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Occupancy of sites of phosphorylation in inactive rat heart pyruvate dehydrogenase phosphate *in vivo*

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1. Inactive pyruvate dehydrogenase phosphate complexes were partially purified from hearts of fed, starved or alloxan-diabetic rats by using conditions that prevent phosphorylation or dephosphorylation. 2. Unoccupied sites of phosphorylation were assayed by incorporation of ^{32}P from [γ-^{32}P]ATP into the complexes. Total sites of phosphorylation were assayed by the same method after complete reactivation, and thus dephosphorylation, of complexes by incubation with pyruvate dehydrogenase phosphate phosphatase. Occupancy is assumed from the difference (total sites − unoccupied sites). Percentage incorporation into individual sites was measured by high-voltage electrophoresis after tryptic digestion. 3. Values (means ± S.E.M., in nmol of phosphate/unit of inactive complex) for total sites, occupied sites and percentage occupancies, with numbers of observations in parentheses were: fed, 2.1 ± 0.04, 1.15 ± 0.04, 54.8 ± 1.6% (39); starved, 2.05 ± 0.03, 1.85 ± 0.03, 90.2 ± 1.4% (28); alloxan-diabetic, 1.99 ± 0.03, 1.72 ± 0.03, 86.4 ± 1.4% (68). 4. Values (means ± S.E.M. for percentage occupancy) for individual sites of phosphorylation in pyruvate dehydrogenase phosphate given in the order sites 1, 2 and 3 were: fed, 100 ± 2.7, 27.8 ± 1.6, 33.9 ± .9; starved, 100 ± 1.4, 76.2 ± 2.0, 92.4 ± 1.5; alloxan-diabetic, 100 ± 1.2, 64.0 ± 1.7, 94.6 ± 1.4. 5. It is concluded that starvation or alloxan-diabetes leads to a 2–3-fold increase in the occupancy of phosphorylation sites 2 and 3 in pyruvate dehydrogenase phosphate in rat heart *in vivo*.

Mammalian pyruvate dehydrogenase complexes (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3 + pyruvate dehydrogenase kinase as an intrinsic component) exist in interconvertible active (dephosphorylated) and inactive (phosphorylated) forms. Dephosphorylation is effected by a loosely associated phosphatase (Linn *et al.*, 1969*a*,*b*). In complexes purified from bovine kidney and heart, or pig heart, sequence analyses of tryptic phosphopeptides have shown that inactivation is correlated with phosphorylation of one specific serine residue (called site 1) in an α chain of pyruvate decarboxylase (EC 1.2.4.1) (subunit composition $\alpha_2\beta_2$) (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). Phosphorylation of sites 2 and 3 occurs mainly after inactivation is complete; relative rates of phosphorylation are site 1 > site 2 > site 3 (approx. 15:3:1; Kerbey *et al.*, 1979). A similar sequence of phosphorylations is seen in complex purified from rat heart mitochondria and in complex *in situ* in intact rat heart mitochondria (Sale & Randle, 1980). Phosphorylation of sites 2 and 3 in addition to site 1 decreases the rate of reactivation of complex by pyruvate dehydrogenase phosphate phosphatase (pig heart complex, Sugden *et al.*, 1978; Kerbey & Randle, 1979; bovine kidney complex, Sugden & Simister, 1980; complex in intact rat heart mitochondria, Sale & Randle, 1980).

In the rat, the proportion of inactive complex in heart muscle is increased by 48 h of starvation or by induction of alloxan-diabetes (Wieland *et al.*, 1971) from 70–75% to 99% (Kerbey *et al.*, 1976). The total amount of the complex (i.e. sum of inactive + active forms) is not changed by diabetes or starvation. It is assumed that the increased proportion of inactive complex in diabetic and starved animals is a consequence of increased phosphorylation of the inactivating site (site 1). The factors involved may include activation of the kinase reaction (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1979) by the increased concentration ratio of acetyl-CoA/CoA in the heart (Garland & Randle, 1964) and activation of the kinase reaction by a further unknown mechanism (Hutson & Randle, 1978). Evidence has also been obtained that the rate of

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activation of inactive complex by phosphatase is decreased in heart mitochondria of starved or diabetic rats (Hutson *et al.*, 1978). As the mechanism apparently involved a change in inactive complex, but did not involve a decrease in phosphatase activity, it was suggested that a more extensive phosphorylation of sites 2 and 3 in inactive complex from hearts of starved or diabetic rats may account for the results (Hutson *et al.*, 1978).

It was therefore important to determine the occupancy of sites of phosphorylation in pyruvate dehydrogenase phosphate complexes (i.e. inactive complexes) *in vivo* in hearts of fed, starved and alloxan-diabetic rats. Three methods of measurement were considered. The first, which would have involved administration of $^{32}P_i$ to rats, was avoided on grounds of safety and the known difficulty of equilibrating intracellular ATP with extracellular $^{32}P_i$. The second, based on analysis of non-radioactive phosphate in the complex, was tried, but proved impracticable because of lack of precision and specificity in sensitive methods of phosphate analysis. The method adopted involved back-titration, i.e. phosphorylation of unoccupied sites with [γ^{32}P]ATP after extraction and partial purification of the complex under conditions that are assumed to prevent phosphorylation or dephosphorylation. This incorporation of ^{32}P was compared with that achieved under comparable conditions with complexes that had been fully reactivated by incubation with pyruvate dehydrogenase phosphate phosphatase. Evidence given by Sugden *et al.* (1978) shows that full reactivation involves complete dephosphorylation. Occupancy of sites of phosphorylation *in vivo* was then obtained by difference. The occupancies *in vivo* in complexes purified from hearts of fed, starved or alloxan-diabetic rats are reported in the present paper.

Experimental

The sources of biochemicals, chemicals and enzymes were as given by Sale & Randle (1980). Pyruvate dehydrogenase phosphate phosphatase was partially purified from frozen ox or pig hearts by the method of Severson *et al.* (1974).

Rats and preparation of rat heart pyruvate dehydrogenase phosphate complex

Details of feeding of rats, induction of alloxan-diabetes and starvation for 48h were as described by Kerbey *et al.* (1977). Preparations of pyruvate dehydrogenase phosphate complex were made from the hearts of 20 rats (rat weight 300–400g). Rats were injected intraperitoneally with Sagatal (May and Baker, Dagenham, Essex, U.K.; 90mg/kg) to induce anaesthesia before rapid removal and freezing of the hearts with a tissue clamp at liquid-N_2 temperature. Freeze-clamped hearts were powdered with a pestle and mortar at liquid-N_2 temperature. The powder was warmed to −18°C by placing in a deep-freeze for 45min, quickly thawed by sprinkling on to 100ml of rapidly stirred buffer at 20°C (30mM-potassium phosphate/50mM-EDTA/2mM-dithiothreitol, pH7.0) and extracted with a Polytron PT 20 homogenizer (3 × 30s bursts, position 6) at about 4°C (the mixture of warm extraction fluid and cold powder was arranged to achieve this final temperature). After centrifuging at 4°C for 30min at 38000*g*, the supernatant (fraction 1 of Table 1) was warmed to 18°C, 0.01 vol. of 5M-NaCl was added, and the pH was adjusted to 6.5 with 10% (v/v) acetic acid. After addition of 0.1 vol. of 50% (w/v) poly(ethylene glycol) the precipitate was collected by centrifugation (30min, 38000*g*, 18°C) and taken up in 7ml of 30mM-potassium phosphate/10mM-EDTA/2mM-dithiothreitol (pH7.0) with a manual glass/glass Potter–Elvehjem homogenizer. At this stage two different methods have been used. In method (A) the pH of the solution was adjusted to 6.2 (micro-electrode) with 10% (v/v) acetic acid and centrifuged (20min, 38000*g*, 4°C). In method (B) the solution was first clarified and stored overnight at 0°C before adjustment to pH6.2 and centrifugation as in method (A). (Method B was abandoned in favour of method A for reasons given in the Results and discussion section.) The pH of the supernatant (obtained by either method) was adjusted to 7.0 (micro-electrode) with 2M-KOH (fraction 2 of Table 1) and the pyruvate dehydrogenase phosphate was collected by centrifuging at 4°C for 2h at 150000*g*. The pellet was dissolved in 2ml of 30mM-potassium phosphate/10mM-EDTA/2mM-dithiothreitol (pH7.0) and further contaminating material was removed by adjusting the pH to 6.2 (micro-electrode) with 10% (v/v) acetic acid and centrifugation at 4°C for 10min at 38000*g*. After returning the pH of the supernatant to 7.0 (micro-electrode) with 2M-KOH (fraction 3 of Table 1) it was layered onto 4ml of 30mM-potassium phosphate/10mM-EDTA/2mM-dithiothreitol (pH7.0) containing 2% (w/v) sucrose and centrifuged at 4°C for 2h at 150000*g* to remove pyruvate dehydrogenase phosphate phosphatase. The pellet, containing pyruvate dehydrogenase phosphate complex, but free of detectable phosphatase (no formation of active complex on incubation for 10min at 30°C in the presence of 25mM-Mg^{2+} and 100μM-Ca^{2+}) and containing only 0.5–2.5% of active complex, was dissolved in 1ml of 30mM-potassium phosphate/2mM-dithiothreitol, pH7.0, (no EDTA) and stored at −18°C (fraction 4 of Table 1). The determinations on any one preparation were completed within 1 week.

When the proportions of active and inactive complexes were assayed in the hearts *in vivo*, the

freeze-clamped powdered heart was extracted into 0.1 M-potassium phosphate/5 mM-EDTA/2 mM-dithiothreitol (pH 7.0) (50 ml/g of heart; Polytron homogenizer, position 5, 30 s), then centrifuged in an Eppendorf 3200 instrument for 2.5 min at 18000 *g* (fraction 0).

Preparation of rat heart pyruvate dehydrogenase complex from pyruvate dehydrogenase phosphate complex, and assay of pyruvate dehydrogenase phosphate complex

Pyruvate dehydrogenase phosphate complex was assayed after complete conversion into pyruvate dehydrogenase complex by using ox or pig heart pyruvate dehydrogenase phosphate phosphatase (enzyme from both sources gave the same results). This method was also used for the preparation of pyruvate dehydrogenase complex. Incubations were at 30°C and contained 1–2 units of pyruvate dehydrogenase phosphate/ml, sufficient phosphatase to effect complete conversion into active complex within 5 min, 10 mM-potassium phosphate, 25 mM-Tris/HCl, 100 μM-$CaCl_2$ and 36 mM-$MgCl_2$ (pH 7.0) (computed free $[Mg^{2+}] = 26$ mM, free $[Ca^{2+}] = 100\,\mu$M). Reaction was initiated by addition of phosphatase after 5 min preincubation at 30°C. The formation of active pyruvate dehydrogenase complex was monitored over 20 min to ensure that maximum conversion into active complex had been achieved. For the preparation of pyruvate dehydrogenase complex, 2–3 units of pyruvate dehydrogenase phosphate were used. After conversion by phosphatase, an equal volume of ice-cold 20 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol (pH 7.0) was added, followed by layering on to 4 ml of 2% (w/v) sucrose in 20 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol (pH 7.0) and centrifugation at 4°C for 2 h at 150000 *g*. The pellet containing pyruvate dehydrogenase complex was dissolved in 200–300 μl of 20 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol and stored at −18°C. Determinations on any one preparation were completed within 3 days.

Phosphorylation of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes with [γ-^{32}P]ATP

Pyruvate dehydrogenase or pyruvate dehydrogenase phosphate complexes (0.3–1.5 units/ml) were incubated at 30°C in 20 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol with 1 mM-$MgCl_2$ and 0.5 mM-[γ-^{32}P]ATP (specific radioactivity varied between 60 and 300 d.p.m./pmol) for up to 30 min. Inactivation of pyruvate dehydrogenase complex was complete (>99%) within 10 min. Concentrations of complex used were dependent on the ATPase activity of the preparation and were chosen such that no more than 40% of the ATP present was hydrolysed in 10 min or 60% in 30 min. ATPase activity was assayed by the method of Cooper *et al.* (1974). Evidence for complete phosphorylation under these conditions is given in the Results and discussion section. Doubling the concentrations of [γ-^{32}P]ATP and $MgCl_2$ did not increase the extent of phosphorylation (results not shown). Incorporation of ^{32}P into protein was measured at various times by spotting duplicate 10 μl samples of the incubations on to 2 cm × 1 cm 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 with [γ-^{32}P]ATP diluted by an equivalent volume of buffer without complex. The blank correction averaged approx. 15% for conditions under which the pyruvate dehydrogenase complex was fully phosphorylated. The validity of this type of blank was examined in two ways, firstly, by mixing buffer containing [γ-^{32}P]ATP with complex at 0°C and immediately spotting samples on to paper squares, and secondly, by mixing buffer containing [γ-^{32}P]ATP with complex fully phosphorylated with non-radioactive ATP. The three types of blank did not differ significantly. The specific radioactivity of [γ-^{32}P]ATP was determined by spectrophotometric assay of the concentration in 1 M-HCl at 257 nm and by assaying samples from the cuvette for radioactivity in methoxyethanol/toluene scintillator (Severson *et al.*, 1974). Incorporation of ^{32}P was calculated as nmol of P/unit of inactive complex.

Pyruvate dehydrogenase complex activity

Pyruvate dehydrogenase complex (active form) was assayed spectrophotometrically by the direct assay of Cooper *et al.* (1974) in the presence of pig heart lipoamide dehydrogenase [10 units/ml; purified rat heart complex is deficient in lipoamide dehydrogenase (Sale & Randle, 1980)]. One unit of complex activity forms 1 μmol of NADH/min at 30°C. One unit of pyruvate dehydrogenase phosphate complex yields one unit of active complex after conversion into the active form by phosphatase.

Paper electrophoresis of tryptic phosphopeptides

For tryptic digestion, 100–200 μl samples of the incubations were removed at various times after complete phosphorylation had occurred and the complex was precipitated by addition of trichloroacetic acid to 10% (w/v). Bovine serum albumin carrier (40 μl of 20 mg/ml) was added to the samples, followed by mixing and cooling to 0°C. The denatured proteins were separated by centrifugation

(Eppendorf 3200 centrifuge, 1 min) and the supernatant was aspirated. The pellets were washed [0.5 ml of 10% (w/v) trichloroacetic acid] by alternate resuspension and centrifugation until the supernatant radioactivity was undetectable (usually five washes). The pellets were dissolved in 30 μl of 8 M-urea/2% (w/v) NH_4HCO_3 and diluted with 90 μl of 2% (w/v) NH_4HCO_3 [it was not necessary to adjust the pH (checked with pH paper) provided that care was taken to remove all the surplus trichloroacetic acid after the final centrifugation]. Trypsin [0.05 vol. of a 20 mg/ml solution in 2 M-urea/2% (w/v) NH_4HCO_3] was added and the samples were incubated for 3–6 h at 30°C. This rendered over 90% of the ^{32}P soluble in 10% trichloroacetic acid. The tryptic digests were then subjected to high-voltage paper electrophoresis on Whatman 3MM paper (2 h at 3 kV) in pH 1.9 buffer [8% (v/v) acetic acid/2% (v/v) formic acid], with N^6-dinitrophenyl-lysine markers. The paper tracks along which the ^{32}P-labelled phosphopeptides had migrated were excised, cut into 1 cm strips and assayed for ^{32}P by liquid-scintillation spectrometry in toluene-based scintillator (Severson *et al.*, 1974). The distribution of ^{32}P between separated [^{32}P]-phosphopeptides was determined by summing the d.p.m. in each peak; recovery of trichloroacetic acid-soluble ^{32}P after electrophoresis was greater than 90%.

Calculations

Overall occupancy in vivo of sites of phosphorylation in pyruvate dehydrogenase phosphate complexes

Pyruvate dehydrogenase phosphate complex, as made, was contaminated with a small amount of active complex. In order to calculate incorporation into inactive pyruvate dehydrogenase phosphate complex (U), it was necessary to correct for incorporation into contaminating active complex. Incorporation of ^{32}P into inactive pyruvate dehydrogenase phosphate complex, in nmol of P/unit of inactive complex, is given by $U = U' - pT$, where U' is incorporation into complex as made (i.e. into inactive complex and contaminating active complex; in nmol of P/unit of inactive complex), p is the concentration of contaminating active complex as a fraction of that of inactive complex, and T is incorporation of ^{32}P into active complex after conversion with phosphatase (in nmol of P incorporated/unit of complex inactivated). The correction for contaminating active complex was small (p was less than 0.026). The occupancy of sites of phosphorylation in inactive complex *in vivo* (X) is given by $X = T - U$, and the percentage occupancy *in vivo* by $100\,X/T$.

Occupancy in vivo of individual phosphorylation sites of pyruvate dehydrogenase phosphate complex

Rat heart pyruvate dehydrogenase complex contains three phosphorylation sites recoverable in three tryptic phosphopeptides: A′ contains site 1 phosphorylated; on complete phosphorylation, A′ is replaced by A, which contains both sites 1 and 2 phosphorylated; B contains site 3 phosphorylated. The three tryptic phosphopeptides may be separated by high-voltage electrophoresis. Fractional incorporations into each site 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) (Sale & Randle, 1980).

If T_1, T_2 and T_3 = ^{32}P incorporation into sites 1, 2 and 3 respectively of pyruvate dehydrogenase complex (nmol of P/unit of inactive complex), then:

$T_1 = T \times$ (fractional incorporation into site 1)

$T_2 = T \times$ (fractional incorporation into site 2)

$T_3 = T \times$ (fractional incorporation into site 3)

U_1, U_2 and U_3 = unoccupied sites (1, 2 and 3 respectively) of pyruvate dehydrogenase phosphate = ^{32}P incorporations (nmol of P/unit of inactive complex) into the sites of pyruvate dehydrogenase phosphate. In pyruvate dehydrogenase phosphate, site 1 is assumed to be fully occupied with unlabelled phosphate ($U_1 = 0$). Thus ^{32}P-labelled phosphopeptide A derived from pyruvate dehydrogenase phosphate contains all its ^{32}P located at site 2. Pyruvate dehydrogenase phosphate complex preparations contained small amounts of pyruvate dehydrogenase complex, which will incorporate ^{32}P into sites 1 and 2 of phosphopeptide A and site 3 of B. As the incorporations into individual sites of pyruvate dehydrogenase are known from the above, a correction may be applied.

Thus:

$U_2 = (U' \times$ fractional incorporation into A$) - p(T_1 + T_2)$

$U_3 = (U' \times$ fractional incorporation into B$) - (p \times T_3)$

O_1, O_2 and O_3 = occupancies of phosphorylation sites 1, 2 and 3 in pyruvate dehydrogenase phosphate (nmol of P/unit of inactive complex).

$O_1 = T_1 - U_1 = T_1$, because $U_1 = 0$

$O_2 = T_2 - U_2$

$O_3 = T_3 - U_3$

Percentage occupancies of individual sites of pyruvate dehydrogenase phosphate were obtained by:

$$\text{Site 1} = \frac{O_1}{T_1} \times 100$$

$$\text{Site 2} = \frac{O_2}{T_2} \times 100$$

$$\text{Site 3} = \frac{O_3}{T_3} \times 100$$

Results and discussion

Preparation of rat heart pyruvate dehydrogenase phosphate complex

Complex purified from hearts of fed normal rats contained on average approx. 2% of active pyruvate dehydrogenase complex and 98% of inactive pyruvate dehydrogenase phosphate complex. The method used to assay the proportions of active and inactive complexes in the frozen tissue yielded a higher proportion of active complex (approx. 30%). The features of the purification which may result in the lower proportion of active complex are discussed below and are set out in detail in Table 1. It was fortuitous that the method of purification used gave preparations of inactive complex from hearts of fed normal rats which contained a low concentration of active complex. The preparation of complex largely free of the active form is not essential, however, for estimating site occupancies in pyruvate dehydrogenase phosphate complex by the method used. The method includes a correction for active pyruvate dehydrogenase complex present.

The optimum method for assay of the proportions of active and inactive complexes in the rat heart (A. L. Kerbey, P. J. Randle & S. Whitehouse, unpublished work) was homogenization of freeze-clamped powdered heart in 0.1 M-potassium phosphate/5 mM-EDTA/2 mM-dithiothreitol (pH 7.0) (50 ml/g of heart), followed by a short centrifugation (2.5 min, 18000 *g*) to clarify the mixture (fraction 0 in Table 1). However, for larger-scale purification of inactive pyruvate dehydrogenase phosphate complex, a different extraction procedure was used to facilitate precipitation and fractionation. This involved a 10-fold decrease in the volume of extraction fluid, balanced by a 10-fold increase in EDTA concentration (i.e. total amount of EDTA added/g of tissue was the same in the two procedures), 30 mM-potassium phosphate buffer (pH 7.0) and a longer centrifugation (30 min) (fraction 1). As shown in Table 1, the method of extraction used in the purification procedure yielded less complex (i.e. active + inactive forms) and a lower proportion of the active form of the complex than did the method used to assay the proportions of active and inactive complexes in the tissue. There was in addition a less marked disproportionate loss of active complex in the final purification step (Table 1). In hearts of fed normal rats, the proportion of active complex was 28.6% of total complex. Pyruvate dehydrogenase phosphate complex purified from the same tissue contained only 2.1% of active complex (Table 1). In hearts of diabetic or starved rats, the proportion of active complex was less than 1% of total complex; purified preparations of pyruvate dehydrogenase phosphate complex also contained less than 1% of active complex.

The yield of pyruvate dehydrogenase phosphate complex from 20 hearts averaged 7.4 units from fed normal animals and 12.8 units from diabetic or starved animals. The percentage yields from approx. 18 g of muscle were 9% (fed normal) and 12%

Table 1. *Yield of active pyruvate dehydrogenase complex and inactive pyruvate dehydrogenase phosphate complex during purification from hearts of fed normal rats by method A*

For full definition of individual fractions (1–4) see the text. Fraction 0 was an extract of frozen powder made under conditions optimal for assay of active and inactive complexes in the heart [50 ml of 0.1 M-potassium phosphate/5 mM-EDTA/2 mM-dithiothreitol (pH 7)/g of heart; Polytron homogenizer, position 5, 30 s]. In fractions 0–3, active complex was assayed spectrophotometrically by coupling to arylamine acetyltransferase (Cooper *et al.*, 1974). Fraction 4 was assayed by the direct method of Cooper *et al.* (1974). Inactive pyruvate dehydrogenase phosphate complex was assayed as active complex after conversion with phosphatase. In fraction 1, 50 mM-EDTA was converted into MgEDTA by addition of 1 M-$MgCl_2$ and pH maintained at 7 with KOH. Lipoamide dehydrogenase was added in assays of fractions 2–4.

Fraction	Step	Activity (units/g wet wt.) for Active complex	Activity (units/g wet wt.) for Inactive complex	Active complex (% of total complex)
0	See the legend	1.19	2.96	28.6
1	Initial extract, supernatant	0.14	1.81	7.7
2	Neutralized pH 6.2 supernatant after poly(ethylene glycol)	0.11	1.59	6.9
3	Neutralized pH 6.2 supernatant after 150000 *g* centrifugation	0.07	1.06	6.6
4	Second 150000 *g* centrifugation	0.01	0.50	2.1

(diabetic or starved) [based on 4.5 units of pyruvate dehydrogenase phosphate/g wet wt. in fed animals and 6 units/g in starved and diabetic animals; see Whitehouse *et al.* (1974) and Kerbey *et al.* (1976)]. In calculating occupancy of phosphorylation sites *in vivo*, it has been assumed that the purified samples used for assays were representative of the total pool of pyruvate dehydrogenase phosphate complex in the heart.

Phosphorylation of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes with Mg–[γ-^{32}P]ATP

General principles. The method adopted for measurement of site occupancies *in vivo* involves the estimation of unoccupied sites by phosphorylation with Mg–[γ-^{32}P]ATP. The occupied sites are then calculated by difference, total sites being estimated on completely dephosphorylated complex. The following conditions must be met if the method is to be valid. No phosphorylation or dephosphorylation must occur during removal of the hearts and extraction and purification of the complex. Phosphorylation of unoccupied sites with Mg-[γ-^{32}P]ATP must be complete; and no exchange or turnover of sites occupied with non-radioactive phosphate must occur during phosphorylation with Mg-[γ-^{32}P]ATP.

Removal and freeze-clamping of hearts took less than 20s. The complex was extracted from the frozen tissue at 4°C with medium containing 50mM-EDTA. Under these conditions, chelation of Ca^{2+} and Mg^{2+} by EDTA prevents dephosphorylation by phosphatase, and chelation of Mg^{2+}, dilution, low temperature and the action of ATPases prevents phosphorylation with Mg–ATP (see Whitehouse *et al.*, 1974; Kerbey *et al.*, 1976). The absence of hydrolysis of pyruvate dehydrogenase phosphate complex during purification was checked by adding pig heart pyruvate dehydrogenase [^{32}P]phosphate complex to the initial extract. The distribution of ^{32}P between sites of phosphorylation in the pig heart complex did not change during co-purification with the rat heart complex (added ^{32}P-labelled complex: site 1, 38.8%; site 2, 33%; site 3, 28.2%; purified complex; site 1, 40.6%; site 2, 33.6%; site 3, 25.8%). Evidence given in the next section indicates that phosphorylation of complexes was complete. Incorporations reached a plateau within 10min, and with pyruvate dehydrogenase complexes incorporations into all three sites were equivalent. Because site-2 and site-3 phosphorylations are slower than site-1 phosphorylation, equivalent incorporation into all three sites in conjunction with complete inactivation may represent unequivocal evidence of complete phosphorylation. The preparations displayed ATPase activity sufficient to hydrolyse up to 40% of the ATP in 10min and 60% in 30min. However, this would not be expected to interfere seriously with phosphorylation, even though ADP is a competitive inhibitor (Linn *et al.*, 1969*a*,*b*). The calculated velocities of phosphorylation after 10 and 30min are 0.72 $V_{max.}$ and 0.55 $V_{max.}$ (initial [ATP] 0.5mM; K_m for ATP 27.4μM; K_i for ADP 60μM; Cooper *et al.*, 1974). With pig heart pyruvate dehydrogenase phosphate complexes, exchange of protein-bound phosphate with the γ-phosphate of ATP, if it occurs, is very slow (<5% in 60min; Radcliffe *et al.*, 1980). Turnover of protein-bound phosphate may occur if complex preparations are contaminated with phosphatase. The preparations of pyruvate dehydrogenase phosphate used in the present study were devoid of phosphatase, as judged by absence of reactivation on incubation of the complexes with Mg^{2+} and Ca^{2+}. However, reactivation of fully phosphorylated complexes is a less sensitive test of phosphatase contamination than release of phosphate, because in ox and pig complexes dephosphorylation of site 2 is much more rapid than reactivation (Sugden *et al.*, 1978; Teague *et al.*, 1979; A. L. Kerbey, P. J. Randle & A. Kearns, unpublished work). As described below, there was evidence of some turnover in preparations of pyruvate dehydrogenase phosphate from starved or diabetic rats obtained by method B, but not by method A.

Incorporations. Typical time courses for incorporation of ^{32}P into pyruvate dehydrogenase complex (obtained by dephosphorylation of pyruvate dehydrogenase phosphate complex with phosphatase) and into pyruvate dehydrogenase phosphate complex (as prepared) in the presence of 0.5mM-[γ-^{32}P]ATP and 1mM-$MgCl_2$ are shown in Fig. 1. For pyruvate dehydrogenase complex, derived from the hearts of normal, diabetic or starved rats, incorporation of ^{32}P reached a plateau within 10min. At this time, greater than 99% inactivation of complex had occurred (results not shown). The maximum incorporations of ^{32}P into pyruvate dehydrogenase complexes, in nmol of P/unit of inactive complex, were: normal, 2.1 ± 0.04 (39 observations on three preparations); diabetic, 1.99 ± 0.03 (68 observations on four preparations) and starved, 2.05 ± 0.03 (28 observations on two preparations) (data for individual preparations are given in Table 2). These values are means $\pm$ S.E.M. of observations taken from the plateau portions of the curves. This method was routinely used to calculate the maximum incorporations of ^{32}P into complex (except where stated otherwise). In a previous study (Sale & Randle, 1980) the maximum total incorporation into rat heart pyruvate dehydrogenase complex, purified by a different method, was 2.20nmol of P/unit of inactive complex. For pyruvate dehydrogenase complex in intact rat heart mitochondria a value of 2.25nmol of P/unit of inactive complex was obtained. These values are

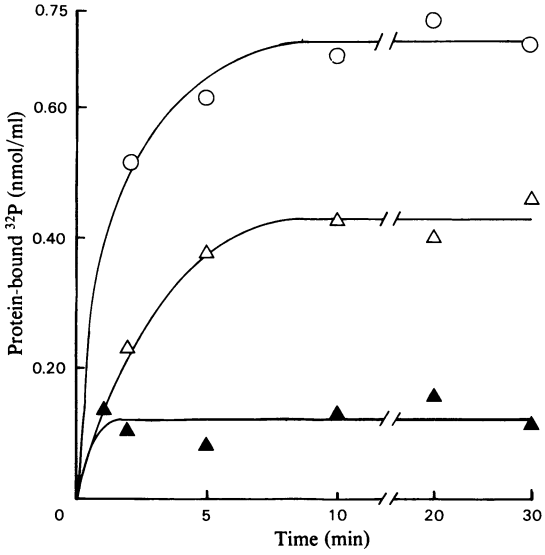

Fig. 1. *Time courses of phosphorylation of rat heart pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes with [γ-^{32}P]ATP*

Pyruvate dehydrogenase phosphate complexes were isolated from hearts of fed normal (△) or alloxan-diabetic (▲) rats by method A (see the Experimental section) and incubated at 30°C at 0.42 or 0.59 unit/ml, respectively, with 0.5 mM-[γ-^{32}P]ATP/20 mM-potassium phosphate (pH 7.0)/1 mM-$MgCl_2$/2 mM-dithiothreitol/10 mM-EGTA. At the times shown, 10 μl samples were taken for assay of protein-bound ^{32}P. Incorporations of ^{32}P into protein were corrected for incorporation into the small amount of pyruvate dehydrogenase complex present. Pyruvate dehydrogenase phosphate complexes were converted into pyruvate dehydrogenase complex by incubation with pyruvate dehydrogenase phosphate phosphatase. After separation from phosphatase by centrifugation, the pyruvate dehydrogenase complexes were incubated with [γ-^{32}P]ATP and incorporation of ^{32}P into protein was assayed as outlined above. The representative data shown (○) are for complex from fed normal rats, incubated at 0.34 unit/ml. All values are the means for duplicate determinations. Incorporations into pyruvate dehydrogenase complexes from starved or diabetic rats, like that into complex from normal rats (○), reached a steady state within 10 min. Incorporations into pyruvate dehydrogenase phosphate complex from starved rats conformed to the time course (▲) shown for diabetic rats. For further details see the Experimental section.

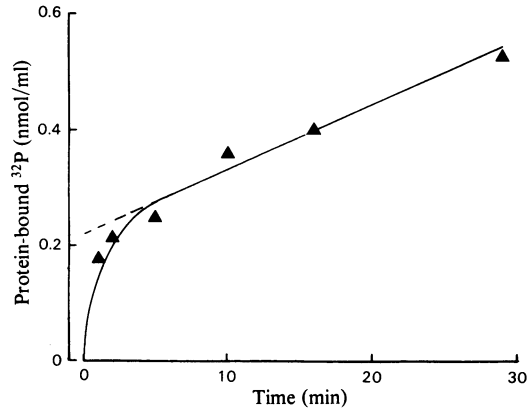

Fig. 2. *Time course of phosphorylation with [γ-^{32}P]ATP of pyruvate dehydrogenase phosphate complex prepared by method B from hearts of 48 h-starved rats*

Pyruvate dehydrogenase phosphate complex was prepared from hearts of 48 h-starved rats by method B and incubated with [γ-^{32}P]ATP, and incorporation of ^{32}P into protein was measured by using the conditions described in the legend to Fig. 1. The concentration of complex was 1.0 unit/ml, and each value is the mean for four observations. The linear portion of the curve was fitted by least-squares linear-regression analysis ($r = 0.980$; slope 0.011 ± 0.001 nmol/min per ml for mean ± S.E.M.). As described in the text, the linear incorporation is assumed to reflect turnover of non-radioactive phosphate; the intercept on the ordinate is assumed to represent incorporation of ^{32}P into unoccupied phosphorylation sites.

similar to those in the present study. Further evidence that phosphorylation was complete was derived from the distribution of ^{32}P between the three sites of phosphorylation. The mean distributions between the three sites in all of the results shown in Table 2 were 0.33 (site 1), 0.33 (site 2), 0.34 (site 3), i.e. equivalent incorporation into all three sites. Further evidence is provided by the complete absence of peptide A′ (Fig. 3); this peptide is present when phosphorylation is incomplete. It is assumed in what follows that phosphorylation of pyruvate dehydrogenase phosphate with [γ-^{32}P]ATP under the same conditions was also complete.

Incorporation of ^{32}P into pyruvate dehydrogenase phosphate complex of fed normal rats prepared by method A or method B was maximal within 10 min (Fig. 1). The maximum total incorporation was 0.961 ± 0.025 nmol of P/unit of inactive complex (mean ± S.E.M. for 39 observations on three preparations). Incorporation of ^{32}P into pyruvate dehydrogenase phosphate complexes from hearts of starved or diabetic rats was substantially lower than with complex from fed normal rats, and reached a plateau within 1–2 min (Fig. 1). The maximum total incorporations (nmol of P/unit of inactive complex; means ± S.E.M.) were diabetic 0.281 ± 0.013 (38 observations, two preparations) and starved 0.166 ± 0.022 (eight observations, one preparation). These data were obtained with complexes prepared by method A.

Table 2. *Occupancy in vivo of sites of phosphorylation in rat heart pyruvate dehydrogenase phosphate complex*
Pyruvate dehydrogenase phosphate complexes were prepared from the hearts of 20 rats either by method A (preparations 3, 4, 5, 8) or by method B (preparations 1, 2, 6, 7, 9), and a portion of each preparation was converted into the corresponding pyruvate dehydrogenase complex with phosphatase. Both types of complex were then incubated with [γ-^{32}P]ATP to phosphorylate maximally available sites (i.e. unoccupied sites). Incorporation of ^{32}P into all sites (total) and individual sites in pyruvate dehydrogenase complex is shown in column (*a*) and into pyruvate dehydrogenase phosphate complex in column (*b*). Incorporation into pyruvate dehydrogenase complex was corrected for incorporation into the small amount of pyruvate dehydrogenase complex present. Incorporations are given in nmol of P/unit of complex inactivated [column (*a*)] or as nmol of P/unit of inactive complex (measured after conversion with phosphatase) [column (b)]. The difference [columns (*a*)–(*b*)] equals the occupancy in the original pyruvate dehydrogenase phosphate complex [column (*c*)]. Percentage occupancies [$100 \times (c)/(a)$] are given in column (*d*). Incorporations of ^{32}P into pyruvate dehydrogenase phosphate complex preparations 6, 7 and 9 had to be corrected for turnover of unlabelled phosphate (for details see the text and Fig. 2; corrected data are shown in this Table). *$P < 0.001$ for difference from any normal fed control (Student's *t* test). Abbreviations: PDH, pyruvate dehydrogenase complex; PDHP, pyruvate dehydrogenase phosphate complex. For further details of methods and calculations see the Experimental section. The total numbers of measurements of incorporation are shown in parentheses.

Preparation no.	Rats	Site(s)	^{32}P incorporation (nmol of P/unit of complex (means ± S.E.M.) for: (*a*) PDH complex	(*b*) PDHP complex	Occupancy of sites of phosphorylation in PDHP complex (means ± S.E.M.) as: (*c*) nmol of P/unit	(*d*) %
1	Normal fed (11)	Total	1.93 ± 0.03	0.96 ± 0.04	0.97 ± 0.05	50 ± 2.1
		1	0.65 ± 0.01	—	0.65 ± 0.01	100 ± 1.5
		2	0.62 ± 0.01	0.45 ± 0.02	0.17 ± 0.02	27 ± 2.6
		3	0.66 ± 0.01	0.52 ± 0.02	0.15 ± 0.02	22 ± 2.7
2	Normal fed (12)	Total	2.33 ± 0.05	1.03 ± 0.04	1.30 ± 0.06	56 ± 2.1
		1	0.76 ± 0.06	—	0.76 ± 0.06	100 ± 8.4
		2	0.76 ± 0.06	0.52 ± 0.02	0.24 ± 0.03	32 ± 3.1
		3	0.81 ± 0.02	0.51 ± 0.02	0.30 ± 0.03	37 ± 2.6
3	Normal fed (16)	Total	2.06 ± 0.04	0.91 ± 0.04	1.15 ± 0.06	56 ± 2.3
		1	0.72 ± 0.01	—	0.72 ± 0.01	100 + 2.0
		2	0.71 ± 0.01	0.53 ± 0.02	0.18 ± 0.03	25 ± 2.7
		3	0.63 ± 0.01	0.38 ± 0.02	0.25 ± 0.02	40 ± 2.7
4	Diabetic (14)	Total	1.97 ± 0.06	0.23 ± 0.02*	1.74 ± 0.06*	88 ± 3.1*
		1	0.66 ± 0.02	—	0.66 ± 0.02	100 ± 3.0
		2	0.66 ± 0.02	0.19 ± 0.02*	0.47 ± 0.03*	72 ± 3.4*
		3	0.66 ± 0.02	0.04 ± 0.01*	0.61 ± 0.02*	94 ± 3.1*
5	Diabetic (24)	Total	1.93 ± 0.04	0.31 ± 0.01*	1.62 ± 0.04*	84 ± 2.0*
		1	0.64 ± 0.01	—	0.64 ± 0.01	100 ± 2.2
		2	0.64 ± 0.01	0.28 ± 0.01	0.37 ± 0.02*	57 ± 2.5*
		3	0.64 ± 0.02	0.03 ± 0.003*	0.61 ± 0.02*	95 ± 2.8*
6	Diabetic (12)	Total	2.24 ± 0.07	0.25 ± 0.05*	1.99 ± 0.08*	89 ± 3.3*
		1	0.74 ± 0.02	—	0.74 ± 0.02	100 ± 3.3
		2	0.74 ± 0.02	0.22 ± 0.04*	0.52 ± 0.04*	71 ± 4.8*
		3	0.77 ± 0.02	0.04 ± 0.01*	0.73 ± 0.02*	95 ± 3.2*
7	Diabetic (18)	Total	1.91 ± 0.03	0.26 ± 0.02*	1.65 ± 0.04*	86 ± 1.8*
		1	0.62 ± 0.01	—	0.62 ± 0.01	100 ± 1.5
		2	0.62 ± 0.01	0.23 ± 0.02*	0.39 ± 0.02*	63 ± 2.7*
		3	0.68 ± 0.01	0.04 ± 0.004*	0.64 ± 0.01*	95 ± 1.7*
8	Normal starved (8)	Total	2.16 ± 0.06	0.17 ± 0.02*	1.99 ± 0.06*	92 ± 2.9*
		1	0.74 ± 0.02	—	0.74 ± 0.02	100 ± 3.0
		2	0.74 ± 0.02	0.15 ± 0.02*	0.58 ± 0.03*	79 ± 3.3*
		3	0.69 ± 0.02	0.01 ± 0.003*	0.67 ± 0.02*	92 ± 3.1*
9	Normal starved (20)	Total	2.00 ± 0.03	0.21 ± 0.02*	1.79 ± 0.03*	89 ± 1.5*
		1	0.65 ± 0.01	—	0.65 ± 0.01	100 ± 1.6
		2	0.65 ± 0.01	0.16 ± 0.01*	0.49 ± 0.01*	75 ± 2.5*
		3	0.70 ± 0.01	0.05 ± 0.004*	0.65 ± 0.01*	93 ± 1.7*

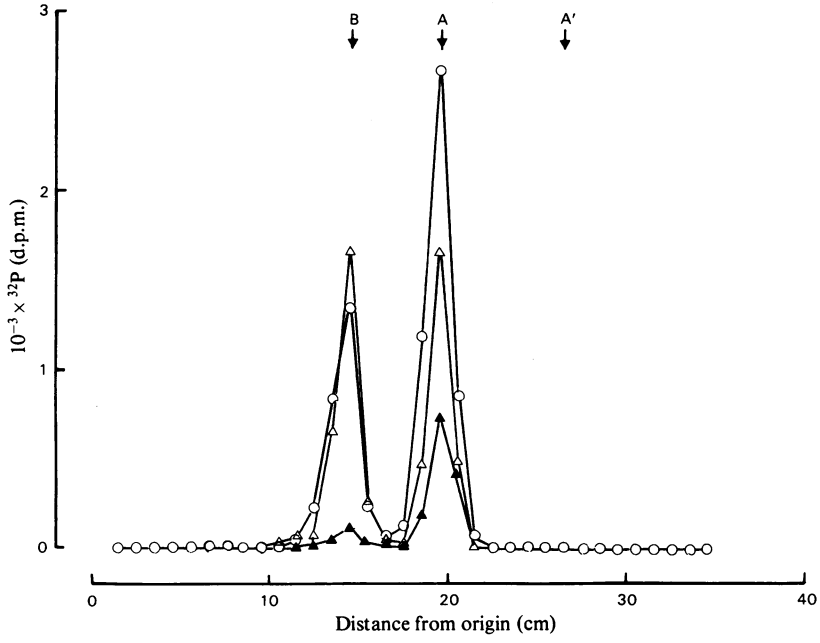

Fig. 3. *High-voltage paper electrophoresis of tryptic digests of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes incubated with [γ-^{32}P]ATP*

Pyruvate dehydrogenase (normal, O) and pyruvate dehydrogenase phosphate complexes (normal, △; alloxan-diabetic, ▲) were incubated with [γ-^{32}P]ATP to label maximally available phosphorylation sites as described in the legend to Fig. 1. Incubations were then terminated and the ^{32}P-labelled proteins were subjected to tryptic digestions followed by high-voltage paper electrophoresis as described in the Experimental section. An N^6-dinitrophenyl-lysine marker migrated 18.5 cm. The expected position of phosphopeptide A′ is assumed from its known electrophoretic mobility relative to the marker.

When pyruvate dehydrogenase phosphate complexes prepared by method B from hearts of starved or diabetic rats (but not fed normal rats) were incubated with [γ-^{32}P]ATP, a plateau of incorporation of ^{32}P was not achieved within 30 min. Instead, an initial rapid incorporation over 1–2 min was followed by a much slower and apparently linear incorporation (Fig. 2). This pattern of incorporation was not seen in pyruvate dehydrogenase complex prepared by dephosphorylation of the same pyruvate dehydrogenase phosphate complexes, nor was it seen with pyruvate dehydrogenase phosphate complexes prepared from hearts of fed normal rats by method B: these complexes gave the pattern of incorporation shown in Fig. 1.

From the recovery (>90%) of ^{32}P in tryptic phosphopeptides, there was no reason to suppose that the slow incorporation of ^{32}P was due to incorporation into anything other than phosphorylation sites of pyruvate dehydrogenase phosphate complex.

These results suggested that slow turnover of non-radioactive phosphate was occurring in pyruvate dehydrogenase phosphate complexes from starved or diabetic rats prepared by method B. This supposition was confirmed in a further experiment (results not shown) in which an 8-fold excess of non-radioactive ATP was added after 45 min of incubation with [γ-^{32}P]ATP (pyruvate dehydrogenase phosphate prepared from hearts of diabetic rats by method B). This resulted in a slow decline in protein-bound ^{32}P at a rate comparable with the slow increase in protein-bound ^{32}P shown in Fig. 2. From this conclusion of turnover, the true incorporation into unoccupied sites was computed by extrapolation of the linear portion of the curve to the ordinate by linear regression analysis (broken line in Fig. 2). The intercept value was taken as the incorporation into unoccupied sites. Values obtained (nmol of P/unit of inactive complex; mean ± S.E.M.) were diabetic 0.259 ± 0.023 (30 observations, two preparations) and starved 0.213 ± 0.015 (20 observations, one preparation) (values for individual preparations are shown in Table 2). These results do not differ significantly from those obtained with complexes prepared by method A, in which turnover was not apparent. The reason for slow turnover of phosphate in pyruvate dehydrogenase phosphate complexes prepared by method B could not be ascertained with certainty. The preparations were

free of phosphatase activity, as assayed by no formation of active complex on incubation for 10min at 30°C in the presence of 25mM-$MgCl_2$ and 100μM-$CaCl_2$. However this assay would not preclude the slow removal of phosphate from site 2 by a low concentration of phosphatase.

Combined values for all data (nmol of P incorporated/unit of inactive complex; mean ± S.E.M.) were: fed normal, 0.961 ± 0.025 (39 observations, three preparations); diabetic, 0.271 ± 0.012 (68 observations; four preparations); starved, 0.200 ± 0.013 (28 observations; two preparations). The occupancies *in vivo* obtained by difference from the incorporations into the corresponding pyruvate dehydrogenase complexes were (nmol of P/unit of inactive complex; mean ± S.E.M.): fed normal, 1.15 ± 0.04; diabetic, 1.72 ± 0.03; starved, 1.85 ± 0.03. As percentages of the incorporations into the corresponding pyruvate dehydrogenase complexes (i.e. the proportion of available sites phosphorylated *in vivo*), the values were (mean ± S.E.M.): fed normal, 54.8 ± 1.6; diabetic, 86.4 ± 1.4; starved, 90.2 ± 1.4. All differences between fed normal and diabetic or starved animals (i.e. as unoccupied sites, occupied sites or percentage occupancy) were significant ($P < 0.001$). It is concluded that the overall occupancy of phosphorylation sites in pyruvate dehydrogenase phosphate complexes *in vivo* is significantly greater in hearts of diabetic or starved rats than in hearts of fed normal rats.

Occupancies of individual phosphorylation sites of pyruvate dehydrogenase phosphate complexes in vivo

Fully phosphorylated rat heart complex would appear to contain three sites of phosphorylation in the α-chains of the decarboxylase component (Sale & Randle, 1980), strictly analogous to those established for bovine kidney and pig heart complexes (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). In fully phosphorylated complex the three phosphorylation sites are recovered in two tryptic phosphopeptides, A (site 1 and site 2) and B (site 3). Partially phosphorylated complex (rat, pig or ox) titrated to inactivation by repetitive small additions of [γ-^{32}P]ATP yields predominantly phosphopeptide A′ (A with only site 1 phosphorylated) together with small amounts of A and B. The three phosphopeptides from rat heart complex may be separated by high-voltage paper electrophoresis at pH 1.9 (Davis *et al.*, 1977) and identified by their mobilities relative to N^6-dinitrophenyl-lysine markers, which are 1.40 (A′), 1.07 (A) and 0.76 (B) (Sale & Randle, 1980). This method has been used to measure ^{32}P incorporation into individual sites in the present study.

For pyruvate dehydrogenase complexes, incorporations of ^{32}P have been computed as: site 1 (incorporation into A′ + 0.5 × incorporation into A); site 2 (0.5 × incorporation into A); and site 3 (incorporation into B). (A′ was absent except for small amounts in preparations 1 and 3 in Table 2.) The expected ratio (A/B) for complete phosphorylation is 2:1; the observed ratio (mean of all results in Table 2) was 1.96:1.

Computation of ^{32}P incorporation into sites 1 and 2 in pyruvate dehydrogenase phosphate complexes is complicated by the presence of non-radioactive phosphate. In purified rat heart and pig heart complexes and in intact rat heart mitochondria, inactivation of the complex is linearly correlated with incorporation of ^{32}P into site 1 (Kerbey *et al.*, 1979; Sale & Randle, 1980). Likewise, reactivation of fully phosphorylated pig heart or rat heart complex with phosphatase is linearly correlated with dephosphorylation of site 1 (A. L. Kerbey, P. J. Randle & G. J. Sale, unpublished work). We have assumed therefore that inactive pyruvate dehydrogenase phosphate complex as prepared contains site 1 fully occupied with non-radioactive phosphate. Thus, for pyruvate dehydrogenase phosphate preparations, incorporations of ^{32}P have been computed as: site 1, no incorporation; site 2 (incorporation into A); site 3 (incorporation into B) (^{32}P-labelled A′ was not found). Representative data for ^{32}P incorporations into tryptic phosphopeptides obtained by electrophoresis are shown in Fig. 3. Full details of the calculation of ^{32}P incorporations into each site of pyruvate dehydrogenase phosphate (including corrections for active pyruvate dehydrogenase complex present) and of site occupancy *in vivo* (i.e. non-radioactive phosphate present in each site) are given in the Experimental section.

The data in Table 2 show that in all preparations of pyruvate dehydrogenase phosphate complex from the hearts of diabetic or starved rats the occupancies of sites 2 and 3 were always significantly higher than in the complex from the hearts of normal fed animals ($P < 0.001$, all differences) (occupancies of site 1 were, by definition, 100% in all cases). Combined mean ± S.E.M. values for different preparations from the same type of animal for the occupancy of individual sites of pyruvate dehydrogenase phosphate complex in terms of nmol of P/unit of inactive complex were for sites 1, 2 and 3, respectively, 0.715 ± 0.021, 0.195 ± 0.015, 0.236 ± 0.017 (normal fed, 39 observations on three preparations) 0.665 ± 0.009, 0.421 ± 0.015, 0.641 ± 0.011 (diabetic; 68 observations on four preparations) and 0.673 ± 0.012, 0.514 ± 0.016, 0.659 ± 0.011 (starved, 28 observations on two preparations). Corresponding percentage occupancies were 100 ± 2.7, 27.8 ± 1.6, 33.9 ± 2.0 (normal), 100 ± 1.2, 64.0 ± 1.7, 94.6 ± 1.4 (diabetic) and

100 ± 1.4, 76.2 ± 2.0, 92.4 ± 1.5 (starved). Occupancies of sites 2 and 3 in the diabetic or starved pyruvate dehydrogenase phosphate complexes are therefore 2–3-fold higher than those found in the normal. The occupancy of site 2 in the starved animal appears to be slightly higher than in the diabetic animal.

General discussion

In the rat, diabetes and starvation increase the proportion of complex in the inactive form in heart from about 70% to 99% (Wieland *et al.*, 1971; Kerbey *et al.*, 1976). The results of the present study show that this is accompanied by a 2–3-fold increase in the occupancies of phosphorylation sites 2 and 3 (i.e. the phosphorylation sites additional to the inactivating site). The values obtained very likely represent those found *in vivo*, as strict precautions were taken to prevent any change in phosphorylation state of the pyruvate dehydrogenase phosphate complexes during their isolation from the hearts.

The results of a recent study of occupancy of phosphorylation sites in the pyruvate dehydrogenase phosphate complex in rat heart mitochondria led us to make predictions about the occupancy of phosphorylation sites *in vivo* in fed normal and diabetic or starved rats (Sale & Randle, 1980). The results obtained in the present study differ mainly in the occupancy of site 2, which was lower than we had predicted in all three categories of heart. As mentioned previously, site 2 is especially sensitive to dephosphorylation by phosphatase in bovine kidney, pig heart and rat heart complexes (Teague *et al.*, 1979; A. L. Kerbey, P. J. Randle & G. J. Sale, unpublished work). The lower occupancy of site 2 found *in vivo* in the present study may reflect higher phosphatase activity *in vivo* because of physiological differences between mitochondria in *in vivo* and *in vitro*. Alternatively it could be due to a short period of hypoxia during the 10–15 s which it took to open the chest and freeze the heart. We have been unable to devise a practicable method of freeze–clamping the heart *in situ* because of anatomical constraints.

Reactivation of complexes by phosphatase is inhibited in mitochondria and in purified pyruvate dehydrogenase phosphate complexes from hearts of starved or alloxan-diabetic rats (Hutson *et al.*, 1978). Inhibition was due to a stable change in the complexes and not to inhibition of the phosphatase *per se*. Because multisite phosphorylation inhibits reactivation of pig heart and bovine kidney complexes by phosphatase (Sugden *et al.*, 1978; Kerbey & Randle, 1979; Sugden & Simister, 1980), it was suggested that multisite phosphorylation in diabetes and starvation may inhibit reactivation by phosphatase. The results of the present study have shown that the occupancies of sites 2 and 3 in hearts of starved or diabetic rats are 2–3-fold higher than in fed normal rats. These results therefore provide direct evidence that multisite phosphorylation may be implicated in the inhibitory effects of diabetes and starvation on reactivation by phosphatase.

Enhanced activity of pyruvate dehydrogenase kinase (Hutson & Randle, 1978) may be a major factor responsible for the increased proportion of inactive complex, for the increased occupancy of phosphorylation sites 2 and 3 *in vivo* in hearts of starved or diabetic rats and, thereby, for mediating inhibition of reactivation of the complex by phosphatase.

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