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Reversible phosphorylation of pyruvate dehydrogenase in rat skeletal-muscle mitochondria

Effects of starvation and diabetes

Stephen J. FULLER and Philip J. RANDLE
Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford OX3 9DU, U.K.

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The total activity of pyruvate dehydrogenase (PDH) complex in rat hind-limb muscle mitochondria was 76.4 units/g of mitochondrial protein. The proportion of complex in the active form was 34% (as isolated), 8–14% (incubation with respiratory substrates) and >98% (incubation without respiratory substrates). Complex was also inactivated by ATP in the presence of oligomycin B and carbonyl cyanide *m*-chlorophenylhydrazone. Ca^{2+} (which activates PDH phosphatase) and pyruvate or dichloroacetate (which inhibit PDH kinase) each increased the concentration of active PDH complex in a concentration-dependent manner in mitochondria oxidizing 2-oxoglutarate/L-malate. Values giving half-maximal activation were 10 nM-Ca^{2+}, 3 mM-pyruvate and 16 μM-dichloroacetate. Activation by Ca^{2+} was inhibited by Na^+ and Mg^{2+}. Mitochondria incubated with [^{32}P]P_i/2-oxoglutarate/L-malate incorporated ^{32}P into three phosphorylation sites in the α-chain of PDH; relative rates of phosphorylation were sites 1>2>3, and of dephosphorylation, sites 2>1>3. Starvation (48 h) or induction of alloxan-diabetes had no effect on the total activity of PDH complex in skeletal-muscle mitochondria, but each decreased the concentration of active complex in mitochondria oxidizing 2-oxoglutarate/L-malate and increased the concentrations of Ca^{2+}, pyruvate or dichloroacetate required for half-maximal reactivation. In extracts of mitochondria the activity of PDH kinase was increased 2–3-fold by 48 h starvation or alloxan-diabetes, but the activity of PDH phosphatase was unchanged.

Glucose is conserved in the rat in starvation and in alloxan-diabetes by diminished flux through the mitochondrial pyruvate dehydrogenase (PDH) complex (EC 1.2.4.1 + 2.3.1.12 + 1.6.4.3). The PDH complex is regulated by reversible phosphorylation catalysed by PDH kinase (EC 2.7.1.99) and PDH phosphatase (EC 3.1.3.43), phosphorylation resulting in inactivation. Starvation or diabetes leads to phosphorylation and inactivation of the complex in heart and skeletal muscle, liver, kidney and adipose tissue, the total concentration of complex (sum of active and inactive forms) being unchanged (reviewed by Reed, 1981; Wieland *et al.*, 1973; Randle *et al.*, 1978). Detailed studies in rat heart indicate that these effects of starvation and diabetes may be mediated by increased activity of PDH kinase through two distinct mechanisms. Oxidation of fatty acids and ketone bodies (which leads to phosphorylation and inactivation of the complex) may activate PDH kinase by increasing mitochondrial ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] (Garland & Randle, 1964; Pettit *et al.*, 1975; Kerbey *et al.*, 1976, 1979; Hansford, 1977; Pearce *et al.*, 1979). This mechanism is rapid in both onset and offset, and may be blocked by inhibitors of fatty acid oxidation (Caterson *et al.*, 1982).

A second mechanism, slower in onset and offset, involves a more stable factor which is present in heart mitochondria and mitochondrial extracts and which is independent of ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] (Hutson & Randle, 1978). This factor appears to be a mitochondrial protein formed by cytoplasmic protein synthesis in response to starvation and diabetes and which accelerates the PDH kinase reaction in heart mitochondria and mitochondrial

Abbreviation used: PDH, pyruvate dehydrogenase.

extracts (Kerbey & Randle, 1981, 1982). The total activity of PDH phosphatase in heart mitochondria is not changed by starvation or diabetes, but its effectiveness in re-activating phosphorylated complex may be decreased by multisite phosphorylation resulting from increased PDH kinase activity (Sugden *et al.*, 1978; Sale and Randle, 1982*a*,*b*). An important consequence of these mechanisms is that effects of starvation and diabetes on the concentration of active complex in the heart persist when the kinase is inhibited by pyruvate or dichloroacetate (Kerbey *et al.*, 1976) or when the phosphatase may be activated by Ca^{2+}, as in increased heart work (Illingworth & Mullings, 1976).

Although the regulation of glucose oxidation by cardiac muscle is of biochemical importance, its overall contribution to glucose conservation in starvation and diabetes is small in relation to the much greater mass of skeletal muscle. It is important therefore to show the relevance to skeletal muscle of mechanisms demonstrated only in cardiac muscle. It is known that flux through the PDH reaction is diminished in rat skeletal muscle by starvation and diabetes (for review see Randle *et al.*, 1978). It is known also that the concentration of active PDH complex in resting or contracting gastrocnemius muscle is decreased by starvation or diabetes (Hennig *et al.*, 1975; Hagg *et al.*, 1976) and in resting, but not in contracting, hindquarter muscle by ketone bodies (Hagg *et al.*, 1976). This apart, nothing has been known of reversible phosphorylation of skeletal-muscle PDH complex or of its regulation.

The objectives of the present study with rat skeletal-muscle mitochondria have been to demonstrate reversible phosphorylation of PDH complex, the main features of its regulation and mechanisms relevant to the effect of starvation and diabetes to decrease the proportion of active complex. Ashour & Hansford (1983) have described interconversion of active and inactive PDH complex in rat skeletal-muscle mitochondria and given evidence for regulation of interconversion by Ca^{2+}, dichloroacetate and [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios. These have not formed part of our own studies, except in so far as they relate to investigations of the effects of diabetes and starvation. Some comments on problems of interpretation of effects of the [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios are given in the General discussion section.

Experimental

Materials

Pig trypsin (type IX), trypsin inhibitor (type I-S) and bovine albumin fraction V were from Sigma (London) Chemical Co., Poole, Dorset; trypsin treated with Tos-Phe-CH_2Cl (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) was from Flow Laboratories, Irvine, Scotland, U.K. Sources of other materials are given in Kerbey *et al.* (1976), Hutson & Randle (1978) and Hutson *et al.* (1978). Bovine albumin was defatted by the method of Chen (1967). PDH complex was purified from pig hearts by the method of Kerbey *et al.* (1979), and partially and fully [^{32}P]phosphorylated complexes were prepared by the method of Sugden *et al.* (1978). PDH phosphatase was partially purified from ox heart by the method of Severson *et al.* (1974). Details of source, feeding, starvation and induction of alloxan-diabetes in male albino Wistar rats are given in Kerbey *et al.* (1977).

Skeletal-muscle mitochondria

The hind-limb muscles (approx. 25g) of 250–350g rats were trimmed of fat and connective tissue, chopped finely and dispersed (Ato-Mix homogenizer, full speed, 12.5s) into 180ml of isolation medium (100mM-Tris/HCl/10mM-EDTA/210mM-mannitol/70mM-sucrose/0.1% bovine albumin, pH7.4) (this step and all subsequent steps were at 0°C). After dilution with 100ml of isolation medium, homogenization was repeated, the homogenate incubated with stirring with 50mg of pig trypsin (type IX) for 15min, filtered through glass wool, centrifuged (10min, 500*g*), and mitochondria were collected from the filtered supernatant by sedimentation (8min, 10000*g*). Mitochondria were washed (twice) by resuspension and centrifugation in isolation medium (as above, but containing 10mM-Tris/HCl) and pooled before the final centrifugation. Mitochondria were suspended in isolation medium (containing 10mM-Tris/HCl) at 10–20mg of protein/ml. Yield was approx. 1mg of protein/g fresh wt. of muscle.

Incubation of mitochondria

Mitochondria (0.5–1mg of protein) were incubated at 30°C in 0.5ml of KCl medium (Kerbey *et al.*, 1976) in Eppendorff centrifuge tubes with further additions as given in the Results section. In incubations with [^{32}P]P_i, [phosphate] was 0.2mM.

Mitochondrial extracts

For assay of PDH complex (active form) and citrate synthase, mitochondria were separated by centrifugation (40s; Eppendorff 3200 centrifuge), the supernatants aspirated, and pellets frozen in liquid N_2. Extracts for assay were prepared by ultrasonic disintegration as described by Kerbey *et al.* (1976). For assay of ATP extracts were prepared with $HClO_4$ (Kerbey *et al.*, 1976). For assay of PDH kinase activity, mitochondrial pellets (after 15min incubation at 30°C without respiratory

substrate) were thawed and dispersed (by syringe aspiration) into 30mM-potassium phosphate/10mM-EGTA/1mM-Tos-Lys-CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one)/5mM-dithiothreitol/oligomycin B (25μg/ml), pH7.0 (2.75mg of mitochondrial protein/ml). The extract was frozen and thawed twice. For assay of PDH phosphatase, extracts were prepared exactly as for PDH kinase, but with 50mM-Tris/HCl/2mM-dithiothreitol, pH7.5, and at 5mg of protein/ml.

Phosphorylation-site occupancy in PDH

Mitochondria were prepared as described above, except that after the first wash inactive complex was converted into active complex by incubation for 5min at 30°C in 15ml of KCl medium. The incubation mixture was then diluted to 50ml with isolation medium (10mM-Tris/HCl, 0°C), and mitochondria were sedimented and resuspended as above. Mitochondria were then incubated in KCl medium (containing 0.2mM-[^{32}P]P_i; sp. radioactivity 1500–8500d.p.m./pmol) and samples taken at prescribed times into Triton medium at 0°C for assay of ^{32}P in PDH complex and in tryptic and tryptic/formic acid [^{32}P]phosphopeptides as described by Sale & Randle (1982*a*).

Analytical methods

PDH complex (active form) was assayed spectrophotometrically by coupling to arylamine acetyltransferase (Coore *et al.*, 1971); citrate synthase was assayed by the method of Srere *et al.* (1963), as modified by Coore *et al.* (1971). PDH kinase activity was assayed in mitochondrial extracts by the rate of ATP-dependent inactivation of PDH complex, and by the rate of incorporation of ^{32}P into PDH complex from [γ-^{32}P]ATP as described by Hutson & Randle (1978).

PDH phosphatase activity in mitochondrial extracts was assayed by the rate of release of [^{32}P]P_i from [^{32}P]phosphorylated pig heart complex (fully phosphorylated complex) and by the rate of conversion of inactive (partially phosphorylated) complex into active complex. Reaction was initiated by mixing, at 30°C, 100μl of mitochondrial extract, 50μl of phosphorylated PDH complex (10units/ml for fully phosphorylated complex; 5.6 or 7.4 units/ml for partially phosphorylated complex), and 50μl of 100mM-Tris/HCl/40mM-EGTA/39.6mM-$CaCl_2$/40mM-$MgCl_2$/4mM-Tos-Lys-CH_2Cl/2mM-dithiothreitol, pH7.5). Active complex and [^{32}P]P_i were assayed at 20s intervals thereafter for up to 80s. Corrections were applied for [^{32}P]P_i released in the absence of extract and for active complex measured both in the absence of extract and in the absence of phosphorylated pig heart complex. In phosphorylated pig heart complexes, typical relative site occupancies were (1:2:3) 0.34:0.33:0.33 (fully phosphorylated) and 0.82:0.12:0.06 (partially phosphorylated).

Mitochondrial protein was assayed by the method of Gornall *et al.* (1949). ATP was assayed in mitochondrial extracts with luciferase as described by Kerbey *et al.* (1977). The specific radioactivity of [γ-^{32}P]ATP was determined as described by Sugden *et al.* (1978). Protein-bound ^{32}P was assayed as described by Sale & Randle (1982*a*,*b*). Tryptic and tryptic/formic acid phosphopeptides were prepared with Tos-Phe-CH_2Cl-treated trypsin, separated by high-voltage paper electrophoresis and assayed for ^{32}P as described by Sale & Randle (1981, 1982*a*,*b*). ATPase activity was assayed by the method of Cooper *et al.* (1974). $CaCl_2$ solutions for preparing Ca–EGTA buffers were standardized by assay of Cl^- (Schales & Schales, 1941). Concentrations of Ca^{2+} were computed by methods given in Severson *et al.* (1974) and Portzehl *et al.* (1964). One unit of enzyme catalyses the conversion of 1μmol of substrate into product in 1min at 30°C.

Results and discussion

Mitochondria and mitochondrial incubations

In initial studies trypsin and Nagarse were compared in the preparation of rat skeletal-muscle mitochondria. Mitochondria prepared with either enzyme were indistinguishable in respect of yield, total concentration of complex (sum of active and inactive forms), proportion of complex in the active form and ATP concentration after incubation for 10min with 5mM-2-oxoglutarate (dipotassium salt)/0.5mM-L-malate (dipotassium salt). In the studies described below trypsin was used routinely. Addition of trypsin inhibitor (1.3-fold excess) at the end of incubation with trypsin had no obvious effect by the criteria given above and was therefore not used (results not shown).

The total concentration of PDH complex in rat skeletal-muscle mitochondria was 76.4$\pm$1.7 units/g of mitochondrial protein (mean$\pm$S.E.M. for 103 preparations) and that of citrate synthase was 921$\pm$23 units/g of protein (mean$\pm$S.E.M. for 50 preparations). These values are comparable with those of heart mitochondria (Kerbey *et al.*, 1976), and total PDH complex activity was comparable with that described for skeletal-muscle mitochondria by Ashour & Hansford (1983). In work described below, total PDH-complex activity was assayed in mitochondria incubated for 10min (or 15min in those from starved or diabetic rats) without respiratory substrate to effect conversion of inactive complex into active complex. Control experiments showed that conversion was complete within this time and that no further increase in

activity was effected in mitochondria by 1mM-potassium dichloroacetate (which inhibits PDH kinase; Whitehouse *et al.*, 1974); or by 4.5mM-EGTA/2mM-$CaCl_2$ (70nM-Ca^{2+}) (which activates PDH phosphatase; Denton *et al.*, 1972) (results not shown). No further increase in activity was observed when extracts of mitochondria were incubated with phosphatase (Table 1).

Concentrations of active complex and of ATP in mitochondria incubated with respiratory substrates are shown in Table 1 and, as discussed more fully in the next section, are comparable with those seen in heart mitochondria. Control experiments showed that steady-state concentrations of active PDH complex and ATP were attained in 5min and were maintained for at least 10min provided that incubation medium was re-aerated every 5min by vortex-mixing (results not shown). It was found that 30s of centrifugation was necessary to achieve full recovery of mitochondria (on the basis of assays of citrate synthase and active complex in no-substrate incubations; results not shown); 40s was used routinely. In mitochondrial extracts PDH complex (active form) was stable for at least 4h, and no interconversion of active and inactive forms was detected (results not shown); assays were completed within 15min of preparation of extracts.

Interconversion of active and inactive forms of PDH complex in skeletal-muscle mitochondria of normal fed rats

The concentration of active PDH complex in freshly prepared skeletal-muscle mitochondria (normal fed rats) was 26 units/g of protein, or 34% of total complex; ATP concentration was 1.87μmol/g of protein. Incubation for 5min at 30°C without respiratory substrate resulted in complete conversion of inactive complex into active complex, but ATP concentration did not change significantly (Table 1). Incubation of mitochondria with 2-oxoglutarate/L-malate or L-glutamate/L-malate or palmitoyl-L-carnitine decreased the concentration of active complex to approx. 6 units/g of protein, or 8% of total complex, and increased ATP to 5.5–5.9μmol/g of protein. With succinate as substrate the concentration of ATP was lower (2.4μmol/g of protein) and the concentration of active complex was higher (11units/g of protein; 14% of total complex) (see Table 1 for individual values and substrate concentrations).

Inactive PDH complex was converted into active complex when skeletal-muscle mitochondria were incubated with oligomycin B and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 10min. Addition of ATP then resulted in inactivation which was essentially complete within 2min. The degree of inactivation was dependent on ATP concentration, and 50% inactivation was achieved with approx. 6mM-ATP (Fig. 1).

The PDH phosphatase reaction is activated by Ca^{2+} (with Ca–EGTA buffers, [Ca^{2+}] for half-maximum activation is approx. 1μM; Randle *et al.*, 1974). In heart mitochondria addition of Ca^{2+} to the incubation medium leads to dephosphorylation and re-activation of PDH complex (Denton *et al.*, 1980; Sale & Randle, 1982*b*). As shown in Fig. 2, the concentration of active complex in rat skeletal-muscle mitochondria oxidizing 2-oxoglutarate/L-malate increased progressively when extra-mitochondrial [Ca^{2+}] was raised from 5.8 to 70nM to reach a maximum of 88±6.9% of total complex. The concentration of active complex fell when [Ca^{2+}] was increased above 70nM. The concentra-

Table 1. *Effect of respiratory substrates on the concentrations of active pyruvate dehydrogenase complex and ATP in skeletal-muscle mitochondria from fed normal rats*

Mitochondria were incubated for 5min in KCl medium with additions as shown and extracts prepared and assayed for complex (active form) and ATP (see the Experimental section). Results are means±S.E.M. for the numbers of incubations shown in parentheses. §Total complex (sum of active and inactive forms) assayed after conversion of inactive complex into active complex with PDH phosphatase. Otherwise assumed from value for active complex in no-substrate incubations. *$P<0.001$ against no-substrate control; †$P<0.001$ and ‡$P<0.02$ against other substrates.

	Concentration of		
	PDH complex (active form)		
	(units/g of protein)	(% of total complex)	ATP (μmol/g of protein)
Not incubated (as made)	26±1.80 (6)*	34±2.36 (6)*	1.87±0.15 (8)
No substrate	75±2.22 (33)	98±2.90§ (33)	1.48±0.18 (18)
5mM-2-Oxoglutarate/0.5mM-L-malate	6±0.77 (8)*	8±0.84 (8)*	5.75±0.16 (28)*
5mM-2-Glutamate/0.5mM-L-malate	6±0.92 (4)*	8±1.20 (4)*	5.50±0.22 (4)*
50μM-Palmitoyl-L-carnitine	6±0.92 (4)*	8±1.29 (4)*	5.91±0.28 (4)*
5mM-Succinate	11±1.33 (6)*‡	14±1.02 (6)*‡	2.38±0.03 (8)*†

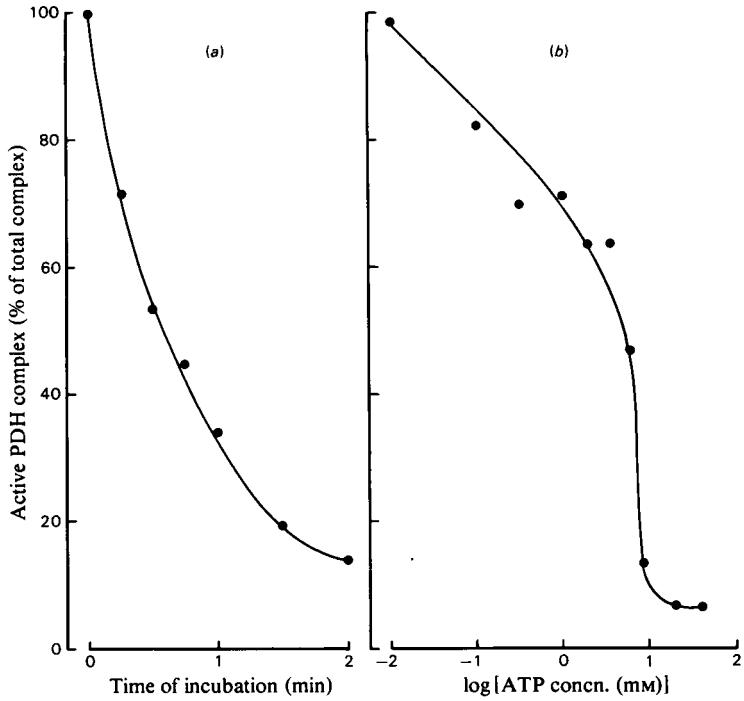


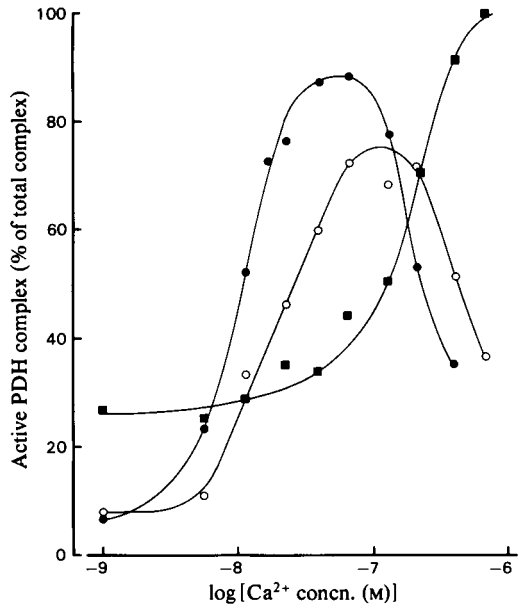
Fig. 1. *Steady-state inactivation of PDH complex in rat skeletal-muscle mitochondria by ATP*
Mitochondria (0.5–1 mg of protein) were incubated for 10 min at 30°C in 0.5 ml of KCl medium, final pH 7.2 (120 mM-KCl/20 mM-Tris/HCl/5 mM-potassium phosphate/2 mM-EGTA) containing 10 μM-carbonyl cyanide *m*-chlorophenylhydrazone and 25 μg of oligomycin B/ml, and then with addition of ATP to 10 mM for the further time shown (*a*) or with the addition of ATP to the concentration shown for an additional 5 min (*b*). For details of extraction and of assay of PDH complex see the Experimental section. Each point is the mean of at least three observations from two mitochondrial preparations.

tion of Ca^{2+} required for 50% active complex ($K_{0.5}$) was approx. 10 nM. The curves relating $[Ca^{2+}]$ to the concentration of active complex were shifted to the right by 15 mM-NaCl or 15 mM-$MgCl_2$, which may lower intramitochondrial $[Ca^{2+}]$ by countertransport (NaCl) or by inhibition of Ca^{2+} entry ($MgCl_2$) (Crompton *et al.*, 1976, 1978). The $K_{0.5}$ for Ca^{2+} was increased 2-fold by NaCl and 20-fold by $MgCl_2$. The effects of Ca^{2+} in rat skeletal-muscle mitochondria in the present study are qualitatively similar to those seen in heart mitochondria by Denton *et al.* (1980), as are the effects of NaCl and $MgCl_2$. The $K_{0.5}$ for Ca^{2+} in skeletal-muscle mitochondria was, however, lower (10 nM as opposed to 40 nM); we have confirmed the higher $K_{0.5}$ (50 nM-Ca^{2+}) in heart mitochondria (results not shown). Direct comparison of $K_{0.5}$ (present study) with values obtained by Ashour & Hansford (1983) is not possible, because incubation conditions were not strictly comparable, but the values appear broadly similar.

The PDH kinase reaction is inhibited by pyruvate (Linn *et al.*, 1969) and by dichloroacetate (Whitehouse *et al.*, 1974), and in heart mitochondria these compounds increase the concentration of active PDH complex (Cooper *et al.*, 1974; Whitehouse *et al.*, 1974). The effects of sodium pyruvate (60 μM–100 mM) and of potassium dichloroacetate (4 μM–5 mM) on the concentration of active complex are shown in Fig. 3. The concentrations giving 50% active complex were 3 mM-sodium pyruvate and 16 μM-potassium dichloroacetate.

Evidence that interconversion of active and inactive forms of PDH complex in rat skeletal-muscle mitochondria is due to reversible phosphorylation

When rat heart mitochondria are incubated with $[^{32}P]P_i$, the γ-phosphate group of ATP becomes fully labelled within 30 s (Hutson *et al.*, 1978). In the following work we have assumed that the equilibration in skeletal-muscle mitochondria is equally rapid. This assumption appears reasonable, because, as in heart mitochondria, approx. 75% of ATP was synthesized *de novo* from $[^{32}P]P_i$ when skeletal-muscle mitochondria were incubated with 2-oxoglutarate/L-malate. Only approx.



25% of ATP was labelled by turnover of its γ-phosphate group.

As shown in Fig. 4, autoradiography of sodium dodecyl sulphate/polyacrylamide gels of proteins from rat skeletal-muscle mitochondria incubated with [^{32}P]P_i/2-oxoglutarate/L-malate showed a single band of radioactivity which co-migrated with the [^{32}P]phosphorylated α-chain of pig heart

Fig. 2. *Effect of Ca^{2+} concentration on steady-state activity of PDH complex in rat skeletal-muscle mitochondria*

Mitochondria (0.5–1 mg of protein) were incubated for 5 min at 30°C in 0.5 ml of KCl medium (see Fig. 1) containing 5 mM-2-oxoglutarate/0.5 mM-L-malate/4.5 mM-EGTA and concentrations of $CaCl_2$ computed to give the free Ca^{2+} concentrations shown (●), or with the further addition of 15 mM-NaCl (○) or 15 mM-$MgCl_2$ (■). Each point is the mean of four observations from three mitochondrial preparations.

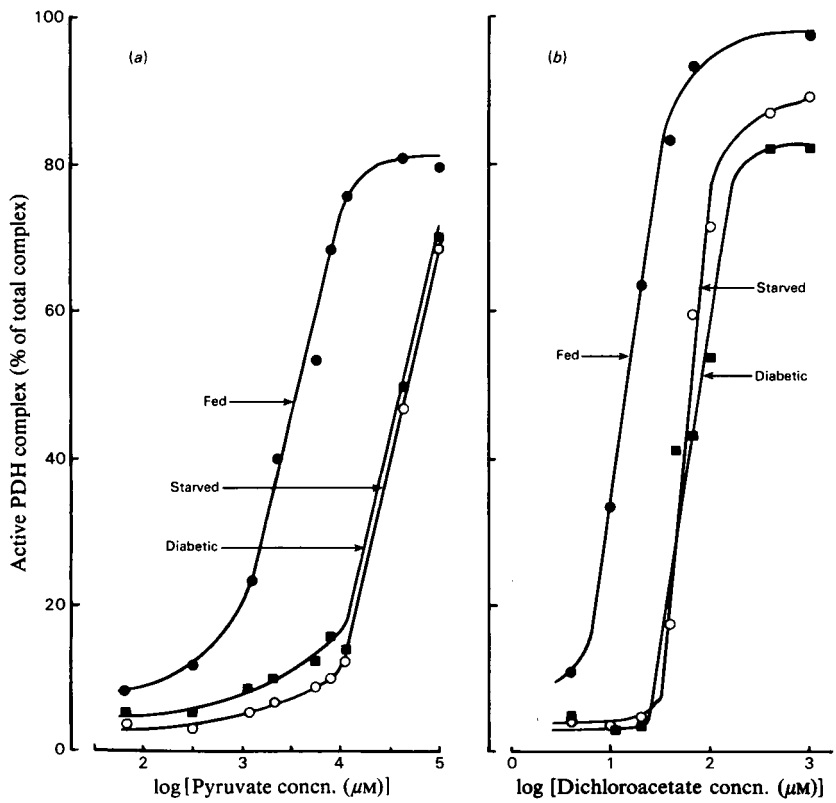


Fig. 3. *Effect of pyruvate or dichloroacetate concentrations on steady-state PDH-complex activity in skeletal-muscle mitochondria from fed (●), 48 h-starved (○) or alloxan-diabetic (■) rats*

Mitochondria (0.5–1 mg of protein) were incubated for 5 min at 30°C in 0.5 ml of KCl medium (see Fig. 1) containing 5 mM-2-oxoglutarate/0.5 mM-malate with the concentrations of pyruvate (*a*) or of dichloroacetate (*b*) shown. Each point is the mean of eight observations (control), six observations (starved) or four observations (diabetic) from four, three, or two mitochondrial preparations respectively.

PDH (distances from origin were: pig heart complex, 4.00cm; rat skeletal-muscle-mitochondrial complex, 3.97cm). The computed M_r values were 44900 (pig heart complex) and 45200 (skeletal-muscle-mitochondrial complex). Further support for the conclusion that the [^{32}P]phosphorylated protein in rat skeletal-muscle mitochondria is the [^{32}P]phosphorylated α-chain of rat skeletal-muscle PDH was obtained by high-voltage electrophoresis at pH 1.9 of [^{32}P]phosphopeptides generated by cleavage with trypsin and by cleavage of tryptic phosphopeptides with formic acid.

In ox kidney and pig heart PDH complexes there are three sites of phosphorylation, which are recovered in two tryptic peptides TA (containing sites 1 and 2) and TB (containing site 3) (for amino acid sequences see Yeaman *et al.*, 1978; Sugden *et al.*, 1979). There are three variants of TA in which site 1 (TA1) or site 2 (TA2) or both sites (TA12) are phosphorylated. Sites 1 and 2 in TA may be separated by cleavage of the Asp^8–Pro^9 bond between the sites with formic acid (Sale & Randle, 1981, 1982*a*). Fully phosphorylated complex yields only TA12 and TB3. Because relative rates of phosphorylation are site 1 > site 2 > site 3, partially phosphorylated complexes yield TA1, with smaller amounts of TA2, TA12 and TB3 (Sale & Randle, 1981, 1982*a*). Rat heart complex is very similar to pig heart complex in respect of the number of sites of phosphorylation and their occupancy (Sale & Randle, 1982*a*,*b*).

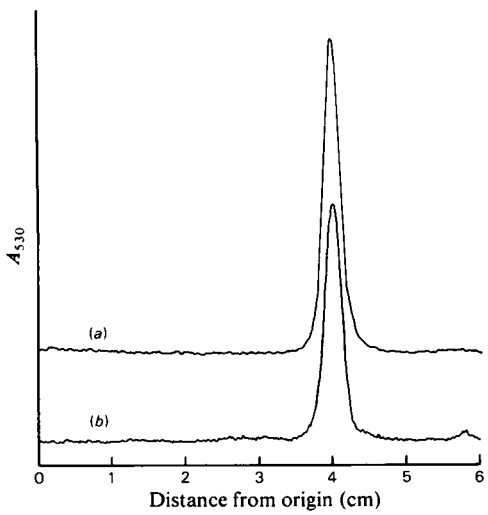


Fig. 4. *Scan of an autoradiograph of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of* (*a*) *proteins of skeletal-muscle mitochondria incubated for 20min with 0.2mM-*[^{32}*P*]P_i *(575d.p.m./pmol)/5mM-2-oxoglutarate/0.5mM-L-malate and* (*b*) [^{32}*P*]*phosphorylated pig heart PDH complex*

When rat skeletal-muscle mitochondria were incubated with [^{32}P]P_i/2-oxoglutarate/L-malate for periods of 10s–20min, three different tryptic [^{32}P]phosphopeptides were detected on high-voltage paper electrophoresis. Their mobilities relative to N^6-dinitrophenyl-lysine were 1.44 ± 0.01, 1.02 ± 0.01 and 0.71 ± 0.01 (means ± S.E.M. for 47 observations). These correspond closely in mobility to the three tryptic phosphopeptides (TA1 + TA2), TA12 and TB3 of pig heart complex (and rat heart complex). After incubation with 90% (v/v) formic acid for 140h at 37°C, phosphopeptide TA12 (purified by electrophoresis at pH 1.9) yielded two phosphopeptides with relative mobilities of 1.33 ± 0.01 and 0.86 ± 0.01 (means ± S.E.M. for 45 observations). These values correspond closely to those of the pig heart PDH-complex phosphopeptides TF1 (containing site 1) and TF2 (containing site 2) (Sale & Randle, 1981, 1982*a*).

The time-dependent changes in the concentrations of tryptic phosphopeptides TA1, TA2, TA12 and TB3 and the occupancies of the three sites during phosphorylation and inactivation of PDH complex in rat skeletal-muscle mitochondria incubated with [^{32}P]P_i/2-oxoglutarate/L-malate are shown in Figs. 5(*a*) and 5(*b*). The results show that the relative rates of phosphorylation were site 1 > site 2 > site 3. Formic acid cleavage of TA12 showed that the relative concentrations of ^{32}P in sites 1 and 2 were $50.0 \pm 0.8\%$ and $50.0 \pm 0.8\%$ (means ± S.E.M. for six observations), showing that sites 1 and 2 in TA12 were phosphorylated to equivalence with [γ-^{32}P]ATP in the mitochondrion. The relationship between inactivation of the complex and the occupancies of the three sites of phosphorylation is shown in Fig. 5(*c*). Inactivation was correlated closely with phosphorylation of site 1, and incorporation into site 1 was 0.54 ± 0.023nmol of P/unit of complex inactivated (mean ± S.E.M. for 13 degrees of freedom). The highest incorporations into sites 2 and 3 relative to site 1 seen in these experiments were 1.0 (site 2) and 0.70 (site 3) (results not shown). As shown in Fig. 5(*a*), there was very little incorporation of ^{32}P into site 2 in the absence of incorporation into site 1 (peptide TA2). By using methods given by Sale & Randle (1982*a*), the contributions of sites 1 and 2 to inactivation were computed to be 98.4% and 1.6% respectively in the steady state in a further experiment (results not shown).

Relative rates of dephosphorylation of the three sites of phosphorylation in rat heart complex by PDH phosphatase are site 2 > site 1 > site 3 (Sale & Randle, 1982*a*). Relative rates of dephosphorylation of the three sites of phosphorylation in rat

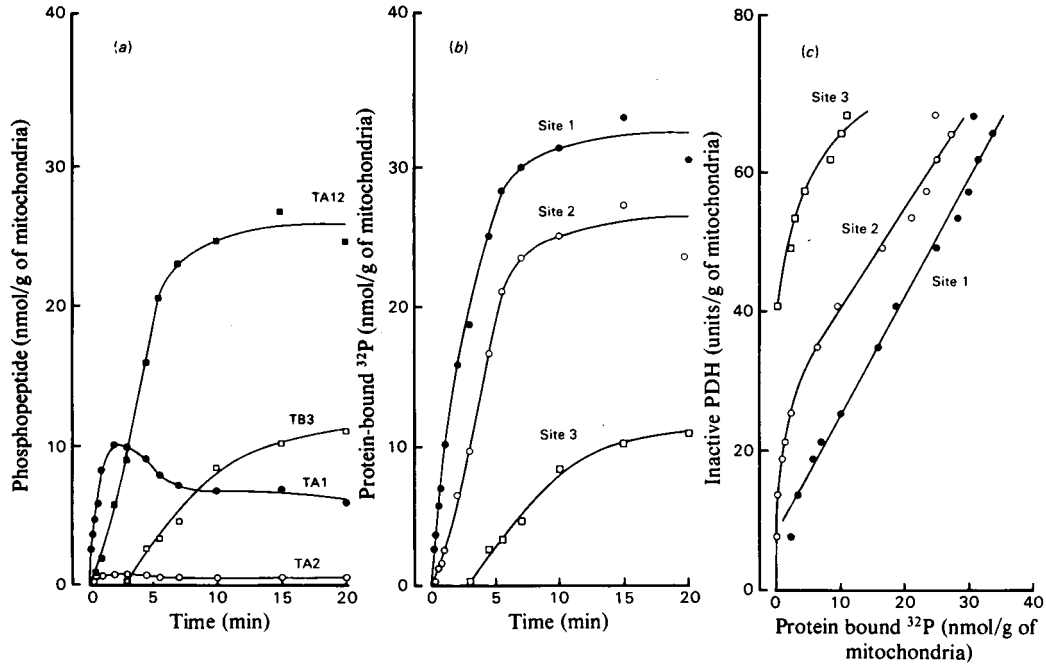


Fig. 5. *Incorporation of ^{32}P from [^{32}P]P_i in rat skeletal-muscle mitochondria into: (a) tryptic [^{32}P]phosphopeptides; (b) sites of phosphorylation in PDH complex; (c) sites of phosphorylation in PDH complex as related to degree of inactivation of the complex*

Inactive PDH complex was converted into active complex during the course of preparation of mitochondria by 5min of incubation at 30°C (see the Experimental section). Mitochondria were then warmed to 30°C (3min) in KCl medium (see Fig. 1) containing 0.2mM-[^{32}P]P_i (3700d.p.m./pmol), and phosphorylation and inactivation of PDH complex were initiated by addition of 2-oxoglutarate (to 5mM) and L-malate (to 0.5mM). For definition of TA1, TA2, TA12 and TB3 in (*a*) and sites 1, 2 and 3 (*b* and *c*) see the text. Activity of PDH complex was assayed in parallel non-radioactive incubations under otherwise identical conditions. Each point is the mean of two to four observations on a single preparation of mitochondria.

skeletal-muscle mitochondria complex have been estimated by measurement of turnover of ^{32}P (i.e. ^{31}P for ^{32}P) as described by Sale & Randle (1982*a*) for heart mitochondria. Apparent first-order rate constants (min^{-1}) for turnover for the three sites were 0.08 ± 0.01 (site 1), 0.18 ± 0.02 (site 2) and 0.02 ± 0.01 (site 3) [means $\pm$ S.E.M., for eight time points (sites 1 and 2) or four time points (site 3)] (results not shown).

In rat skeletal-muscle mitochondria incubated with [^{32}P]P_i/2-oxoglutarate/L-malate, steady-state incorporation of ^{32}P into the PDH complex was inhibited progressively by concentrations of potassium dichloroacetate over the range 2–10μM. Analysis of site occupancy showed that the degree of inactivation of the complex was correlated with occupancy of site 1 and that occupancy of sites 2 and 3 was less than that of site 1 (site 3 < site 2) (results not shown).

Effects of 48h starvation and alloxan-diabetes on the concentration of active PDH complex in rat skeletal-muscle mitochondria

The total concentration of PDH complex in skeletal-muscle mitochondria was unaffected by 48h starvation or alloxan-diabetes. Values (units/g of protein) were 76.6 ± 2.3 (71) (control), 77.3 ± 3.1 (19) (48h-starved), 73.7 ± 2.8 (13) (alloxan-diabetic) (means $\pm$ S.E.M. for the numbers of mitochondrial preparations in parentheses). Citrate synthase activity was also unchanged (results not shown). Therefore activity of either enzyme may be used as an index of recovery in comparing different batches of mitochondria, and in work described below use of either index led to the same conclusions.

The concentration of active complex was decreased by starvation or diabetes in mitochondria incubated for 5min with 2-oxoglutarate/malate.

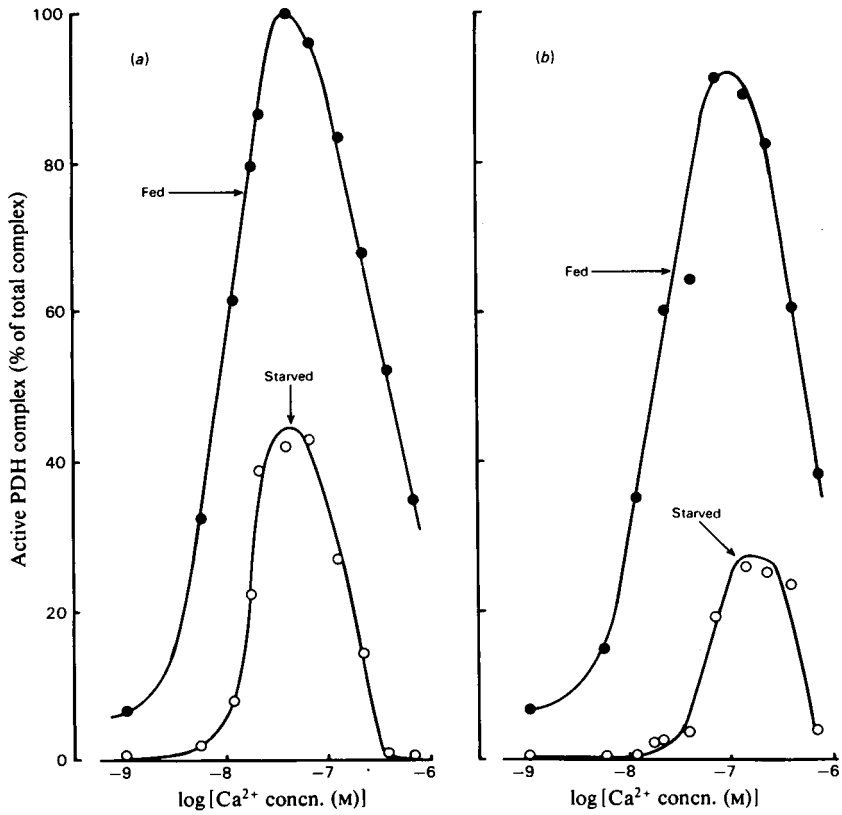


Fig. 6. *Effect of Ca^{2+} concentration on activity of PDH complex in skeletal-muscle mitochondria; effect of 48h starvation of the rat*

Mitochondria were incubated as described in the legend to Fig. 2 in the absence (*a*) or presence (*b*) of 15mM-NaCl. Each point is the mean of two observations on a single preparation of mitochondria.

Values (% of total) were 9.6 ± 0.6 (8) (control), 3.5 ± 0.5 (6) (starved) and 5.8 ± 0.5 (4) (diabetic) (means $\pm$ S.E.M., for the numbers of observations in parentheses; two observations per preparation; $P < 0.01$ for starved or diabetic versus control). As shown in Fig. 3, this difference between fed and starved and control and diabetic was maintained when the proportion of active complex was increased by the PDH kinase inhibitors sodium pyruvate and potassium dichloroacetate. The concentrations of the effectors required for 50% active complex were increased from 3mM-pyruvate and 16μM-dichloroacetate (control) to 35mM-pyruvate and 68μM-dichloroacetate (starved) and to 32mM-pyruvate and 87μM-dichloroacetate (diabetic). Results given in Fig. 6 show that the difference between fed and starved rats in respect of the concentration of active complex in skeletal-muscle mitochondria persisted in the presence of Ca-EGTA buffers computed to give Ca^{2+} concentrations in the range 5.8–713nM. The effect of starvation was to lower the concentration of active complex at all concentrations of Ca^{2+} tested, in the presence (Fig. 6*b*) and absence (Fig. 6*a*) of NaCl, and to decrease the peak concentration of active complex without significant alteration in the concentration of Ca^{2+} at which this peak was achieved.

Effects of 48h starvation and alloxan-diabetes on the activity of PDH kinase and of PDH phosphatase in extracts of rat skeletal-muscle mitochondria

The effect of starvation or diabetes to lower the concentration of active complex in skeletal-muscle mitochondria could result from an increase in PDH kinase activity, or from a decrease in PDH phosphatase, or from a combination of the two. The purpose of the experiments described below was to ascertain whether diabetes or starvation have effects on the activities of PDH kinase or PDH phosphatase in extracts of mitochondria under defined conditions. Mitochondria were

incubated for 15min at 30°C in the absence of respiratory substrate before extraction to convert all of the complex into the active (dephosphorylated) form.

Kinase activity. As shown in Fig. 7(*a*) the rate of inactivation by ATP of PDH complex in extracts of rat skeletal-muscle mitochondria from 48h-starved or alloxan-diabetic rats was increased relative to that in fed normal controls. This increased rate of inactivation was associated with an increased rate of incorporation of ^{32}P from [γ-^{32}P]ATP into the complex (Fig. 7*b*). Inactivation was a pseudo-first-order reaction, and the apparent first-order rate constants (min^{-1}; means $\pm$ S.E.M. for three mitochondrial preparations in each group) were: control, 0.51 ± 0.02 (14 observations each at 1, 2, 3 and 6min); starved, 1.82 ± 0.11 (9 observations each at 20, 40, 60 and 90s); diabetic, 1.0 ± 0.05 (10 observations each at 20, 40, 60 and 90s); no-ATP control, 0.01 (combined means of three preparations from each group). Increased incorporation of ^{32}P in starved and diabetic rats after 6min (when inactivation was approaching completion in all three types of extract) may be due to increased phosphorylation of sites (2+3) in starved and diabetic rats (results not shown).

Control experiments showed no differences in the rate of ATP hydrolysis under the conditions of the kinase assay in the three groups (12% in 6min), and no activity of PDH phosphatase in mitochondrial extracts under the conditions of the kinase assay (assay based on release of [^{32}P]P_i from

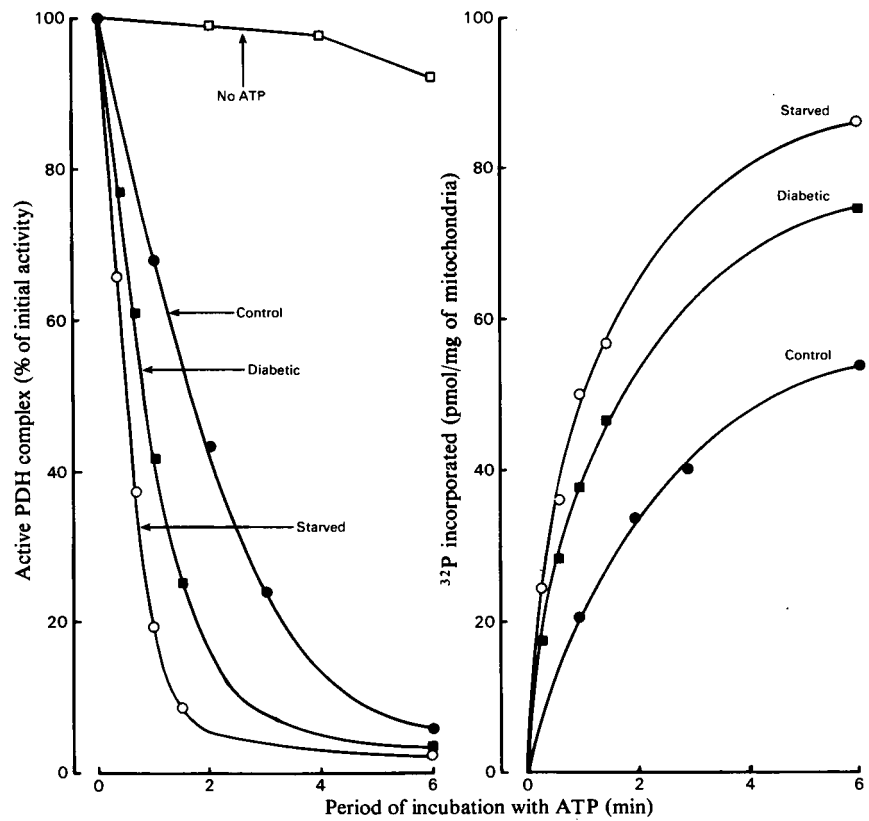


Fig. 7. *Effect of 48h starvation or alloxan-diabetes on the activity of PDH kinase in extracts of rat skeletal muscle mitochondria*

Extracts (see the Experimental section) were prepared from mitochondria in which inactive PDH complex had been converted into active complex by incubation in KCl medium (see Fig. 1) for 15min at 30°C. The PDH kinase reaction was initiated by addition of [γ-^{32}P]ATP (60–90 d.p.m./pmol) to 0.3mM, and samples were taken for assay of PDH complex and ^{32}P in phosphorylated complex at the times shown. Each point is the mean of nine or more observations from three mitochondrial preparations (complex activity) or of six observations from two mitochondrial preparations (^{32}P incorporation). Initial concentrations of PDH complex in incubations were (means $\pm$ S.E.M., in munits/ml) 180 ± 7.3 (fed control), 169 ± 3.6 (48h starved) and 171 ± 4.6 (alloxan-diabetic) (27 observations for each).

[^{32}P]phosphorylated pig heart complex, 1.7 units/ml) (results not shown). Because of the use of extracts of mitochondria and because mitochondria were preincubated without respiratory substrates before extraction, known metabolite activators of kinase (NADH, acetyl-CoA) were absent (results not shown).

PDH phosphatase activity. The activity of PDH phosphatase in extracts of rat skeletal-muscle mitochondria measured with phosphorylated pig heart PDH complex was not changed by starvation or induction of alloxan-diabetes in the rat. In assays based on release of [^{32}P]P_i from [^{32}P]phosphorylated pig heart complex, values (in nmol [^{32}P]P_i released/min per mg of mitochondrial protein in the assay) were 0.34 ± 0.05 (control), 0.34 ± 0.02 (starved) and 0.33 ± 0.06 (diabetic) (means $\pm$ S.E.M., for four preparations of mitochondria and three or four observations at 20, 40 and 60s per preparation). Progress curves for samples taken at 20, 40 and 60s were linear. In assays based on release of active complex, reaction was pseudo-first-order and the apparent first-order rate constants (min^{-1}) were 0.93 ± 0.06 (control), 0.96 ± 0.05 (starved) and 0.99 ± 0.13 (diabetic) (means $\pm$ S.E.M., for four preparations of mitochondria and three observations on each at 20, 40, 60 and 80s).

General discussion and conclusions

The mitochondria used in the present study were derived from a mixture of red and white muscles, because essentially all of the hind-limb muscles were used. The features of reversible phosphorylation in the PDH complex in skeletal-muscle mitochondria of normal fed rats in the present study showed no gross differences from those seen in heart mitochondria. The concentrations of total complex and of active complex in freshly prepared mitochondria and in mitochondria incubated with respiratory substrates were strictly comparable under the same conditions (cf. Kerbey *et al.*, 1976, 1977; Sale & Randle, 1980, 1982*a*). The conversion of inactive complex into active complex induced by pyruvate, dichloroacetate or Ca^{2+} was also similar, except that $K_{0.5}$ for pyruvate was higher and $K_{0.5}$ for Ca^{2+} was lower in skeletal-muscle mitochondria than in heart mitochondria under comparable conditions (cf. Whitehouse *et al.*, 1974; Kerbey *et al.*, 1976; Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981). The concentrations of active complex in mitochondria incubated with glutamate/malate or with palmitoylcarnitine were lower than those observed in skeletal-muscle mitochondria by Ashour & Hansford (1983). The reason for this difference is not apparent, except that the incubation time was 5min in the present study and 10min in the study by Ashour & Hansford (1983). In our studies, extending the incubation time to 10min had no effect, provided that the medium was re-aerated after 5min. Failure to re-aerate resulted in an increase in active complex.

The incorporation of ^{32}P into site 1 in skeletal-muscle mitochondrial PDH (in nmol of P/unit of complex inactivated) was at the lower limit of values obtained in heart mitochondrial complex (Sale & Randle, 1980, 1981, 1982*a*,*b*). The relative rates of phosphorylation and of turnover (^{31}P for ^{32}P) of the three sites of phosphorylation, the relationship between occupancy of phosphorylation sites and the fraction of complex in the inactive form, and the relative contributions of phosphorylation of sites 1 and 2 to inactivation of the complex in skeletal-muscle mitochondria, were similar to values obtained with heart mitochondria by Sale & Randle (1980, 1982*a*), as were the electrophoretic mobilities of tryptic and tryptic/formic acid [^{32}P]phosphopeptides.

The effects of 48h starvation or alloxan-diabetes on reversible phosphorylation in the PDH complex in rat skeletal-muscle mitochondria were comparable with those seen in heart mitochondria and indicative of similar mechanisms. Two findings appear to be of particular significance. The activity of PDH phosphatase in extracts of mitochondria measured at $3\mu M$-Ca^{2+} was unaffected by starvation or diabetes. Moreover, the shape of the curves relating the concentration of active complex in skeletal-muscle mitochondria to Ca^{2+} concentration was not affected by starvation, although at each concentration of Ca^{2+} the concentration of active complex was lowered. This might suggest that the activity of PDH phosphatase in mitochondria is not changed by starvation or diabetes. The activity of PDH kinase in extracts of skeletal-muscle mitochondria was increased 2–3-fold by starvation or diabetes, as in extracts of heart mitochondria (cf. Kerbey & Randle, 1981, 1982). This finding is especially important. It indicates that the stable mechanism of increased PDH kinase activity which is independent of acetyl-CoA and NADH may be of more general significance and perhaps fundamental to the conservation of glucose in starvation and in diabetes. It is capable, for example, of providing an explanation of the effect of starvation and of diabetes to lower the concentration of active complex in both resting and contracting skeletal muscle and at high and low work loads in cardiac muscle (Hennig *et al.*, 1975; Hiraoka *et al.*, 1980; McCormack & Denton, 1981; Sale & Randle, 1982*b*).

The role of fatty acid (and ketone-body) oxidation in the effect of starvation and diabetes on the

concentration of active complex in skeletal muscle is less clear than in heart muscle. In heart muscle enhanced fatty acid oxidation is obligatory because the effect of starvation or diabetes was reversed when fatty acid oxidation was inhibited by 2-tetradecylglycidate (Caterson *et al.*, 1982). In skeletal-muscle mitochondria 2-tetradecylglycidate failed to reverse effects of starvation or diabetes on the concentration of active complex (Caterson *et al.*, 1982). This may well have been due to failure to inhibit fatty acid oxidation, and further study is required. Ashour & Hansford (1983) found that oxidation of palmitoylcarnitine by skeletal-muscle mitochondria may decrease the proportion of active complex and increase [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] in the absence of Ca^{2+}, but not in its presence. There is a need for a systematic study of the effects of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios on the concentration of active complex in skeletal-muscle mitochondria. Such a study should take account of the finding that activation of the PDH kinase reaction by acetyl-CoA is most evident at [acetyl-CoA]/[CoA] ratios between 0 and 0.6 (and may reach a plateau at unit ratio) and at low [NADH]/[NAD^+] ratios (Kerbey *et al.*, 1976, 1979).

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