-potassium phosphate/5 mM-EDTA/5 mM-2-mercaptoethanol. Samples of extract (50 μl) were assayed for protein bound radioactivity (Corbin & Reiman, 1974) and for active pyruvate dehydrogenase (Coore *et al.*, 1971). Samples were then incubated with pig heart pyruvate dehydrogenase phosphate phosphatase and samples assayed for active complex at 5 and 10 min. When conversion was complete (usually 5 min) samples (50 μl) were taken for assay of protein bound radioactivity (see above). The correlation coefficient (r) for the regression of protein-bound ^{32}P on pyruvate dehydrogenase activity (active form) was -0.92 . The slope yielded an estimate of $1.65 \pm 0.16 \text{ nmol of P released/unit of active pyruvate dehydrogenase formed in the phosphatase reaction (mean} \pm \text{s.e.m.)}$.

chondria incubated with respiratory substrates. This change appears between the first and sixth minutes of incubation, whereas inactivation of the pyruvate dehydrogenase complex by phosphorylation takes only 1–2 min. We have studied the incorporation of $^{32}\text{P}_i$ into ATP and protein in rat heart mitochondria. The incorporation of $^{32}\text{P}_i$ into the γ -phosphate of ATP was complete in 30 s; the incorporation of $^{32}\text{P}_i$ into protein took 6 min to completion (not shown). Hughes & Denton (1976) have already summarized evidence that the incorporation is due solely to phosphorylation of the α -chains of the pyruvate decarboxylase component of pyruvate dehydrogenase. Our own studies confirm their observations and conclusions in this regard (not shown). In our studies with rat heart mitochondria (see Fig. 10 for details) the extent of phosphorylation at completion

was $1.65 \pm 0.16 \text{ nmol of P/unit of complex inactivated}$. If the rat heart complex has the same subunit composition and specific enzyme activity as the pig heart complex, this incorporation would correspond to 3–4 mol of P/mol of decarboxylase tetramer. It seems possible, therefore, that the rat heart complex may exhibit multi-site phosphorylation. We have not been able to ascertain the degree of phosphorylation of the complex in mitochondria at earlier times. This is because freshly prepared mitochondria contain at least 30% of unlabelled pyruvate dehydrogenase phosphate. We have been unable to make satisfactory experiments with mitochondria containing only active complex.

The results of these studies suggest that phosphorylation of sites additional to an inactivating site in the pyruvate dehydrogenase complex may regulate the rate of conversion of inactive complex into active complex in rat heart mitochondria. It seems to us very likely that inhibitory effects of diabetes, starvation and metabolism of acetate on re-activation of the complex by the phosphatase in heart muscle are mediated by phosphorylation of these additional sites. This conclusion is provisional, because it has to be confirmed by chemical methods of assay in addition to the bioassay methods used here.

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References

- Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) *Biochem. J.* **143**, 625–641
- Cooper, R. H., Randle, P. J. & Denton, R. M. (1975) *Nature (London)* **257**, 808–809
- Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–127
- Corbin, J. D. & Reiman, E. M. (1974) *Methods Enzymol.* **38**, 287–299
- Davis, P. F., Pettit, F. H. & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun.* **75**, 541–549
- Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
- Denton, R. M., Randle, P. J., Bridges, B. J., Cooper, R. H., Kerbey, A. L., Pask, H. T., Severson, D. L., Stansbie, D. & Whitehouse, S. (1975) *Mol. Cell. Biochem.* **9**, 27–53
- Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **93**, 678–687
- Hansford, R. G. (1976) *J. Biol. Chem.* **251**, 5483–5489
- Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **26**, 471–473
- Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348

- Kerbey, A. L., Radcliffe, P. M. & Randle, P. J. (1977) *Biochem. J.* **164**, 509–519
- Linn, T. C., Pettit, F. H. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241
- Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 327–342
- Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582
- Portenhauser, R. & Wieland, O. H. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 647–658
- Randle, P. J., Denton, R. M., Pask, H. T. & Severson, D. L. (1974) *Biochem. Soc. Symp.* **39**, 75–87
- Severson, D. L., Denton, R. M., Bridges, B. J. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237
- Sugden, P. H. & Randle, P. J. (1978) *Biochem. J.* **173**, 659–668
- Sugden, P. H., Hutson, N. J., Kerbey, A. L. & Randle, P. J. (1978) *Biochem. J.* **169**, 433–435
- Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774
- Wieland, O. H. (1975) *FEBS Lett.* **52**, 44–47
- Wieland, O. H., von Funcke, H. & Löffler, G. (1971a) *FEBS Lett.* **15**, 295–298
- Wieland, O. H., Siess, E. A., Schulze-Wethmar, F. H., von Funcke, H. & Winton, B. (1971b) *Arch. Biochem. Biophys.* **143**, 593–601