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Incorporation of [^{32}P]Phosphate into the Pyruvate Dehydrogenase Complex in Rat Heart Mitochondria

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1. Evidence is given for three sites of phosphorylation in the α-chains of the decarboxylase component of purified rat heart pyruvate dehydrogenase complex, analogous to those established for procine and bovine complexes. Inactivation of rat heart complex was correlated with phosphorylation of site 1. Relative initial rates of phosphorylation were site 1 > site 2 > site 3. 2. Methods are described for measurement of incorporation of $^{32}P_i$ into the complex in rat heart mitochondria oxidizing 2-oxoglutarate + L-malate (total, sites 1, 2 and 3). Inactivation of the complex was related linearly to phosphorylation of site 1 in mitochondria of normal or diabetic rats. The relative initial rates of phosphorylation were site 1 > site 2 > site 3. Rates of site-2 and site-3 phosphorylation may have been closer to that of site 1 in mitochondria of diabetic rats than in mitochondria of normal rats. 3. The concentration of inactive (phosphorylated) complex was varied in mitochondria from normal rats by inhibiting the kinase reaction with pyruvate at concentrations ranging from 0.15 to 0.4 mM. The results showed that the concentration of inactive complex is related linearly to incorporation of $^{32}P_i$ into site 1. Inhibition of $^{32}P_i$ incorporations with pyruvate at all concentrations over this range was site 3 > site 2 > site 1. 4. With mitochondria from diabetic rats, pyruvate (0.15–0.4 mM) inhibited incorporations of $^{32}P_i$ into site 3, but it had no effect on the concentration of inactive complex or on incorporations of $^{32}P_i$ into site 1 or site 2. It is concluded that site-3 phosphorylation is not required for inactivation of the complex in rat heart mitochondria. 5. Evidence is given that phosphorylation of sites 2 and 3 may inhibit reactivation of the complex by dephosphorylation in rat heart mitochondria.

Bovine and porcine pyruvate dehydrogenase complexes (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) are inactivated by phosphorylation of a single serine residue in an α-chain of the pyruvate decarboxylase component (EC 1.2.4.1; subunit composition $\alpha_2\beta_2$). Complete phosphorylation results in phosphorylation of two further serine residues (Sugden & Randle, 1978; Yeaman *et al.*, 1978; Sugden *et al.*, 1979). Phosphorylation of these two additional serine residues has been shown, in the pig heart complex, to inhibit reactivation of the complex by pyruvate dehydrogenase phosphate phosphatase (Sugden *et al.*, 1978). In rat heart mitochondria the proportion of active complex is determined by the relative activities of kinase and phosphatase. Pyruvate, which inhibits the kinase reaction, increases the proportion of active complex; this effect of pyruvate is substantially inhibited in heart mitochondria from alloxan-diabetic or starved rats (Kerbey *et al.*, 1976, 1977; Hutson *et al.*, 1978).

Abbreviation used: SDS, sodium dodecyl sulphate.

In the present study we have investigated the incorporation of $^{32}P_i$ into pyruvate dehydrogenase in heart mitochondria from normal and alloxan-diabetic rats. Methods have been developed for studying total incorporation and incorporation into three sites of phosphorylation; and to assess their functional significance in regulation of the rat heart complex. Evidence for incorporation of $^{32}P_i$ into the α-chain of the complex in rat-epididymal-fat-pad mitochondria and into multiple sites of phosphorylation in rat heart and rat fat-pad mitochondria has been given by Hughes & Denton (1976, 1978).

Experimental

Materials

The sources of biochemicals, chemicals and enzymes were as given by Kerbey *et al.* (1976, 1977, 1979), Hutson & Randle (1978), Sugden & Randle

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(1978) and Sugden *et al.* (1979). Pig heart pyruvate dehydrogenase complex was purified by the method of Kerbey *et al.* (1979) and converted into pyruvate dehydrogenase phosphate (fully phosphorylated form) as described by Sugden *et al.* (1978). [γ-^{32}P]ATP and $^{32}P_i$ (1–10mCi/ml in HCl, pH 2–3) were from The Radiochemical Centre, Amersham, Bucks, U.K. Whatman 3MM paper for high voltage electrophoresis was washed with 200ml of 1mM-EDTA followed by 200ml of electrophoresis buffer and air dried at room temperature. Lipoamide dehydrogenase was from BCL, Lewes, East Sussex, U.K.

Rats and rat heart mitochondria

Details of feeding of rats and of induction of alloxan-diabetes were as given by Kerbey *et al.* (1977). Mitochondria were prepared from rat hearts with Nagarse as described by Chappell & Hansford (1978). Rat-heart mitochondrial incubations were made at 30°C in KCl medium [125mM-KCl/20mM-Tris/HCl(pH 7.4)/2mM-EDTA/0.2mM-potassium phosphate]. Other additions were as given in text, Tables or Figures. Incubations with $^{32}P_i$ were made in capped 2ml tubes containing KCl medium, 0.3ml (where only protein-bound $^{32}P_i$ was measured) or 1ml (where $^{32}P_i$ in tryptic phosphopeptides was to be measured) and approximately 2mg of mitochondrial protein/ml. The specific radioactivity of phosphate varied between 0.15–1.25mCi/μmol (for details of individual experiments see Tables and Figures). The HCl in the $^{32}P_i$ was approximately neutralized by addition of 20μl of 0.5M-$KHCO_3$/ml of $^{32}P_i$ added to KCl medium. For assays of pyruvate dehydrogenase, parallel incubations of mitochondria were made in non-radioactive medium (0.5ml) in Eppendorf centrifuge tubes under otherwise identical conditions. In all incubations aeration was renewed at 5-min intervals by vortex mixing the solution.

Measurement of protein-bound ^{32}P in mitochondria

Duplicate samples of incubated mitochondria (10μl) were spotted on to 2cm × 1cm pieces of Whatman 3MM paper and phosphorylation was terminated by dropping them into ice-cold 10% (w/v) trichloroacetic acid. The procedure was then as described by Corbin & Reimann (1974). Radioactivity on paper squares was assayed by liquid scintillation spectrometry with toluene-based scintillator (Severson *et al.*, 1974). Blanks were incorporated, using medium without mitochondria. For determination of the specific activity of $^{32}P_i$ in KCl medium samples were assayed for radioactivity in methoxyethanol/toluene scintillator (Severson *et al.*, 1974).

Measurement of ^{32}P in tryptic phosphopeptides from mitochondrial protein

After taking samples for assay of protein-bound $^{32}P_i$ (see preceding section) 0.1vol. of 100% (w/v) trichloroacetic acid was added to the mitochondrial incubate. After mixing and cooling to 0°C, the denatured protein was separated by centrifuging (5min, 1400*g*) and the supernatant aspirated. The pellets were washed (2ml of 10% trichloroacetic acid) by alternately resuspending and centrifuging until the supernatant radioactivity was less than 5% of that of the pellet (usually six washes). The pellets were dissolved in 0.2ml of formic acid, cooled to 0°C, and 0.4ml of ice-cold performic acid added (formic acid incubated at room temperature for 2h with 0.05vol. of H_2O_2, 20vol. strength and cooled to 0°C). After 2.5h of incubation at 0°C, 10ml of water was added and the samples were freeze-dried overnight. These operations were completed on the day of mitochondrial incubation.

The freeze-dried solids were dissolved in 50μl of 8M-urea/2% (w/v) NH_4HCO_3, diluted with 150μl of 2% (w/v) NH_4HCO_3, and 0.05vol. of trypsin solution was added [20mg/ml in 2M-urea/2% (w/v) NH_4HCO_3] and incubated for 6h at 30°C. This rendered >90% of the ^{32}P soluble in 10% trichloroacetic acid. The tryptic digests were then subjected to high-voltage electrophoresis on Whatman 3MM paper (2h at 3kV) in pH 1.9 buffer [8% (v/v) acetic acid/2% (v/v) formic acid] with N^6-dinitrophenyl-lysine markers. In preliminary experiments the phosphopeptides were located by radioautography (Kodak Blue Band BB5 X-ray film). In all experiments the paper tracks were excised, cut into 1cm strips and assayed for ^{32}P by liquid scintillation spectrometry. The profile of radioactivity was plotted on graph paper and the distribution of ^{32}P between the three fractions determined by excising and weighing the peaks and/or summing the d.p.m. in samples contributing to each peak. The two methods of calculation gave near-identical distributions.

Measurement of ^{32}P in tryptic phosphopeptides from rat heart pyruvate dehydrogenase [^{32}P]phosphate

Pyruvate dehydrogenase complex (active form) free of pyruvate dehydrogenase phosphate phosphatase was isolated from rat hearts by scaling down the method described for pig hearts by Kerbey *et al.* (1979). The total yield was 4.6 units of active complex. On SDS/polyacrylamide disc gel electrophoresis (see Sugden & Randle, 1978) four major bands were seen corresponding to the four subunits of the purified pig heart complex, together with a number of bands of higher molecular weight, and one band of lower molecular weight. The four major bands accounted for approximately 60% of the

material staining with Coomassie Blue (based on scan at 580nm). Fully phosphorylated pyruvate dehydrogenase phosphate (2.20nmol of phosphate/unit of complex inactivated) was prepared by incubation of active complex (0.6 unit/ml) with [γ-^{32}P]ATP (0.5mM; 214d.p.m./pmol) and 1mM-$MgCl_2$ for 34min at 30°C (incorporation was complete at this time). Partially phosphorylated pyruvate dehydrogenase phosphate (0.89nmol of phosphate/unit of complex inactivated) was prepared by nine additions at 5-min intervals of 5nmol of [γ-^{32}P]ATP to 0.5ml of reaction mixture containing 0.3 unit of active complex with 0.2mM-$MgCl_2$ (see Sugden *et al.*, 1979). The partially phosphorylated pyruvate dehydrogenase phosphate contained approximately 9% of active complex. The ATP additions used in its preparation were somewhat greater than in the corresponding preparation with pig heart complex because of the comparatively high ATPase activity of the rat heart preparation (initial rate of hydrolysis with 0.5mM-ATP and 1mM-$MgCl_2$ approximately 10%/min).

Protein-bound [^{32}P]phosphate was measured by the method of Corbin & Reimann (1974) (see previous section). Tryptic phosphopeptides were prepared (after precipitation of protein with 10% trichloroacetic acid) and analysed by high-voltage electrophoresis by the methods given in the preceding section.

Assay of pyruvate dehydrogenase complex (active form)

Rat heart pyruvate dehydrogenase complex was assayed spectrophotometrically by following NAD^+ reduction (see Cooper *et al.*, 1974). Pyruvate dehydrogenase (active form) was assayed in extracts of mitochondria by coupling to arylamine acetyltransferase (Coore *et al.*, 1971). Mitochondria were separated and extracts prepared as described by Kerbey *et al.* (1976). Total pyruvate dehydrogenase (sum of active and inactive forms) was assayed in extracts of mitochondria incubated without substrate for not less than 10min (Kerbey *et al.*, 1976).

Sources of error and calculations

Duplicate estimates of protein-bound ^{32}P in mitochondria (paper squares method) agreed to within 13% of their mean. Tryptic digestion of denatured mitochondrial proteins rendered more than 90% of the ^{32}P trichloroacetic acid soluble. After high voltage electrophoresis >90% of the ^{32}P rendered trichloroacetic-acid-soluble by tryptic digestion was recovered in three peaks of radioactivity (see Results & Discussion section for mobilities of these peaks). Duplicate determinations of the distribution of radioactivity between these peaks (i.e. duplicate electrophoreses) agreed to within 7% provided that a peak contained more than 10% of the total radioactivity. With smaller peaks of radioactivity, error between duplicates was relatively greater.

Mitochondria incubated without an oxidizable substrate for 10–45min showed trichloroacetic-acid-insoluble ^{32}P. It is known that inactive pyruvate dehydrogenase phosphate is converted (>95%) into active pyruvate dehydrogenase under these conditions (Kerbey *et al.*, 1976). Incorporation of $^{32}P_i$ into pyruvate dehydrogenase phosphate in mitochondria was calculated as the difference (incorporation in experimental − incorporation in no-substrate incubation) for each time period. This correction was approximately 12% for conditions under which the pyruvate dehydrogenase complex was fully phosphorylated, and greater when phosphorylation was incomplete. The identity of the trichloroacetic-acid-insoluble ^{32}P in mitochondria incubated without substrate has not been established. Results of incorporation of $^{32}P_i$ into pyruvate dehydrogenase phosphate have been calculated as pmol or nmol of phosphate/mg or g of mitochondrial protein (from the specific radioactivity of medium P_i). The concentration of pyruvate dehydrogenase phosphate (as units equivalent of active complex) has likewise been calculated from the difference (active complex in no-substrate incubation − active complex in experimental)/mg of mitochondrial protein.

Results and Discussion

Phosphorylations in purified rat heart pyruvate dehydrogenase complex

Fully phosphorylated rat heart pyruvate dehydrogenase [^{32}P]phosphate complex gave a single band of radioactivity on SDS/polyacrylamide disc gel electrophoresis. This coincided with a protein band corresponding in electrophoretic mobility to the α-chain of the decarboxylase component (EC 1.2.4.1) of the pig heart complex (not shown).

Data published by Sugden *et al.* (1979) and Kerbey *et al.* (1979) show that fully phosphorylated pig heart complex contains three phosphorylation sites (all serine residues). These are recovered in two tryptic phosphopeptides: A (site 1, serine A5; site 2, serine A12) and B (site 3, serine B6). In the holocomplex the relative initial rates of phosphorylation are approximately 15:3:1 (site 1:site 2:site 3). Pig heart complex inactivated by repetitive small additions of [γ-^{32}P]ATP (partially phosphorylated complex) yields two tryptic phosphopeptides, A and A′ (A′ is peptide A with only serine A5 phosphorylated). The ratio A′/A was approx. 5.2:1. Cleavage of A′ with CNBr showed that not less than 85% of the phosphate in A′ is in site 1. The percentage of A′ containing phosphate in site 2 is thus likely to be small. The electrophoretic mobilities

of the three peptides relative to N^6-dinitrophenyl lysine markers on high voltage paper electrophoresis at pH 1.9 were 1.40 (A'), 0.99 (A) and 0.61 (B).

Three analogous tryptic phosphopeptides were obtained from purified rat heart pyruvate dehydrogenase [^{32}P]phosphate complexes; their electrophoretic mobilities relative to N^6-dinitrophenyl lysine markers were 1.40 (A'), 1.07 (A) and 0.76 (B). Fully phosphorylated rat heart complex gave A and B in the ratio of 1.8:1 (A/B). Rat heart complex inactivated by repetitive small additions of [γ-^{32}P]ATP gave A', A and B in the ratio 13.6:10.9:1 (A':A:B; all ratios based on $^{32}P_i$). It is assumed (by analogy with the pig heart complex) that the rat heart complex contains three phosphorylation sites in α-chains of the decarboxylase component. Peptide A' is assumed to be phosphorylated in site 1, the major inactivating site; peptide A in sites 1 and 2, and peptide B in site 3. The points of evidence are as follows. The electrophoretic mobilities correspond to those of tryptic phosphopeptides obtained from the pig heart complex under comparable conditions of phosphorylation, tryptic digestion and electrophoresis. The difference in electrophoretic mobility between phosphopeptides A' and A from the rat heart complex corresponded to the change induced by incorporation of one phosphate (established for the corresponding pig heart complex phosphopeptides by Sugden *et al.*, 1979). Peptide A' was only recovered in tryptic digests of partially phosphorylated rat heart complex; on full phosphorylation peptide A' disappeared and was replaced by A. In the fully phosphorylated complex, peptide A contained approximately twice as much $^{32}P_i$ as did peptide B. The relative initial rates of phosphorylation in the rat heart holo-complex (as in the pig heart complex) were site 1 > site 2 > site 3. The small difference in electrophoretic mobility between phosphopeptide B from rat heart and pig heart complexes may reflect species differences in amino-acid composition. It has not been practicable to obtain sufficient rat heart muscle to purify phosphopeptides for amino acid analysis or sequence determination.

Incorporation of $^{32}P_i$ into pyruvate dehydrogenase phosphate in rat heart mitochondria; total incorporation, incorporation into tryptic phosphopeptides and correlation with pyruvate dehydrogenase complex activity

Interpretation of $^{32}P_i$ incorporations. In rat heart mitochondria the specific radioactivity of the γ-phosphate of ATP is equivalent to that of medium $^{32}P_i$ within 30s (minimum time investigated; A. L. Kerbey, P. J. Randle & P. H. Sugden, unpublished work). Calculations based on oxygen consumption show that turnover of the γ-phosphate of mitochondrial ATP requires less than 1s at full coupling (Sugden & Kerbey, 1978). It is assumed that incorporation of $^{32}P_i$ into mitochondrial protein in the present study is not dependent on the rate of labelling of the γ-phosphate of ATP.

We also assume in what follows that respiration-dependent incorporation of $^{32}P_i$ into trichloroacetic-acid-insoluble material in rat heart mitochondria is incorporation into pyruvate dehydrogenase phosphate complex. The evidence is as follows. In heart mitochondria from normal rats this incorporation is inhibited by known inhibitors of the pyruvate dehydrogenase kinase reaction, e.g. dichloroacetate (A. L. Kerbey, P. J. Randle & P. H. Sugden, unpublished work) and pyruvate (the present paper). Concentrations of pyruvate that completely prevented inactivation of the complex in mitochondria from normal rats (5mM-pyruvate) completely prevented this incorporation of $^{32}P_i$ (not shown). The $^{32}P_i$ incorporated in mitochondrial incubations can be rendered trichloroacetic-acid-soluble by incubation of extracts of mitochondria with pig heart pyruvate dehydrogenase phosphate phosphatase. The solubilization of $^{32}P_i$ correlates with the appearance of active pyruvate dehydrogenase complex (Hutson *et al.*, 1978). On SDS/polyacrylamide disc gel electrophoresis (or on Sepharose 6B gel filtration in SDS) the trichloroacetic-acid-insoluble ^{32}P migrates as a single band at a molecular weight corresponding to that of the α-subunit of pig heart pyruvate decarboxylase (Sugden & Kerbey, 1978). The maximum total incorporation of $^{32}P_i$ into this band (30min incubation with 2-oxoglutarate + malate; disc gel electrophoresis) was 2.1nmol of phosphate/unit of complex inactivated (A. L. Kerbey, P. J. Randle & P. H. Sugden, unpublished work). This incorporation was very similar to those obtained in the present study by the paper squares method (see later). Lastly, more than 90% of the trichloroacetic-acid-insoluble ^{32}P is solubilized by trypsin. Not less than 90% of this solubilized ^{32}P is recovered in three tryptic phosphopeptides with electrophoretic mobilities indistinguishable from those derived from purified rat heart pyruvate dehydrogenase [^{32}P]phosphate complexes (see preceding section, Fig. 1 and next paragraph).

Fig. 1 shows the distribution of ^{32}P after high-voltage paper electrophoresis of tryptic digests of mitochondria incubated with $^{32}P_i$. These two runs were selected because one shows predominantly peaks A' and A, the other peaks A and B. In more than 50 separate experiments only three major peaks of radioactivity (A', A and B) and one minor peak (X) were detected together with a small amount of radioactivity at the origin (not shown). Electrophoretic mobilities relative to N^6-dinitrophenyl lysine markers were 1.36 (A'), 1.00 (A) and 0.72 (B).

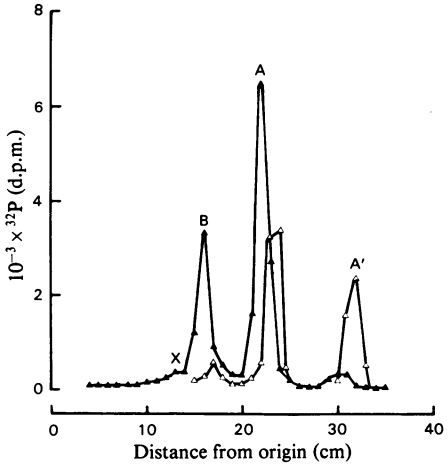

Fig. 1. *High-voltage electrophoresis of tryptic digest of rat heart mitochondria incubated with $^{32}P_i$; distribution of $^{32}P_i$*

Heart mitochondria of normal rats incubated for 0.5 min (△) or 10 min (▲) with 5 mM-2-oxoglutarate + 0.5 mM-L-malate. Radioactivity data omitted from (△) were essentially identical with (▲). For details of incubations, termination of incubations, tryptic digestion, paper electrophoresis and assay of radioactivity see the legend to Fig. 5 (and also Fig. 3), and the Experimental section. The two traces are from different electrophoresis runs, which explains why the peaks are displaced, relatively, by 1 cm.

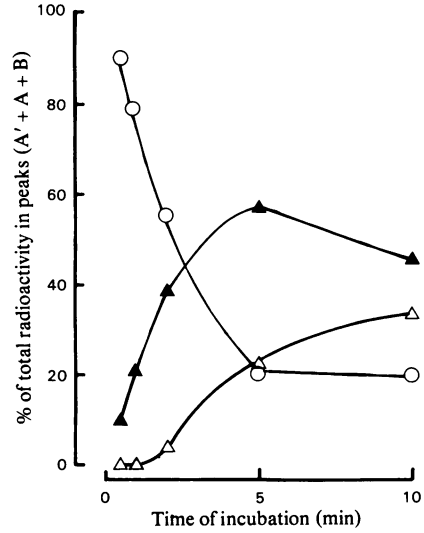

Fig. 2. *Time course for incorporation of $^{32}P_i$ into peaks A' (○), A (▲) and B (△) in electrophoretograms of tryptic digests of rat heart mitochondria*

Heart mitochondria of normal rats were preincubated for 10 min without substrate and then were incubated for the time shown with 5 mM-oxoglutarate + 0.5 mM-L-malate. Mitochondria from hearts of alloxan-diabetic rats gave qualitatively similar results (not shown). Values are means of results from two experiments (i.e. two batches of mitochondria). The differences between individual values were <10% of their mean value. The points shown at 10 min are means of values at 10, 20 and 35 min which did not differ significantly. Peaks A', A and B are as shown in Fig. 1. For other details see the legend to Fig. 3 and the Experimental section.

Peaks A and B predominated in mitochondria in which $^{32}P_i$ incorporation and complex inactivation were complete. Peaks A' and A predominated in mitochondria in the early stages (0.5–2 min) of $^{32}P_i$ incorporation and complex inactivation.

As shown in Fig. 2, peak A' declines and is replaced by peak A as phosphorylation proceeds. Peak B appears after a lag and is slower to steady state than peaks A' and A. The apparent decline in peak A between 5 and 10 min of incubation is due to the increase in peak B, which diminishes the percentage contribution of peak A to the total incorporation. These results were obtained with mitochondria preincubated without substrate (for discussion of reasons, see next section). The sequence of incorporations of radioactivity into peaks A', A and B is thus in accordance with what was observed during phosphorylation and inactivation of purified rat heart pyruvate dehydrogenase complex. It is concluded that peaks A', A and B are tryptic phosphopeptides derived from pyruvate dehydrogenase [^{32}P]phosphate in rat heart mitochondria. It is assumed in what follows that these correspond to three sites of phosphorylation which are computed as site 1 [incorporation into A' + (0.5 × incorporation into A)]; site 2 (0.5 × incorporation into A); site 3 (incorporation into B). Incorporation into each site was then calculated as the product of (total incorporation) × (fractional incorporation into each site).

The origin of radioactivity in peak X is not known. The radioactivity in this peak accounted for no more than 2% of the radioactivity in peaks (A' + A + B) in mitochondria in which $^{32}P_i$ incorporation was complete. It was present in all electrophoretograms, including those in which pyruvate dehydrogenase inactivation was completely inhibited with 5 mM-pyruvate (not shown). One possibility is that it is derived from phosphorylated branched-chain 2-oxoacid dehydrogenase complex (Parker & Randle, 1978).

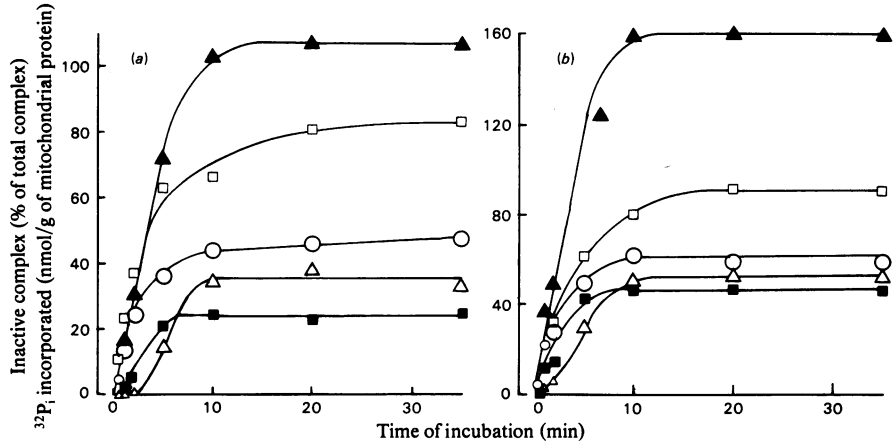

Fig. 3. *Inactive pyruvate dehydrogenase complex* (□) *and incorporation of* $^{32}P_i$ *into pyruvate dehydrogenase phosphate* (▲, *total;* ○, *site 1;* ■, *site 2;* △, *site 3*) *in rat heart mitochondria preincubated without substrate*

(*a*) Non-diabetic, (*b*) alloxan-diabetic. Values are means of duplicate determinations in each of two experiments (i.e. two batches of mitochondria). In (*a*) one point of (■) and (▲) close to the origin have beem omitted through lack of space. In (*b*) one point of (△), one point of (▲) and two points of (□) have been omitted. Rat heart mitochondria (approximately 2mg of protein) were preincubated for 10min at 30°C in 1ml of KCl medium without substrate to effect dephosphorylation of pyruvate dehydrogenase phosphate. Potassium 2-oxoglutarate (to 5mM) and potassium L-malate (to 0.5mM) were then added and incubation made for the time shown. Incubation was completed by removal of duplicate 10μl samples to paper for assay of total protein-bound ^{32}P followed by addition of 0.1vol. of 100% (w/v) trichloroacetic acid. Tryptic phosphopeptides were generated from the precipitated protein, separated by electrophoresis on paper and assayed for ^{32}P by liquid scintillation spectrometry. The specific radioactivities of $^{32}P_i$ in KCl medium (present during preincubation and incubation) were 2200d.p.m./pmol (0.5, 1 and 2min) or 440d.p.m./pmol (5, 10, 20, 35min). A no-substrate incubation was included as a control; duplicate 10μl samples were spotted onto paper at each time period and used as a blank correction. Pyruvate dehydrogenase was assayed in extracts prepared from mitochondria incubated under otherwise identical conditions in the absence of $^{32}P_i$. Total pyruvate dehydrogenase complex (sum of active and inactive forms) was assumed from mitochondria preincubated for 10min without substrate (see Kerbey *et al.*, 1976). Pyruvate dehydrogenase phosphate concentration was calculated as the difference (total complex − active complex). Total complex activities in units/g of mitochondrial protein were 121 (non-diabetic) and 84 (diabetic). For further details of preparation of mitochondria, incubation medium and assays, see the Experimental section.

Incorporation of $^{32}P_i$ *with 2-oxoglutarate* + L-*malate; time course, correlation with complex inactivation, and effect of alloxan-diabetes.* Rat heart mitochondria, as made, contain approximately 39% of pyruvate dehydrogenase complex in the inactive phosphorylated form (normal rats) or 69% (alloxan-diabetic rats; Hutson *et al.*, 1978). Incorporation of $^{32}P_i$ into pyruvate dehydrogenase in these mitochondria involves both phosphorylation *de novo* and turnover of existing pyruvate dehydrogenase phosphate. Conversion of inactive complex into active complex can be effected (>95%) by preincubation for 10min without respiratory substrate (Kerbey *et al.*, 1976). It would appear that this results in >95% dephosphorylation of all sites, because complete reactivation of fully phosphorylated pig heart complex is associated with complete removal of ^{32}P (Sugden *et al.*, 1978). Incorporation of $^{32}P_i$ following addition of respiratory substrate is thereafter dependent (>95%) upon phosphorylation *de novo*. Both techniques have been used. In pilot experiments, performed in duplicate with two mitochondrial preparations, total incorporation of $^{32}P_i$, incorporation of $^{32}P_i$ into sites 1, 2 and 3, and the concentration of inactive complex was measured after 0.5, 1, 2, 5, 10, 20 and 35min of incubation. These results are shown in Figs. 3–6. The statistical significance of potential findings in pilot experiments was then established by replicate assays in further experiments.

The results of experiments in which phosphorylation *de novo* was followed in mitochondria preincubated for 10min without respiratory substrate are shown in Figs. 3 and 4. Fig. 3 shows the formation of inactive complex, the total incorporation of $^{32}P_i$ into pyruvate dehydrogenase phosphate, and the incorporation of $^{32}P_i$ into sites 1, 2 and 3. Fig. 3(*a*) shows in the non-diabetic rat that

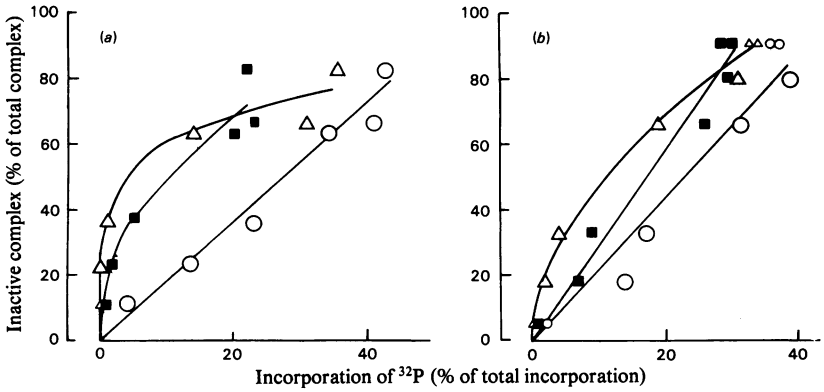

Fig. 4. *Relationship between inactivation and incorporation of $^{32}P_i$ into sites 1, 2 and 3 of pyruvate dehydrogenase complex in rat heart mitochondria preincubated without substrate*

O, Site 1; ■, site 2; △, site 3; (*a*) non-diabetic; (*b*) alloxan-diabetic. The plots are based on data from Fig. 3 and the details of the experiments are as given in the legend to Fig. 3 and in the Experimental section. Total incorporations (nmol/g of mitochondrial protein) were 106 (non-diabetic) and 158 (diabetic).

inactivation of the complex and site 1 incorporation were substantially maximal by 5 min; total incorporation was completed by 10 min. Incorporation of $^{32}P_i$ into site 2 appeared after 0.5–1 min, whereas incorporation into site 3 appeared between 2 and 5 min; in both cases the incorporation was maximal by 10 min. Essentially similar results were obtained with mitochondria from diabetic rats (Fig. 3*b*) except that incorporation into sites 2 and 3 may have been initially faster. To establish statistically that the relative initial rates of $^{32}P_i$ incorporation are site 1 > site 2 > site 3 replicate measurements were made at the end of 1 min of incubation (five observations for non-diabetic; four for diabetic; single batches of mitochondria). The percentage of distributions of $^{32}P_i$ between sites were (means ± S.E.M.; non-diabetic, diabetic) 85.6 ± 0.28, 72.9 ± 0.57 (site 1); 14.4 ± 0.28, 27.1 ± 0.57 (site 2); 0 ± 0, 0 ± 0 (site 3). The differences (site 1 − site 2; site 2 − site 3) for non-diabetic and for diabetic were significant ($P < 0.001$) confirming that the relative rates of initial incorporation are site 1 > site 2 > site 3.

The maximum incorporation of $^{32}P_i$ into the complex was compared in mitochondria of diabetic and non-diabetic rats after 20 min of incubation (five observations in each group; single batches of mitochondria). The mean values ± S.E.M. in nmol of phosphate/unit of complex inactivated were 2.7 ± 0.24 (non-diabetic) and 2.1 ± 0.19 (diabetic) ($P > 0.1$). This finding is supported by the combined means for maximum incorporation from all of our experiments. These were 2.16 ± 0.13 (non-diabetic, 20 observations) and 2.31 ± 0.12 (diabetic, 19 observations); 10, 20 and 35 min incubations, seven mitochondrial preparations in each group. There were thus no detectable differences between heart mitochondria from non-diabetic or diabetic rats in terms of maximal incorporation of $^{32}P_i$, the rate at which this was attained or the rate of inactivation of the complex.

Fig. 4 shows the relationship between the incorporation *de novo* of $^{32}P_i$ into sites 1, 2 and 3 (as a percentage of the maximal total incorporation) and the percentage of pyruvate dehydrogenase complex inactivated. For both diabetic and non-diabetic mitochondria incorporation of $^{32}P_i$ into site 1 was related linearly to inactivation of the complex. The slopes (means ± S.E.M.) were 1.74 ± 0.11 (non-diabetic) and 2.36 ± 0.20 (diabetic); the intercepts were 1.03 ± 2.99 (non-diabetic) and −4.09 ± 5.22 (diabetic); and the correlation coefficients (r) were 0.991 (non-diabetic) and 0.980 (diabetic). This plot shows clearly in the non-diabetic (*a*) that site-3 and site-2 phosphorylations lag behind site-1 phosphorylation and inactivation of the complex. The curves for site-2 and site-3 phosphorylations are closer to the curve for site-1 phosphorylation in the diabetic (*b*) than in the non-diabetic. For example, at 30% inactivation of the complex the occupancy of sites 2 and 3 are 3-fold and 8-fold greater in the diabetic than in the non-diabetic.

Figs. 5 and 6 show equivalent data for experiments in which the preincubation in the absence of substrate was omitted (*a*, non-diabetic; *b*, diabetic). These incorporations involve both phosphorylation *de novo* of active complex and turnover of inactive complex. This point is established by the maximal incorporations which were (nmol of phosphate/unit of complex inactivated) 2.25 (non-diabetic) and 2.64

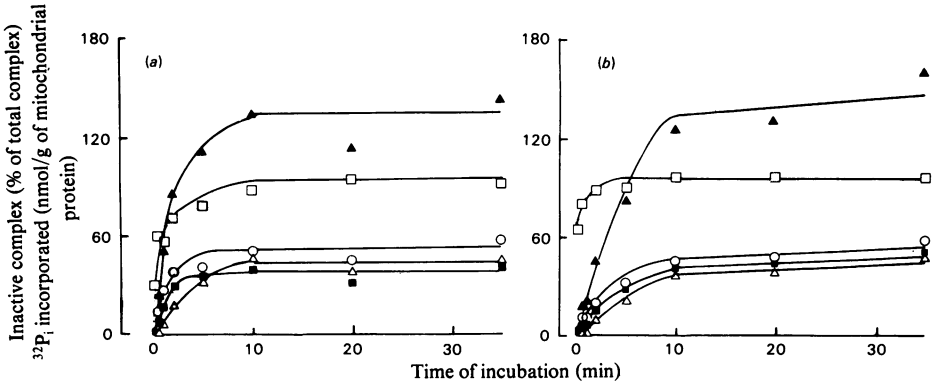

Fig. 5. *Inactive pyruvate dehydrogenase complex* (□) *and incorporation of* $^{32}P_i$ *into pyruvate dehydrogenase phosphate* (▲, *total;* ○, *site 1;* ■, *site 2;* △, *site 3*) *in rat heart mitochondria* (*no preincubation without substrate*)
(*a*) Non-diabetic; (*b*) alloxan-diabetic. Values are means of duplicate determinations in each of two experiments (i.e. two batches of mitochondria). Details were as given in the legend to Fig. 3 except that mitochondria were not preincubated (i.e. 2-oxoglutarate + malate were present throughout). The total activities of pyruvate dehydrogenase (in units/g of mitochondrial protein) were 63.6 (non-diabetic) and 67.6 (diabetic).

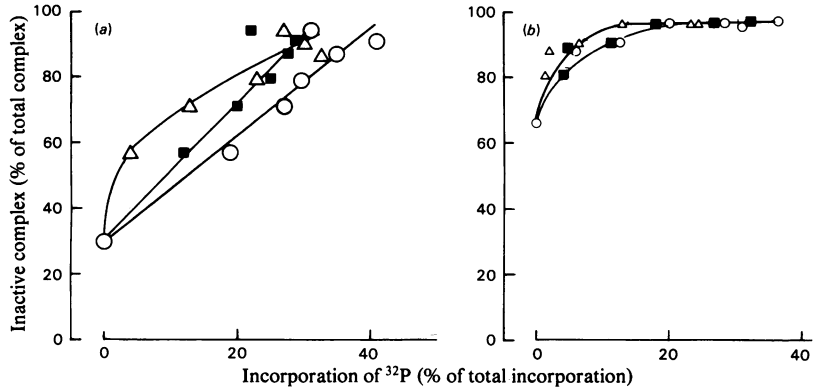

Fig. 6. *Relationship between inactivation and incorporation of* $^{32}P_i$ *into sites 1, 2 and 3 of pyruvate dehydrogenase complex in rat heart mitochondria* (*no preincubation without substrate*)
○, Site 1; ■, site 2; △, site 3. (*a*) Non-diabetic; (*b*) alloxan-diabetic. The plots are based on data from Fig. 5 and the details of the experiments are as given in the legend to Fig. 5 and in the Experimental section. Total incorporations (nmol/g of mitochondrial protein) were 141 (non-diabetic) and 161 (diabetic).

(diabetic), results that are very similar to those obtained with no substrate preincubations. At maximum incorporations and inactivation (attained between 5 and 10min) the percentage distributions of total ^{32}P between sites 1, 2 and 3 were 39, 28 and 33 (non-diabetic) and 37, 33 and 30 (diabetic) which both approximate to 1:1:1 distribution. The initial rates of phosphorylation of individual sites and of total incorporation (Fig. 5) were lower in the diabetic than in the non-diabetic. This may be correlated with the presence of a much greater proportion of inactive complex in the diabetic at the beginning of incubation with $^{32}P_i$ (66% in this experiment as compared with 30% in the non-diabetic). Incorporation in the diabetic is thus dependent to a much greater extent on turnover than in the non-diabetic.

Fig. 6 shows the correlation between incorporation of $^{32}P_i$ into sites 1, 2 and 3 (as a percentage of maximum total incorporation) and the percentage of complex in the inactive form. The data shown in Fig. 6 for phosphorylation *de novo* plus turnover may be compared with data in Fig. 4 for

phosphorylation *de novo* alone. The curves in Fig. 6 are displaced upwards relative to those in Fig. 4 because of the initial presence of inactive non-radioactive complex. The linear relationship between inactive complex and site-1 phosphorylation is retained in the results with non-diabetic mitochondria (Fig. 6*a*; slope 1.65 ± 0.21; intercept $29.7 \pm 6.0\%$ of inactive complex; $r = 0.962$). The relationship in the diabetic (Fig. 6*b*) is not linear and suggests that phosphorylation *de novo* of site 1 may be more rapid than turnover in the diabetic; i.e. that the phosphatase reaction is rate-limiting during turnover.

Effect of pyruvate on activity of complex and incorporation of $^{32}P_i$ into phosphorylation sites 1, 2 and 3 in rat heart mitochondria

The results shown in Fig. 4(*a*) suggest that in normal rat heart mitochondria, phosphorylation of site 3 (and to a lesser extent site 2) is only significant when the proportion of inactive complex rises above 40–50%. The objective of these experiments with pyruvate was to investigate further the effect of varying the proportion of inactive complex on the phosphorylation of individual sites. Relevant background information is as follows. Pyruvate inhibits the pyruvate dehydrogenase kinase reaction in bovine, porcine and rat heart complexes, but has no effect on the phosphatase reaction (Linn *et al.*, 1969; Cooper *et al.*, 1974). Pyruvate inhibits both site-1 and site-(2 + 3) phosphorylations in the pig heart complex, the K_i being lowest for site-(2 + 3) phosphorylations (Kerbey *et al.*, 1979). In rat heart mitochondria oxidizing 2-oxoglutarate + malate, the proportion of active complex is decreased by the presence of pyruvate over the range 0.2–5 mM (Kerbey *et al.*, 1976). This effect of pyruvate is substantially decreased in mitochondria from diabetic rats (Kerbey *et al.*, 1976, 1977). This is due partly to failure of pyruvate to inhibit the kinase reaction (Hutson & Randle, 1978) and partly to inhibition of reactivation of the complex by the phosphatase reaction (Hutson *et al.*, 1978). In mitochondria of non-diabetic rats the effect of pyruvate is substantially reduced by pre-incubation of mitochondria (for 5–10 min) with 2-oxoglutarate + malate or succinate prior to addition of pyruvate (Hutson *et al.*, 1978).

Heart mitochondria of normal fed rats. Fig. 7 shows the relationship between the percentage of pyruvate dehydrogenase complex in the inactive (phosphorylated) form and the incorporation of $^{32}P_i$ into sites 1, 2 and 3. The data were obtained with rat heart mitochondria incubated for 10 min and 15 min with (2-oxoglutarate + L-malate) ± pyruvate (concentrations ranging from zero to 0.4 mM). Incorporation of $^{32}P_i$ into site 1 was linearly related to the concentration (or percentage) of inactive complex [slope 0.97 ± 0.06; intercept 4.35 ± 4.39 (means ± S.E.M); $r = 0.980$]. Incorporation of $^{32}P_i$ into site 3, however, was substantially inhibited by concentrations of pyruvate that had comparatively smaller effects on the concentration of inactive complex. For example, pyruvate at 0.4 mM inhibited incorporation of $^{32}P_i$ into site 3 by 95% whereas inactivation of complex was inhibited by only 45%. Site-2 phosphorylation was intermediate between site-1 and site-3 phosphorylations. These data may suggest that site-1 (and perhaps site-2) phosphorylation determines inactivation and that site-3 phosphorylation has no role in inactivation. This has previously been demonstrated in the pig heart complex (Sugden & Randle, 1978; Sugden *et al.*, 1979; Kerbey *et al.*, 1979). The results shown in Fig. 7 were from a single experiment which has been duplicated (not shown).

In the pig heart complex phosphorylation of sites 2 and 3 inhibits reactivation of the complex by pyruvate dehydrogenase phosphate phosphatase (Sugden *et al.*, 1978). The data shown in Fig. 7 suggest an experiment to test this in mitochondria. With 0.25 mM-pyruvate, 75% of complex was inactive and $^{32}P_i$ incorporations (as percentage of incorporation with zero pyruvate) were 87 (site 1), 69 (site 2) and 24 (site 3). If site-(2 + 3) phosphorylations inhibit reactivation by phosphatase, mitochondria incubated with 0.25 mM-pyruvate should exhibit more rapid reactivation of the complex on addition of an inhibitor of the kinase reaction than mitochondria incubated with 2-oxoglutarate + malate alone. This expectation was realized in an experiment, the results of which are summarized in Table 1. In this experiment pyruvate was added to 2 mM to inhibit the kinase reaction after 10 min of incubation with (2-oxoglutarate + malate) ± 0.25 mM-pyruvate. The concentration of active complex (measured at 16 min) was significantly greater when 0.25 mM-pyruvate was present in the first 10 min of incubation than in its absence.

The effects of 5 mM-pyruvate were also investigated by means of a time course using conditions that were otherwise as shown in Fig. 5. There was no detectable inactivation of pyruvate dehydrogenase complex or total incorporation of $^{32}P_i$ (not shown). High-voltage electrophoresis of a tryptic digest of mitochondria incubated for 35 min with 5 mM-pyruvate showed small peaks A′ and A (no B). The incorporation of $^{32}P_i$ into (A′ + A) was 2.6% of that incorporated into (A′ + A + B) after 35 min with 2-oxoglutarate + L-malate.

Heart mitochondria from alloxan-diabetic rats. Table 2 shows the effects of pyruvate on the concentration of inactive complex and incorporations of $^{32}P_i$ in heart mitochondria of diabetic rats. In Expt. 1, pyruvate at all concentrations tested

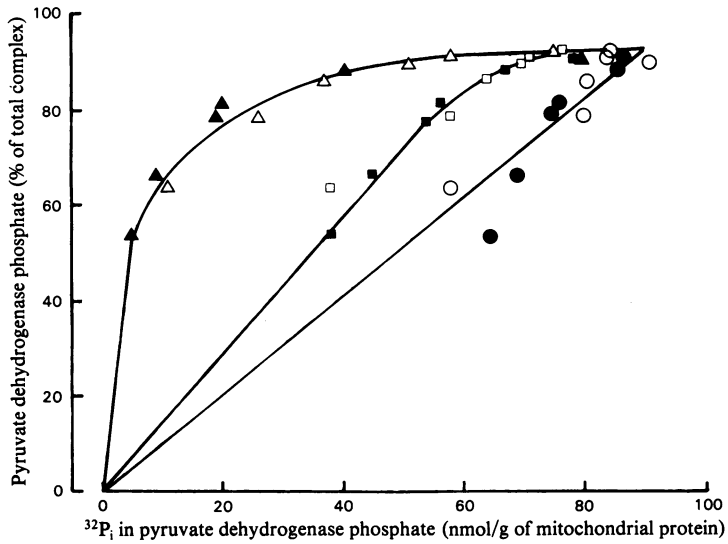

Fig. 7. *Correlation between the concentration of inactive pyruvate dehydrogenase complex and incorporation of $^{32}P_i$ into sites 1, 2 and 3 in heart mitochondria of fed normal rats with various pyruvate concentrations*

Rat heart mitochondria (approx. 2mg of protein) were incubated at 30°C for 10min (closed symbols) or 15min (open symbols) in 1ml of KCl medium (see the Experimental section) containing $^{32}P_i$ (0.2mM; 1000d.p.m./pmol), 5mM-2-oxoglutarate and 0.5mM-L-malate. Aeration of incubation medium was renewed every 5min by vortex mixing the solution. The concentration of sodium pyruvate was 0, 0.15, 0.20, 0.25, 0.30 or 0.40mM (identifiable for each type of symbol by reading from right to left; i.e. each point represents a single pyruvate concentration). At the end of incubation duplicate samples were taken for assay of protein-bound phosphate and the reaction was terminated by addition of 0.1vol. of 100% (w/v) trichloroacetic acid. The precipitated protein was subjected to tryptic digestion and the tryptic phosphopeptides were assayed for ^{32}P after high-voltage electrophoresis (see the Experimental section). Pyruvate dehydrogenase (active form) was assayed in duplicate extracts of mitochondria incubated in parallel in non-radioactive medium. Total pyruvate dehydrogenase (sum of active and inactive forms) was assayed in extracts of mitochondria incubated for 15min without added oxidizable substrate. The concentration of pyruvate dehydrogenase phosphate was calculated as the difference (total − active form). ●,○, Site-1 phosphorylation; ■,□, site-2 phosphorylation; ▲,△, site-3 phosphorylation. Each point is a single mitochondrial incubation in a single experiment. The results were confirmed in a second experiment (not shown). The total concentration of pyruvate dehydrogenase in mitochondria was 94.4 units/g of protein. For site-1 data the slope was (mean ± S.E.M.) 0.973 ± 0.06nmol of protein-bound phosphate/unit of pyruvate dehydrogenase phosphate and the correlation coefficient (r) was 0.980. The intercepts for sites 1 and 2 (linear regression analysis) did not differ significantly from zero ($P > 0.4$). The conventions for site numbers and assumptions in calculations are given in the Introduction and in the text of the Results section.

Table 1. *Effect of pyruvate on the concentration of active pyruvate dehydrogenase complex in rat heart mitochondria*

Rat heart mitochondria (approximately 1mg protein) were incubated in 0.5ml of KCl medium at 30°C for 16min as shown in the Table. Mitochondria were then separated by centrifugation, and extracted and assayed for pyruvate dehydrogenase complex (active form). For details of preparation of mitochondria (non-diabetic rats), composition of KCl medium, extraction and assay of pyruvate dehydrogenase complex see the Experimental section. The total concentration of pyruvate dehydrogenase complex (sum of active and inactive forms) was 66 units/g mitochondrial protein; $*P < 0.001$ for $(d-c)-(b-a)$. 5mM-2-oxoglutarate and 0.5mM L-malate were used. There were eight observations in groups 1, 2 and 3 and six observations in group 4. Values are given as means ± S.E.M.

	Incubation period (min) and additions			
Group	0–10	10–16	Active complex (units/g of mitochondrial protein)	Differences
1	2-Oxoglutarate + malate	2-Oxoglutarate + malate	1.94 ± 0.16 (a)	($b-a$)
2	2-Oxoglutarate + malate	2-Oxoglutarate + malate + 2mM-pyruvate	13.0 ± 0.82 (b)	11.06 ± 0.84
3	2-Oxoglutarate + malate + 0.25mM-pyruvate	2-Oxoglutarate + malate + 0.25mM-pyruvate	9.1 ± 0.90 (c)	($d-c$)
4	2-Oxoglutarate + malate + 0.25mM-pyruvate	2-Oxoglutarate + malate + 2mM-pyruvate	31.2 ± 0.71 (d)	22.08 ± 1.14*

Table 2. *Correlation between the concentration of inactive pyruvate dehydrogenase complex and incorporation of ^{32}P into sites 1, 2 and 3 in heart mitochondria of alloxan-diabetic rats with varying pyruvate concentration*

In Expt. 1 values for the concentration of inactive complex and for total incorporation of $^{32}P_i$ are means of duplicate determinations. The distribution between sites is derived in each case from a single high-voltage paper electrophoresis. In Expt. 2 there were four observations in each group (i.e. four incubations) and values are means ± S.E.M. The total concentrations of pyruvate dehydrogenase in mitochondria (sum of active and inactive forms in units/g of mitochondrial protein) were 74 (Expt. 1) and 65 (Expt. 2). The experimental details were otherwise as given in the legend to Fig. 7, except that incubations were for 10 min only. In all incubations medium contained 5 mM-2-oxoglutarate + 0.5 mM-L-malate. *$P < 0.001$, †$P < 0.05 > 0.02$, against zero pyruvate. $P > 0.1$ for other differences in Expt. 2. § ± half the difference between duplicate assays.

		Incorporation of $^{32}P_i$ (nmol/g of mitochondrial protein)				
Expt.	Pyruvate concn. (mM)	Total	Site 1	Site 2	Site 3	Inactive complex (units/g of mitochondrial protein)
1	0	107	39	35	33	61
	0.15	129	59	54	16	68
	0.20	113	53	49	11	67
	0.25	104	49	46	9	67
	0.30	126	62	56	8	67
	0.40	122	61	54	7	66
2	0	119 ± 1.68	47 ± 0.96	45 ± 0.95	27.0 ± 0.25	61 ± 0§
	0.3	92 ± 4.10†	45 ± 1.85	42 ± 1.93	4.7 ± 0.13*	59 ± 1.4§

(range 0.15–0.4 mM) had no effect on the concentration or proportion of inactive complex. The incorporation of $^{32}P_i$ into site 3 decreased progressively with increasing pyruvate concentration to reach approximately 20% of the zero-pyruvate incorporation at 0.4 mM-pyruvate. There was no decrease in site-1 or site-2 incorporations at any of the pyruvate concentrations. In Expt. 2 the statistical significance of the effect of pyruvate at 0.3 mM on site-3 incorporation was established. The fall in site-3 incorporation was matched by a corresponding fall in total incorporation; there was no significant change in the concentration (or proportion) of inactive complex or in site 1 or site 2 incorporations. The results confirm the diminished effect of pyruvate on the activity of the pyruvate dehydrogenase complex in heart mitochondria of diabetic rats (Kerbey *et al.*, 1976, 1977). They show further that pyruvate has no effect on site-1 or site-2 phosphorylations in contra-distinction to its effects in mitochondria of non-diabetic rats. The diminution in site 3 phosphorylation induced by pyruvate in mitochondria of diabetic rats may show, unequivocally, that site-3 phosphorylation makes no contribution to inactivation of the complex. Thus loss of 80% of the ^{32}P in site 3 led to no detectable increase in complex activity. With 5 mM-pyruvate alone, 42% of the complex was active (22.7 units/g of mitochondrial protein out of a total of 54.4); total incorporation was 17.2 nmol of phosphate/g of protein (11.6 in site 1; 5.5 in site 2). These results are means of duplicate determinations at 10, 20 and 35 min of incubation; there was no detectable site-3 incorporation.

General Discussion and Conclusions

In an earlier study (Randle *et al.*, 1978) it was observed that addition of $^{32}P_i$ to rat heart mitochondria following inactivation of the pyruvate dehydrogenase complex by preincubation with 2-oxoglutarate + L-malate led to rapid incorporation of $^{32}P_i$ into the complex. The steady-state incorporation (2.25 nmol of phosphate/unit of inactive complex) was comparable to values obtained in the present study where $^{32}P_i$ was present throughout. These results show that the phosphorylated complex turns over in mitochondria, i.e. that kinase and phosphatase reactions operate simultaneously. Therefore in the present study $^{32}P_i$ incorporations into the complex in rat heart mitochondria are the resultant of both kinase and phosphatase reactions.

Current evidence suggests that kinase reactions are sequential in the order sites 1, 2 and 3; that site-1 phosphorylation is faster than site-2 and site-3 phosphorylations; and that phosphatase reactions may be random (Sugden & Randle, 1978; Yeaman *et al.*, 1978; Kerbey *et al.*, 1979; Teague *et al.*, 1979). In the present study the relative initial rates of phosphorylation in rat heart mitochondria were site 1 > site 2 > site 3 under conditions where only phosphorylation *de novo* was measured. In these experiments the kinase reaction(s) may predominate under initial-rate conditions because of the low

concentration of pyruvate dehydrogenase phosphate. The low rate of site-2 and site-3 phosphorylations relative to that of site 1 in mitochondria *in situ* thus conforms to the pattern seen with purified porcine, bovine and rat heart complexes.

The maximum total incorporation of ^{32}P into the pyruvate dehydrogenase complex in rat heart mitochondria in the present study was 2.25 ± 0.13 nmol of phosphate/unit of inactive complex (mean ± S.E.M. for combined results with 14 mitochondrial preparations). This value and that for purified rat heart complex (see below) is greater than the value of 1.32 nmol of phosphate/unit for the fully phosphorylated pig heart complex. There were no significant differences between mitochondria from diabetic or non-diabetic rats or with or without no substrate preincubation. Maximum total incorporation into the purified rat heart complex was 2.20 nmol of phosphate/unit of inactive complex. This value is based on rat heart complex supplemented with lipoamide dehydrogenase complex, because purified rat heart complex, unlike rat heart mitochondrial extracts and pig heart complex, was deficient in lipoamide dehydrogenase. This was demonstrated by the small peak of lipoamide dehydrogenase on SDS/polyacrylamide disc gel electrophoresis (not shown); and by the 40% increase in activity of the complex on addition of lipoamide dehydrogenase (1–10 unit/ml in the cuvette). These results suggest either that phosphorylation of the complex in mitochondria was substantially complete notwithstanding the presence of phosphatase, or alternatively that phosphorylation of purified rat heart complex was incomplete (there was substantial adenosine triphosphatase activity). Some significant differences between mitochondria as made, and mitochondria preincubated without substrate were noted. After 10, 20 and 35 min of incubation the proportion of active complex (Figs. 3 and 5) and the proportion of peak A′ (not shown) were higher in mitochondria preincubated without substrate.

In alloxan-diabetic rats the proportion of active complex in freeze-clamped hearts is substantially lower than in non-diabetic controls (1.5% against 27.1%; Kerbey *et al.*, 1976). This difference between diabetic and control can persist in heart mitochondria incubated with 2-oxoglutarate + L-malate but is not always found (Kerbey *et al.*, 1976, 1977). Heart mitochondria of diabetic rats are invariably less well coupled than mitochondria of non-diabetic rats and show lower concentration ratios of ATP/ADP and $NADH/NAD^+$ and lower respiratory control ratios (Kerbey *et al.*, 1976, 1977). These lower ratios inhibit pyruvate dehydrogenase kinase reactions in purified ox and pig heart complexes (Linn *et al.*, 1969; Pettit *et al.*, 1975; Cooper *et al.*, 1974, 1975; Kerbey *et al.*, 1979) and in rat heart mitochondria (Hansford, 1976; Kerbey *et al.*, 1977). Metabolite ratios in heart mitochondria of diabetic rats may thus be expected to result in a lower rate of kinase reactions than in mitochondria of normal rats. In the present study there were no obvious differences between normal and diabetic mitochondria in the initial rate of overall $^{32}P_i$ incorporation into the pyruvate dehydrogenase complex. It may be inferred that the rate would be higher in the diabetic mitochondria if metabolite ratios were comparable to those of normal mitochondria. It has been shown that the rate of phosphorylation and inactivation of the complex in extracts of diabetic mitochondria incubated with [γ-^{32}P]ATP is much greater than in extracts of non-diabetic mitochondria (Hutson *et al.*, 1978). It was concluded that the kinase reaction is activated in diabetes by a novel and unknown mechanism. The present findings are not at variance with this conclusion. Moreover it was noted in the present study that the initial rate of phosphorylation *de novo* of site 2 and the maximum degree of inactivation were greater in diabetic mitochondria.

The pyruvate dehydrogenase kinase reaction in extracts of diabetic mitochondria is resistant to inhibition by pyruvate (Hutson & Randle, 1978) and the proportion of active complex in perfused hearts or heart mitochondria of diabetic rats exposed to pyruvate is lower than in non-diabetic controls (Kerbey *et al.*, 1976, 1977). This observation in mitochondria has been confirmed in the present study and it has been shown, additionally, that this resistance to pyruvate is accompanied by greater incorporation of $^{32}P_i$ into sites 1 and 2 of the complex. However, site-3 incorporation was inhibited by pyruvate as effectively in the diabetic as in the non-diabetic and this inhibition was not associated with any detectable reactivation of the complex in the diabetic. This would appear to exclude a role for site 3 in inactivation of the complex. The alternative explanation, namely that pyruvate specifically inhibits removal of non-radioactive phosphate from site 3 seems improbable. With 5 mM-pyruvate, inhibition of site-3 incorporation accompanied a partial reactivation of the complex that correlated with diminished incorporations into sites 1 and 2.

In heart mitochondria of normal rats the ratio (site-1 incorporation)/(site-2 + site-3 incorporations) was related inversely to the concentration of inactive complex in the present study. This was not unexpected because phosphorylation of site 1 is more rapid than that of sites 2 and 3 and in the pig heart complex the K_i for pyruvate is lower for sites 2 and 3 than for site 1 (Kerbey *et al.*, 1979). If the results shown in Fig. 7 (and Fig. 3) are generally applicable then they predict that in the heart of normal fed rats

the ratios of phosphorylation of site 1 : site 2 : site 3 will be 5 : 3.6 : 1 (for 28% of active complex). In the diabetic the predicted ratios will approximate to 1 : 1 : 1 (for 1.5% of active complex). These predictions are susceptible to investigation if technical difficulties associated with purification of pyruvate dehydrogenase phosphate from rat heart can be overcome.

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References

Chappell, J. B. & Hansford, R. G. (1978) in *Subcellular Components* (Birnie, G. D., ed.) 2nd edn., p. 77, Butterworths, London

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. & Reimann, E. M. (1974) *Methods Enzymol.* **38**, 287–299

Hansford, R. G. (1976) *J. Biol. Chem.* **251**, 5483–5489

Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473

Hughes, W. A. & Denton, R. M. (1978) *Biochem. Soc. Trans.* **6**, 1228–1230

Hutson, N. J. & Randle, P. J. (1978) *FEBS Lett.* **92**, 73–76

Hutson, N. J., Kerbey, A. L., Randle, P. J. & Sugden, P. H. (1978) *Biochem. J.* **173**, 669–680

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

Kerbey, A. L., Radcliffe, P. M., Randle, P. J. & Sugden, P. H. (1979) *Biochem. J.* **181**, 427–433

Linn, T. C., Pettit, F. H., Hucho, F. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241

Parker, P. J. J. & Randle, P. J. (1978) *FEBS Lett.* **95**, 153–156

Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582

Randle, P. J., Hutson, N. J. & Kerbey, A. L. (1978) *FEBS–Symp.* **42**. 3–12

Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237

Sugden, P. H. & Kerbey, A. L. (1978) *FEBS–Symp.* **42**, 71–80

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

Sugden, P. H., Kerbey, A. L., Randle, P. J., Waller, C. A. & Reid, K. B. M. (1979) *Biochem. J.* **181**, 419–426

Teague, W. M., Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1979) *Biochem. Biophys. Res. Commun.* **87**, 244–252

Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C. & Dixon, G. H. (1978) *Biochemistry* **17**, 2364–2370