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Regulation of Pig Heart Pyruvate Dehydrogenase by Phosphorylation

STUDIES ON THE SUBUNIT AND PHOSPHORYLATION STOICHEIOMETRIES

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1. The molecular weights of the subunits of purified pig heart pyruvate dehydrogenase complex were determined by sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis and were: pyruvate decarboxylase, α-subunit 40600, β-subunit 35100; dihydrolipoyl acetyltransferase 76100; dihydrolipoyl dehydrogenase 58200. 2. Inactivation of the pyruvate dehydrogenase complex by its integral kinase corresponded to the incorporation of 0.46nmol of P/unit of complex activity inactivated. 3. Further incorporation of phosphate into the complex occurred to a limit of 1.27nmol of P/unit of complex inactivated (approx. 3 times that required for inactivation). 4. Phosphate was incorporated only into the α-subunit of the decarboxylase. 5. The molar ratio of phosphate to α-subunits of the decarboxylase was estimated by radioamidination of amino groups of pyruvate dehydrogenase [^{32}P]phosphate complex by using methyl [1-^{14}C]acetimidate, followed by separation of α-subunits by sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis. Inactivation of the complex (0.46nmol of P/unit of complex inactivated) corresponded to a molar ratio of one phosphate group per two α-chains (i.e. one phosphate group/$\alpha_2\beta_2$ tetramer). Complete phosphorylation corresponded to three phosphate groups per $\alpha_2\beta_2$ tetramer. 6. Subunit molar ratios in the complex were also estimated by the radioamidination technique. Results corresponded most closely to molar ratios of 4 α-subunits:4 β-subunits:2 dihydrolipoyl acetyltransferase subunits:1 dihydrolipoyl dehydrogenase subunit.

Mammalian pyruvate dehydrogenase complexes catalyse the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2. Three individual enzymes in the complex (pyruvate decarboxylase, EC 1.2.4.1, dihydrolipoyl acetyltransferase, EC 2.3.1.12 and dihydrolipoyl dehydrogenase, EC 1.6.4.3) catalyse the overall reaction of the complex through mobile lipoyl residues attached to the acetyltransferase (for review, see Reed & Cox, 1970). (The recommended trivial name for EC 1.2.4.1 is pyruvate dehydrogenase, but to avoid confusion with pyruvate dehydrogenase complex we have used the name pyruvate decarboxylase for EC 1.2.4.1. Because of this confusion, P.J.R. has mistakenly referred to the complex as EC 1.2.4.1 in a number of earlier papers.) The complex contains an intrinsic kinase (no EC number), which, with $MgATP^{2-}$, catalyses phosphorylation and inactivation of the complex; dephosphorylation and re-activation is catalysed by a more loosely associated phosphatase (no EC number) (Linn *et al.*, 1969*a*,*b*, 1972). The isolated pyruvate decarboxylase component of the bovine kidney and heart and pig heart complexes is a tetramer of two α- and two β-subunits (Barrera *et al.*, 1972; Hamada *et al.*, 1976). It is the α-subunit that is phosphorylated in bovine kidney and heart and pigeon breast muscle (Barrera *et al.*, 1972; Severin & Feigina, 1977). The stoicheiometry of phosphorylation in the complex is uncertain, having been variously reported as 2mol of phosphate per mol of decarboxylase tetramer ($\alpha_2\beta_2$) in the bovine kindey complex (Linn *et al.*, 1969*a*) and inactivation by 1mol of phosphate per mol of decarboxylase tetramer (Reed *et al.*, 1974). More recent work showed the presence of three sites of phosphorylation in α-chains of the decarboxylase of bovine kidney complex (Davis *et al.*, 1977).

The subunit composition of the bovine heart and kidney and pig heart complexes has hitherto been determined by methods based on molecular-weight determinations of the whole complex and of its subunits (Hayakawa *et al.*, 1969; Barrera *et al.*, 1972; Hamada *et al.*, 1975, 1976). Because the complex is large (mol.wt. approx. 10^7) and contains many subunits (approx. 75–150), the calculated composition is sensitive to errors in molecular-weight determination. In the present paper, the subunit stoicheiometry and the stoicheiometry of phosphorylations in the pig heart complex have been investigated by radio-

Abbreviations used: SDS, sodium dodecyl sulphate; QAE-Sephadex, quaternary aminoethyl-Sephadex.

amidination of free amino groups in the complex with methyl [1-^{14}C]acetimidate (Bates *et al.*, 1975*a*).

Experimental

Materials

Pyruvate dehydrogenase phosphate phosphatase was prepared from pig heart as described by Severson *et al.* (1974). Pyruvate dehydrogenase complex was purified from pig heart as described by Cooper *et al.* (1974) with the following modifications. Mercaptoethanol (5mM) was replaced by dithiothreitol (2mM). On the second day and immediately before precipitation of pyruvate dehydrogenase complex at pH5.4 [in 1.5% poly(ethylene glycol)], additional contaminating material was removed by centrifugation at 18°C for 20min at 32000*g* at pH6.1 (the pH being lowered by 1M-$MgCl_2$). The pyruvate dehydrogenase preparations were free of pyruvate dehydrogenase phosphate with the phosphate in the inactivating site (shown by no increase in activity after incubation for 20min at 30°C in 50mM-Tris/HCl/20mM-$MgCl_2$/9.95mM-$CaCl_2$/10mM-EGTA, pH7.0, with phosphatase). Preparations of pyruvate dehydrogenase [^{32}P]phosphate (see below for preparation) were free of detectable phosphatase activity (shown by measurement of release of trichloroacetic acid-soluble ^{32}P after incubation for 30min at 30°C in 50mM-Tris / HCl / 20mM-$MgCl_2$ / 9.95mM-$CaCl_2$ / 10mM-EGTA, pH7.0, as described by Denton *et al.*, 1972). Dithiothreitol, 2,4,6-trinitrobenzenesulphonic acid, trypsin (diphenylcarbamoyl chloride-treated, type XI), human transferrin, bovine serum albumin (recrystallized), ovalbumin and bovine pancreatic ribonuclease were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Pig heart lactate dehydrogenase was from Boehringer Corp. (London), London W5 2TZ, U.K. Materials for polyacrylamide-disc-gel electrophoresis were from Bio-Rad Laboratories, Bromley, Kent BR2 9LY, U.K. QAE-Sephadex A-25 was from Pharmacia, London W5 5SS, U.K. Sources of other chemicals, radiochemicals and biochemicals were as given by Kerbey *et al.* (1976) or Cooper *et al.* (1974). Methyl [1-^{14}C]acetimidate was prepared by the method of Bates *et al.* (1975*b*), specific radioactivity 85.1 d.p.m./nmol.

Methods

Pyruvate dehydrogenase activity. This was measured by the direct spectrophotometric assay as given by Cooper *et al.* (1974). One unit of enzyme activity produces 1μmol of NADH/min at 30°C.

SDS/polyacrylamide-disc-gel electrophoresis. Disc gels (5% or 7.5%, 0.5cm × 9cm) were run in 0.1M-sodium phosphate/0.1% (w/v) SDS in a Pharmacia GE-4 gel electrophoresis system with a Pharmacia EPS 500/400 power supply essentially as described previously (Sugden *et al.*, 1976; Weber & Osborn, 1969) at 4mA/tube. Protein loading was approx. 50–200μg/gel. Gels were standardized with 10–20μg of the following proteins: human transferrin (mol.wt. 88000), bovine serum albumin and its dimer (mol.wts. 68000 and 136000 respectively), ovalbumin (mol.wt. 43000), pig heart lactate dehydrogenase (mol.wt. 36000) and bovine pancreatic ribonuclease (mol.wt. 13700). Molecular weights were taken from Schulze *et al.* (1955) and Weber & Osborn (1969).

Proteins were detected by immersion of gels in 0.2% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:5, by vol.) for 16h and destaining in methanol/acetic acid/water (5:1:5, by vol.) by diffusion in a Pharmacia GD-4 destainer with a Pharmacia DPS power supply. Coomassie Blue-stained gels were scanned at 580nm in a Gilford 2410-S linear-transport attachment. In some cases, protein bands were detected by immersion of gels in 50% (w/v) trichloroacetic acid for 16h and then in 10% (w/v) trichloroacetic acid for 24h. Proteins were clearly visible as white bands when lit obliquely by a spotlight on a black background.

Protein-bound radioactivity in gels was measured either by slicing with a Bio-Rad model 190 gel slicer (2mm slices) or by excision of the discrete protein bands. Two methods of gel solubilization were used. In the first, protein bands were excised, macerated with forceps and dried under vacuum in plastic scintillation vials. The dried gels were dissolved by heating in 0.5ml of 100-vol. H_2O_2 (30%, w/v) at 50°C for 15h. After this time, 0.3ml of 5% (w/v) sodium dithionite was added (to destroy excess H_2O_2, which interfered with liquid-scintillation counting) and radioactivity measured by liquid-scintillation counting in 20ml of methoxyethanol/toluene scintillation fluid (Severson *et al.*, 1974) in a Searle Isocap 300 scintillation counter. ^{14}C radioactivity was corrected for quenching by the external-standard method. Counting efficiency was about 80%. Recoveries of ^{14}C from [^{14}C]protein (denatured, The Radiochemical Centre) and of ^{32}P from pyruvate dehydrogenase [^{32}P]phosphate which had been previously set in small gels were 93.2±2.0% and 99.7±1.6% respectively (mean±S.E.M., three observations). In the second method, macerated gel slices were incubated with 5% (v/v) Protosol in Econofluor (New England Nuclear, Winchester, Hants, U.K.) at 40°C for 24h. Gels in which protein bands were detected with trichloroacetic acid were sometimes difficult to dissolve, in which case Protosol concentration was increased to 10% (v/v). Recoveries of ^{14}C from denatured [^{14}C]protein and ^{32}P from pyruvate dehydrogenase [^{32}P]phosphate set in small gels were 97.3±1.2% and 100±1.0% respectively (mean ±S.E.M., three observations). Efficiency of counting

of ^{14}C was estimated by the external-standard method and was about 85%.

Phosphorylation of pyruvate dehydrogenase. Phosphorylation was carried out as described previously (Sugden *et al.*, 1978) either by repeated addition of limiting amounts of [γ-^{32}P]ATP (specific radioactivity 25–100 c.p.m./pmol) or by allowing phosphorylation to go to completion in the presence of excess [γ-^{32}P]ATP. The specific radioactivity of [γ-^{32}P]ATP was estimated from its A_{257} at pH2 and liquid-scintillation counting. Removal of non-protein-bound ^{32}P by dialysis was as described by Sugden *et al.* (1978). Incorporation of ^{32}P into protein-bound radioactivity was measured by the method of Corbin & Reimann (1974). It was shown that there was complete recovery of protein-bound ^{32}P by this method by the fact that direct counting of radioactivity of pyruvate dehydrogenase [^{32}P]phosphate gave identical results.

Radioamidination of pyruvate dehydrogenase and pyruvate dehydrogenase [^{32}P]phosphate. Radioamidination of pyruvate dehydrogenase complex was carried out essentially as described by Bates *et al.* (1975*a*), by using the amino-group-specific reagent methyl [1-^{14}C]acetimidate. In some cases proteins were reduced and *S*-carboxymethylated as described by Riley & Perham (1970), but this operation did not affect the results. Suitable controls were incubated in the absence of methyl [1-^{14}C]acetimidate to determine unmodified amino groups (see below). After incubation with methyl [1-^{14}C]acetimidate for 6h at room temperature (19°C), the samples were placed in dialysis tubing and incubation tubes were rinsed out with 0.3vol. of dialysis buffer. Samples were dialysed for 48h at room temperature against 10mM-sodium phosphate/0.1% (w/v) SDS, pH7.2, with three changes of dialysis buffer. Appearance of label in the dialysis buffer was monitored and had fallen to undetectable values at the end of dialysis. Samples were removed from the dialysis tubing and warmed for 10min at 65–70°C in the presence of 10% (v/v) glycerol, 0.28M-2-mercaptoethanol and 0.002% (w/v) Bromophenol Blue. The pyruvate dehydrogenase subunits were separated by SDS/polyacrylamide-disc-gel electrophoresis, stained, destained (see above) and the protein bands excised. Gels were solubilized and radioactivity in the protein bands was measured as described above. The molar ratios of the subunits were calculated from the measured ^{14}C radioactivity in the protein bands and the lysine contents of the subunits [from data of Massey *et al.* (1962) and Hamada *et al.* (1975, 1976)].

To measure the molar ratio of phosphate incorporation to α-subunit (see the Results and Discussion section), pyruvate dehydrogenase (200 units, 10 units/ml) was titrated to inactivation by addition of limiting quantities (approx. 10 serial additions of 0.1μmol) of [γ-^{32}P]ATP (10mM, 25c.p.m./pmol). When about 97% inactivated, trichloroacetic acid (100%, w/v) was added to a concentration of 5% (w/v) and the preparation was dialysed against 2 litres of 2mM-EDTA (adjusted to pH7.0 with KOH) at 4°C for 60h with four dialysis-buffer changes. In some experiments, pyruvate dehydrogenase [^{32}P]-phosphate preparations were not denatured and were dialysed after the addition of 0.05vol. of 200mM-EDTA (pH7.0). There was no difference between results with these two methods, nor was there any detectable loss of protein-bound ^{32}P from denatured protein (the native preparation may lose up to 4% of the ^{32}P during dialysis).

Pyruvate dehydrogenase [^{32}P]phosphate preparations were freeze-dried and radioamidinated (Bates *et al.*, 1975*a*). Subunits were separated by SDS/polyacrylamide-disc-gel electrophoresis as described above. The α-subunit band (see the Results and Discussion section) was excised and solubilized as described above. The molar ratio of ^{32}P to α-subunit was calculated from the ratio of $^{32}P/^{14}C$ radioactivity and from the specific radioactivity of [γ-^{32}P]ATP used and the lysine content of the pig heart α-subunit (Hamada *et al.*, 1976). The ratio of $^{32}P/^{14}C$ radioactivity was determined on channel 6 of a Searle Isocap 300 liquid-scintillation spectrometer. A quench curve was constructed for ^{14}C in channel 6A and for ^{32}P in channel 6B. Over the observed range of external-standard channel ratios, the efficiency of ^{14}C counting in channel 6A was about 75–85% and for ^{32}P in channel 6B was 100%. Samples containing the solubilized α-subunit protein band were counted on channel 6. Channel overlap was corrected by storing the samples at 4°C in the dark for about 2 weeks (to allow ^{32}P to decay) and then re-counting. There was no change in counting efficiency on storage. From the decay constant for ^{32}P (0.04846 day^{-1}), ^{14}C radioactivity in channel 6A could be corrected for ^{32}P overlap and then corrected for quenching. Similarly, ^{32}P radioactivity in channel 6B could be calculated from the decrease in radioactivity and thus corrected for ^{14}C overlap. (Efficiency of counting of ^{32}P in channel 6A was about 10% and ^{14}C in channel 6B was about 50%.) The practicability of this method was tested by using a sample containing known amounts of ^{14}C and ^{32}P radioactivity. Suitable controls of gels run in the absence of added protein were subtracted as appropriate.

The extent of amino-group modification, after radioamidination and dialysis, was estimated by the trinitrobenzenesulphonic acid method of Habeeb (1966) as described by Ludwig & Hunter (1972). Suitable unmodified control preparations of pyruvate dehydrogenase or pyruvate dehydrogenase phosphate were included and all measurements included a protein blank and a reagent blank. Radioamidination of amino groups was more than 97% complete in all experiments.

Results and Discussion

Characteristics of the pyruvate dehydrogenase complex preparations

When stored at −15°C in 20mM-potassium phosphate/2mM-dithiothreitol, pH7.0, pyruvate dehydrogenase activity was stable for at least 2 months. This was in contrast with the more rapid loss of activity when 5mM-2-mercaptoethanol replaced dithiothreitol (Cooper *et al.*, 1974). On SDS/polyacrylamide-disc-gel electrophoresis, four major protein bands were observed (Fig. 1). These were identified by means of their predominance and relative migration. The molecular weights (given as means±S.E.M. with numbers of observations in parentheses) were dihydrolipoyl acetyltransferase, 76100±600 (36); dihydrolipoyl dehydrogenase, 58200±600 (37); pyruvate decarboxylase α-subunit, 40600±400 (36); pyruvate decarboxylase β-subunit, 35100±400 (36). These values are in close agreement with the values previously given for the α- and β-subunits and the acetyltransferase subunit of the pig heart enzyme (Hamada *et al.*, 1975, 1976) and with the values given for the bovine kidney or heart and pigeon breast-muscle pyruvate dehydrogenase complex subunits when examined by SDS/polyacrylamide-disc-gel electrophoresis (Barrera *et al.*, 1972; Severin & Feigina, 1977). Two additional minor protein bands were often seen [band A, mol.wt. 104100±1100 (21); band B, mol.wt. 26200±400 (9); see Fig. 1]. Whether these proteins are components of the complex or whether they are contaminants is not known. In this regard, it has been reported that the molecular weights of the phosphatase and the kinase for bovine kidney pyruvate dehydrogenase complex are about 100000 and 50000 respectively (Reed *et al.*, 1974). It is possible that band A is the phosphatase and band B is the monomer of a native kinase dimer. From Fig. 1, however, it is clear that the four major subunits represent more than 95% of protein present.

Phosphorylation of the pyruvate dehydrogenase complex

When incubated with 0.3–0.5mM-$MgATP^{2-}$, pyruvate dehydrogenase was rapidly inactivated and rapidly incorporated the γ-phosphate group of ATP into protein-bound phosphate (Fig. 2). Phosphorylation was approx. 10 times more rapid with 2mM-dithiothreitol as thiol reagent than with 5mM-2-

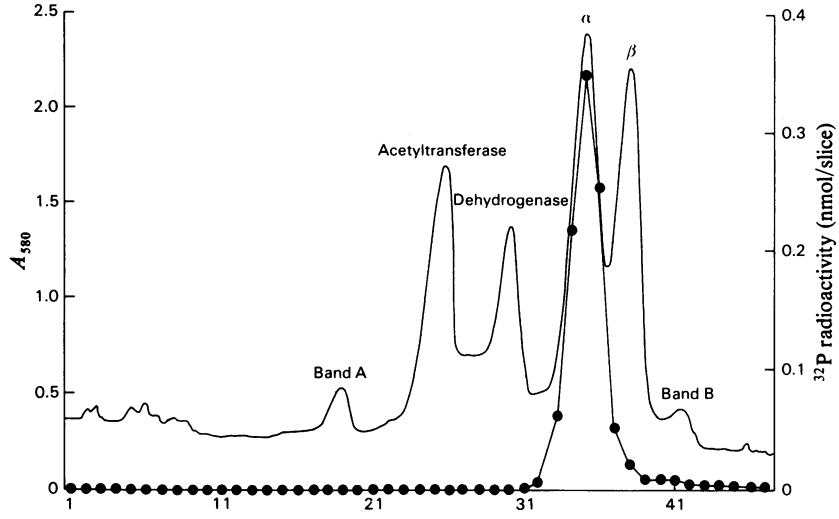

Fig. 1. *SDS/polyacrylamide-disc-gel electrophoresis of purified pig heart pyruvate dehydrogenase [^{32}P]phosphate*
Pyruvate dehydrogenase (1ml, 5 units/ml) was phosphorylated in the presence of 0.5mM-[γ-^{32}P]ATP as described in the Experimental section. After 30min, 0.1ml of 100% (w/v) trichloroacetic acid was added and the mixture was centrifuged at 3000 rev./min for 5min in a bench centrifuge. Incorporation of protein-bound ^{32}P was 1.14nmol of ^{32}P/unit of enzyme activity. The precipitated protein was washed with 3×5ml of 10% (w/v) trichloroacetic acid and resuspended in 10mM-sodium phosphate/0.28M-2-mercaptoethanol/2% (w/v) SDS. The mixture was adjusted to pH7 with 8M-NaOH (by using BDH Universal indicator) and was then warmed at 65–70°C for 10min. Glycerol (0.1vol.) and Bromophenol Blue (final concn. 0.005%, w/v) were added. SDS/polyacrylamide-disc-gel electrophoresis, staining, destaining and scanning of gels was performed as described in the Experimental section. Gels were sliced (2mm slices) and ^{32}P in each slice was measured (●). Slice no. 1 is the top of the gel (i.e. that in the cathode chamber) and slice no. 47 is the bottom of the gel (i.e. that in the anode chamber).

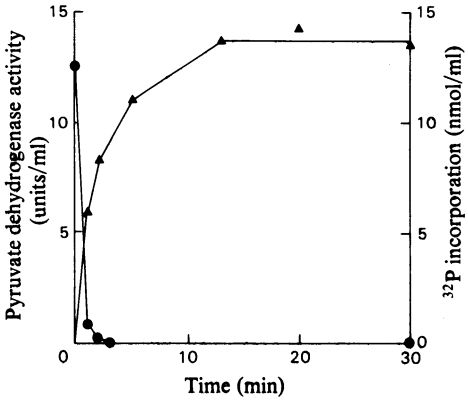

Fig. 2. *Time course of phosphorylation of pyruvate dehydrogenase by [γ-^{32}P]ATP*
Pyruvate dehydrogenase (1 ml, 12.5 units/ml) was incubated with 0.5 mM-[γ-^{32}P]ATP as described in the Experimental section. At the times indicated, samples (5 μl or 20 μl respectively) were withdrawn and assayed for enzyme activity (●) and ^{32}P incorporation into protein (▲) as described in the text.

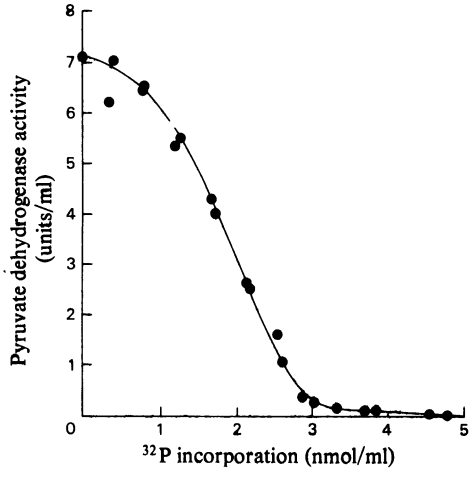

Fig. 3. *Incorporation of ^{32}P into pyruvate dehydrogenase at limiting concentrations of [γ-^{32}P]ATP*
Pyruvate dehydrogenase (3 ml, 7 units/ml) was incubated with limiting amounts of [γ-^{32}P]ATP. At 9 min intervals [γ-^{32}P]ATP (5 μl, 7.5 nmol) was added. Pyruvate dehydrogenase activity and ^{32}P incorporation in samples (5 and 20 μl respectively) were measured at 5 and 8 min after each addition of [γ-^{32}P]ATP. For the final two ATP additions, the amounts of [γ-^{32}P]ATP added were increased to 15 nmol and 30 nmol.

mercaptoethanol. Although inactivation was more than 90% complete within 1 min, phosphate incorporation was only 80% complete in 5 min. Inactivation was complete before phosphate incorporation. It was shown that after 30 min incubation phosphate incorporation was at least 90% of maximal, and that the lack of further phosphate incorporation after 30 min incubation was not caused by depletion of ATP or decrease in the ATP/ADP concentration ratio with concomitant inhibition of pyruvate dehydrogenase kinase. This was demonstrated as follows. After 30 min incubation (Fig. 2), pyruvate dehydrogenase [^{32}P]phosphate was dialysed for 60 h against 2 litres of 10 mM-potassium phosphate/1 mM-EDTA/0.1 mM-dithiothreitol at pH 7.0 with three buffer changes. There was less than 4% loss of protein-bound phosphate and trichloroacetic acid-soluble ^{32}P was less than 2% of the total after dialysis. After this time, the dialysed enzyme solution was incubated for 30 min with 2 mM-$MgCl_2$, 10 mM-EGTA and 0.3 mM-[γ-^{32}P]ATP (these reagents were added in a total volume that was 10% of that of the enzyme so as to cause relatively small changes in the buffer concentration). Phosphate incorporation increased from 1.14 to 1.23 nmol/unit of enzyme inactivated (an increase of only about 8%). When pyruvate dehydrogenase complex was incubated with 0.3–0.5 mM-$MgATP^{2-}$ for 30 min (no dialysis), incorporation proceeded to the extent of 1.27±0.04 nmol of phosphate/unit of enzyme inactivated (mean±S.E.M., 13 observations on nine different pyruvate dehydrogenase preparations).

However, from the time course of inactivation and phosphorylation of pyruvate dehydrogenase, it is evident that phosphate incorporation continues after inactivation of the enzyme is complete. Since inactivation and phosphorylation of the enzyme is so rapid under these conditions, it is not possible to estimate the extent of phosphate incorporation required for 100% inactivation from a time course of phosphorylation such as that in Fig. 2. It is possible to obtain this by 'titrating' enzyme activity against the addition of limiting quantities of [γ-^{32}P]ATP and incubating for about 5 min. Phosphorylation (and thus inactivation) stops as the incubation mixture becomes depleted of [γ-^{32}P]ATP. At this stage, activity and phosphate incorporation are measured. Addition of further [γ-^{32}P]ATP then produces further inactivation and phosphorylation of the enzyme. A typical experiment is shown in Fig. 3. The plot of pyruvate dehydrogenase activity against ^{32}P incorporation was sigmoid. Initially phosphate incorporation proceeded with only a relatively small degree of inactivation. As phosphate incorporation increased, so the gradient of the line became steeper. As the enzyme neared complete inactivation, the gradient became less steep. Phosphate incorporation continued after inactivation of the enzyme was more than 97% complete, as implied in Figs. 2 and 3. The remaining enzyme activity (3% of total) also decreased. The ratio of the intercepts on the abscissa

obtained by extrapolation was about 1.7:1 (two experiments, range 1.6–1.8). This implies that twice as much phosphate may have to be incorporated into the enzyme to produce inhibition of 100% compared with that required for 97% inactivation. The pyruvate dehydrogenase may thus be similar to glycogen synthetase (EC 2.4.1.11) in this regard (Soderling, 1975). The separate additions of [γ-^{32}P]ATP described in Fig. 3 were greater than required to phosphorylate the pyruvate dehydrogenase complex. This is because pyruvate dehydrogenase kinase and ATPase activities, which are present in most pyruvate dehydrogenase preparations, cause depletion of [γ-^{32}P]ATP and concomitant production of ADP. These factors inhibit the pyruvate dehydrogenase kinase reaction.

The phosphate incorporation required for the initial inactivation was 0.46 ± 0.03 nmol of phosphate/unit of enzyme inactivated (mean $\pm$ S.E.M. for nine observations on seven different pyruvate dehydrogenase preparations). The complete phosphorylation of the enzyme involved incorporation of 1.27 nmol of phosphate/unit, or 2.8 times that required for 97% inactivation.

Phosphate incorporation occurred only into the α-subunit of pyruvate decarboxylase, even in a completely phosphorylated pyruvate dehydrogenase preparation containing 1.14 nmol of phosphate/unit of enzyme inactivated (Fig. 1). This is also the case with pyruvate dehydrogenase from bovine heart or kidney or pigeon breast muscle (Barrera *et al.*, 1972; Severin & Feigina, 1977). However, these workers did not carefully define the extent of incorporation of phosphate into the enzyme. There was no evidence that the phosphorylated α-subunit migrates differently from the non-phosphorylated α-subunit on SDS/polyacrylamide-disc-gel electrophoresis; both preparations gave identical values for molecular weight.

It is important to establish that phosphate is incorporated only into a single site during inactivation of the enzyme in experiments using limiting quantities of [γ-^{32}P]ATP. This was investigated by tryptic hydrolysis of preparations of pyruvate dehydrogenase containing 0.40 nmol of phosphate/unit of enzyme inactivated (87% enzyme inactivation) and containing 1.31 nmol of phosphate/unit of enzyme inactivated (100% enzyme inactivation) and subsequent QAE-Sephadex chromatography (see Fig. 4). Initial pyruvate dehydrogenase concentrations in both preparations were equal (7.5 units/ml). For the preparation containing 0.40 nmol of phosphate/unit, a single peak of [^{32}P]phosphopeptide was eluted at an ammonium formate concentration of 0.41 M. For the preparation containing 1.31 nmol of phosphate/unit, there were two major [^{32}P]phosphopeptide peaks eluted at ammonium formate concentrations of 0.22 and 0.49 M, and a minor peak at 0.27 M. The area of the peak eluted at 0.49 M-ammonium formate was approximately twice that of the peak eluted at 0.41 or 0.22 M. These findings suggest that phosphorylation of the α-subunit under conditions of limiting ATP concentrations occurs initially at a single site until it is completely phosphorylated. Enzyme activity at this stage is probably about 3% of original activity (see Fig. 3). The α-subunit is then phosphorylated on two other sites, one of which is on the same tryptic phosphopeptide as the initial inactivating phosphorylation and the other of which is on a different tryptic peptide. Such a scheme would correspond to that proposed for the bovine kidney complex α-subunit (Davis *et al.*, 1977), where two tryptic phosphopeptides were isolated. One of these contained the phosphoserine residue concerned with inactivation and a second phosphoserine residue, and the other contained a single phosphoserine residue. This scheme also explains why the tryptic digest of the pyruvate dehydrogenase phosphate containing 1.31 nmol of phosphate/unit did not show a phosphopeptide peak eluted at 0.41 M-ammonium formate. A second phosphorylation of this phosphopeptide in the α-subunit apparently altered its elution to 0.49 M-ammonium formate. The evidence from the tryptic phosphopeptide studies also suggests that the pyruvate dehydrogenase kinase acts sequentially, phosphorylating the inactivating site preferentially and then phosphorylating the remaining sites, at least under conditions of limiting ATP concentration. It should also be noted that all phosphate in completely phosphorylated pyruvate dehydrogenase phosphate can be removed by pyruvate dehydrogenase phosphate phosphatase with complete recovery of enzyme activity (Sugden *et al.*, 1978).

It is interesting to speculate over what range on the phosphorylation curve (Fig. 3) pyruvate dehydrogenase functions *in vivo*. It has been reported that the complex is about 30% active (expressed as a percentage of total activity) in the rat heart on perfusion with glucose+insulin, and this decreases to approx. 2% in the alloxan-diabetic or 7% on perfusion with glucose+insulin+acetate (Kerbey *et al.*, 1976). Somewhat higher percentages of active complex have been reported in rat heart by Wieland *et al.* (1971*a*,*b*). These experiments indicate that pyruvate dehydrogenase *in vivo* is regulated over the steepest portion of the activity/phosphorylation curve (Fig. 3). This would ensure that the activity change/unit of ATP hydrolysis is maximal.

Ratio of subunits in the pyruvate dehydrogenase complex

The subunit stoicheiometry of the pyruvate dehydrogenase complex may be investigated by scanning SDS/polyacrylamide disc gels of the complex and measuring the area under the peaks (Table 1). However, because of the known inaccuracies involved in this type of approach (Fishbein, 1972),

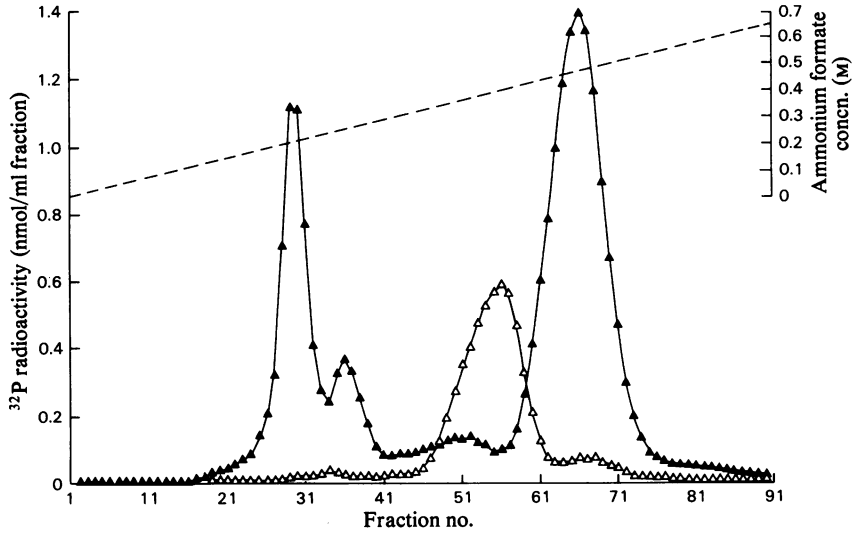

Fig. 4. *QAE-Sephadex chromatography of tryptic [^{32}P] phosphopeptides of pyruvate dehydrogenase [^{32}P] phosphate*
Pyruvate dehydrogenase [^{32}P]phosphate (initial enzyme activity 7.5 units/ml) containing 0.40nmol of ^{32}P/unit of enzyme activity (87% inactivated) or 1.31nmol of ^{32}P/unit of enzyme activity (100% inactivated) was prepared and dialysed extensively as described in the Experimental section. Each preparation (1.5ml) was incubated with diphenylcarbamoyl chloride-treated trypsin (10mg/ml) for 20h at 35°C. Hydrolysis was complete as shown by complete recovery of ^{32}P radioactivity in a trichloroacetic acid (10%, w/v)-soluble form. After 20h, 65μl of 70% $HClO_4$ (BDH) was added and the mixture incubated at 0°C for 15min. Precipitated protein was removed by centrifugation at 3000 rev./nim for 5min in a bench centrifuge and the supernatants were adjusted to pH7.5 with 5M-KOH (BDH Universal Indicator). After incubation at 0°C for 15min, $KClO_4$ was removed by centrifugation at 3000 rev./min for 5min in a bench centrifuge and 0.35ml of each supernatant was applied to a column (0.9cm×6cm) of QAE-Sephadex A-25, equilibrated with 0.02M-ammonium formate (pH8.5, adjusted with aq. 18M-NH^3). Tryptic [^{32}P]phosphopeptides derived from pyruvate dehydrogenase [^{32}P]phosphate containing 0.40 (△) or 1.31 (▲) nmol of ^{32}P/unit were eluted by a linear gradient of ammonium formate, pH8.5 (total volume 100ml, 0.02–0.7M; ----). Fractions (approx. 1ml) were collected and ^{32}P radioactivities therein were measured in methoxyethanol/toluene scintillation fluid (Severson *et al.*, 1974).

Table 1. *Subunit molar proportions of the pyruvate dehydrogenase complex*
SDS/polyacrylamide-disc-gel electrophoresis was performed as described in the Experimental section. Three different preparations of pyruvate dehydrogenase were used for both methods. Gels were scanned and the areas under the peaks were determined by weighing the traces. These values were divided by the appropriate subunit molecular weight (see the text) to calculate molar proportions. Results are means±S.E.M. for the numbers of determinations in parentheses.

	Gel-scanning method (mol/mol)	Radioamidination method (mol/mol)
α-Subunit/β-subunit	0.96±0.05 (8)	0.97±0.03 (18)
α-Subunit/dihydrolipoyl acetyltransferase	3.13±0.24 (8)	2.07±0.10 (18)
α-Subunit/dihydrolipoyl dehydrogenase	3.30±0.36 (8)	3.89±0.11 (13)

it was decided to investigate the subunit stoicheiometry by the radioamidination method of Bates *et al.* (1975*a*). The lysine contents of the component proteins of the pig heart complex are as follows: α-chain, 18.4mol of lysine/mol of α-chain (Hamada *et al.*, 1976); β-chain, 14.8mol of lysine/mol (Hamada *et al.*, 1976); dihydrolipoyl acetyltransferase, 38mol of lysine/mol (Hamada *et al.*, 1975). The value of lysine residues for the acetyltransferase is one less than that in Hamada *et al.* (1975), owing to the presence of one blocked amino group as lipoyl-lysine. The lysine content of dihydrolipoyl dehydrogenase was calculated as follows. From the data of Massey *et al.* (1962) as corrected by Sober (1970), the mean residue weight of the dehydrogenase was calculated. The mol.wt. (58200, see above) was divided by the mean residue weight to give the total amino acid residues/dehydrogenase chain and this value was multiplied by the lysine content in mol/100mol to give 45.6 lysine residues/chain. This value is somewhat higher than those previously calculated. Since

there is no evidence of blocked *N*-terminal amino groups for these proteins (Massey *et al.*, 1962; Hamada *et al.*, 1975, 1976), the total number of amino groups is equal to number of lysine residues+1.

Results (Table 1) show that the agreement between the radioamidination method and the gel-scanning method are reasonable, except for the α-subunit/acetyltransferase ratio. It is suggested that the dye-binding method underestimates the amount of acetyltransferase subunit.

The probable molar proportions of α-subunit/β-subunit/acetyltransferase/dehydrogenase from radioamidination experiments is 4:4:2:1. This means that there is one decarboxylase tetramer ($\alpha_2\beta_2$) per acetyltransferase monomer per 0.5 dihydrolipoyl dehydrogenase monomer. This stoicheiometry is difficult to reconcile with symmetry considerations. For strict symmetry, the molar proportions of decarboxylase ($\alpha_2\beta_2$)/acetyltransferase/dihydrolipoyl dehydrogenase must by 1:1:1. In the isolated pig heart complex, the ratio is 1:1:0.5. This may imply that 50% of the dihydrolipoyl dehydrogenase component is lost on each enzyme purification or that the complex is not strictly symmetrical. Furthermore, the pig heart dihydrolipoyl dehydrogenase is a dimer of mol.wt. 110000 as isolated (Hayakawa *et al.*, 1969). Further anomalies are apparent on consideration of the molecular weight of the complex (about 7.5×10^6 by sedimentation equilibrium; Hayakawa *et al.*, 1969). If the molecular weight of the binding unit, i.e. decarboxylase+acetyltransferase+dihydrolipoyl dehydrogenase, is 256000 (1:1:0.5 molar proportions, respectively) or 285700 (1:1:1 molar proportions, respectively), then from a consideration of molecular weights the complex would most closely fit an octahedrally symmetrical (432) model. Such a particle would have 24 binding units and hence a mol.wt. of 6.16×10^6–6.86×10^6. This is a slight underestimate, since this calculation does not include the associated pyruvate dehydrogenase kinase. A pentagonal dodecahedral complex (532 symmetry) requires 60 binding units and hence the complex mol.wt. would be 15.40×10^6–17.14×10^6. Similarly, a 24-subunit acetyltransferase 'core' (calculated mol.wt. 1.83×10^6) is in better agreement with the empirical mol.wt. of 1.8×10^6 (Hayakawa *et al.*, 1969; Hamada *et al.*, 1975) than a 60-subunit core. Thus octahedral symmetry of the acetyltransferase core is favoured on this basis. However, electron micrographs of the pig heart and bovine kidney complex and acetyltransferase core apparently show pentagonal dodecahedral symmetry (Ishikawa *et al.*, 1966; Reed & Oliver, 1968; Hayakawa *et al.*, 1969).

Stoicheiometry of phosphorylation of the pyruvate dehydrogenase complex

From a comparison with data given in Walsh *et al.* (1976), it is found that the amount of phosphate incorporated into the complex by titration to inactivation with limiting amounts of ATP (0.46±0.03 nmol of P/unit of enzyme inactivated, see above) is equal to acetylation of the complex by [3-^{14}C]pyruvate+thiamin pyrophosphate (0.57±0.03 nmol of acetyl group/unit of enzyme activity; mean±S.E.M. for ten observations) and also equal to thiamin pyrophosphate binding by the complex (0.49±0.07 nmol bound/unit of enzyme activity, mean±S.E.M.; Walsh *et al.*, 1976). There is one lipoyl residue/acetyltransferase chain (Hamada *et al.*, 1975; it is this that is acetylated by pyruvate+thiamin pyrophosphate in the absence of other substrates) and there is one decarboxylase tetramer ($\alpha_2\beta_2$) per acetyltransferase monomer (Table 1). Thus there must be one mol of phosphate incorporated/mol of $\alpha_2\beta_2$ tetramer at inactivation and one thiamin pyrophosphate-binding site/$\alpha_2\beta_2$ tetramer. It has, however, been reported by Butler *et al.* (1977) that there are two thiamin pyrophosphate-binding sites/$\alpha_2\beta_2$ tetramer in the bovine kidney decarboxylase.

The above conclusion about the phosphorylation stoicheiometry is supported by a simple calculation. The molar proportions of decarboxylase/acetyltransferase/dihydrolipoyl dehydrogenase in the isolated complex are 1:1:0.5 (Table 1). The molecular weight of such a binding unit is 256000 (from the values above and ignoring the kinase, which is a minor component of the complex). The specific activity of the complex is 7–9 units/mg (Walsh *et al.*, 1976). Thus ^{32}P incorporation for the formation of $\alpha_2\mathbf{P}\cdot\beta_2$ tetramer is 0.43–0.56 nmol/unit, a range within which the empirically determined phosphate incorporation (0.46 nmol/unit) lies. It should be noted that this calculation is independent of the molecular weight of the holocomplex.

Because such arguments provide only relatively indirect evidence, it was decided to determine the stoicheiometry of phosphorylation by a more direct method. Pyruvate dehydrogenase complex was 'titrated' to inactivation by using limiting amounts [γ-^{32}P]ATP (phosphate content=0.47 nmol/unit, 97% inactivated), then radioamidinated and the subunits were separated by SDS/polyacrylamide-disc-gel electrophoresis. From a knowledge of the lysine content of the α-subunit (Hamada *et al.*, 1976) and the decay constant of ^{32}P, the molar ratio of phosphate/α-chain was calculated. It was found that for 97% inactivation, 0.47±0.01 nmol of phosphate was incorporated/nmol of α-chain (mean±S.E.M. for 25 observations on one pyruvate dehydrogenase preparation). Two other preparations of pyruvate dehydrogenase gave similar results. There is no evidence that protein-bound ^{32}P is hydrolysed on electrophoresis, since recovery of ^{32}P from the α-subunit band was often in excess of 90%.

These findings indicate that inactivation of pyruvate dehydrogenase involves the incorpora-

tion of 1 mol of phosphate/mol of $\alpha_2\beta_2$ tetramer:

$$(a_2\beta_2)_n + n\ \text{ATP} \rightarrow (a\text{P}\cdot\alpha\beta_2)_n + n\ \text{ADP}.$$

Two further phosphorylations on the $\alpha_2\beta_2$ tetramer are then possible (since fully phosphorylated complex contains three times as much ^{32}P as that 'titrated' to inactivation; see above):

$$(\alpha\text{P}\cdot\alpha\beta_2)_n + 2n\ \text{ATP} \rightarrow (\alpha_2\text{P}_3\cdot\beta_2)_n + 2n\ \text{ADP}.$$

It is not clear whether the second and third phosphorylation sites are on the same α-chain as the first phosphorylation site, but experiments involving tryptic hydrolysis (Fig. 4) and also evidence from the bovine kidney enzyme (Davis *et al.*, 1977) suggest that at least one of the additional sites of phosphorylation is on the same α-chain as the inactivating phosphorylation. From these data, it is possible to propose a model consistent with the above facts (Fig. 5). In this model, all three phosphorylations occur on the same α-subunit. Other models are possible, but will not be considered here, e.g. two phosphorylations on one α-chain and the third on the other. In any event, the pyruvate dehydrogenase kinase displays a degree of specificity in determining the sites of phosphorylation. This may not be determined by the kinase itself, but could also possibly be influenced by the relative orientations of the decarboxylase and kinase in the assembled multienzyme complex.

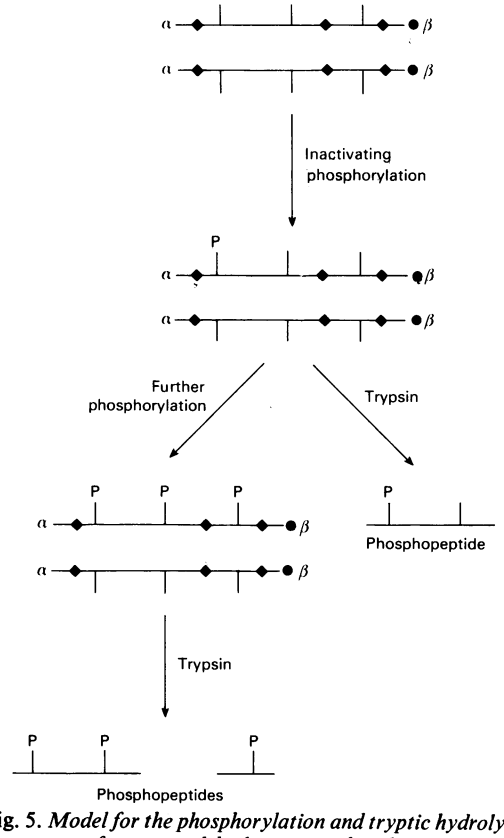

Fig. 5. *Model for the phosphorylation and tryptic hydrolysis of pyruvate dehydrogenase phosphate*

The sites sensitive to tryptic hydrolysis on the α-chain of the pyruvate decarboxylase $\alpha_2\beta_2$ tetramer are denoted by diamonds (♦). Only the decarboxylase is shown, but this is meant to represent the complex.

It has been proposed that the decarboxylase binding unit is not the $\alpha_2\beta_2$ tetramer but the $\alpha\beta$ dimer in the bovine heart or kidney complex (Barrera *et al.*, 1972). It is difficult to reconcile this proposal in the pig heart complex with the findings that only 0.5 mol of P/mol of $\alpha\beta$ dimer is incorporated at inactivation and that such incorporation inhibits acetyl-CoA formation from pyruvate and pyruvate decarboxylation by at least 95% (Walsh *et al.*, 1976).

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