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Studies on the incorporation of [^{32}P]phosphate into pyruvate dehydrogenase in intact rat fat-cells

Effects of insulin

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1. Intact rat epididymal fat-cells were incubated with $^{32}P_i$, and the intracellular proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. One of the separated bands of phosphorylated proteins had an apparent subunit mol.wt. of 42000, which is the same as that of the α-subunit of the pyruvate dehydrogenase complex. By using a combination of subcellular fractionation, immunoprecipitation with antiserum raised against pyruvate dehydrogenase complex and two-dimensional electrophoresis it was apparent that the incorporation into α-subunits accounted for 35–45% of the total incorporation into this band of phosphoproteins. 2. The increase in the initial activity of pyruvate dehydrogenase that follows brief exposure of fat-cells to insulin was shown to be associated with a decrease in the steady-state incorporation of ^{32}P into the α-subunits of pyruvate dehydrogenase. 3. Tryptic peptide analysis of pyruvate dehydrogenase [^{32}P]phosphate, labelled in intact fat-cells, indicated that three serine residues on the α-subunit were phosphorylated, corresponding to the three sites phosphorylated when purified pig heart pyruvate dehydrogenase was incubated with [γ-^{32}P]ATP. The relative phosphorylation of all three serine residues appeared to be similar in ^{32}P-labelled α-subunits in both control and insulin-treated fat-cells.

Pyruvate dehydrogenase complex from mammalian tissues has been shown to be inactivated by phosphorylation of the α-subunit of the decarboxylase component (EC 1.2.4.1; subunit composition $\alpha_2\beta_2$) catalysed by a tightly bound ATP-linked kinase activity (Linn *et al.*, 1969*a*,*b*; Reed, 1974; Denton *et al.*, 1975). Studies with components of the purified bovine kidney complex have shown that phosphorylation may occur on three different serine residues contained in two phosphopeptides derived by cleavage of the α-subunit with trypsin (Davis *et al.*, 1977; Yeaman *et al.*, 1978). The amino acid sequences of the phosphopeptides were found to be as follows: peptide A, Tyr-His-Gly-His-Ser(P)[site 1]-Met-Ser-Asn-Pro-Gly-Val-Ser(P)[site 2]-Tyr-Arg (referred to as T-1 and T-2 for the monophosphorylated and diphosphorylated forms respectively); peptide B, Tyr-Gly-Met-Gly-Thr-Ser(P)[site 3]-Val-Glu-Arg (referred to as T-3 for the monophosphorylated form). Phosphorylation at site 1 was found to proceed rather faster than at sites 2 and 3 and to correlate closely with the loss of overall catalytic activity. Very similar findings have also been reported for the purified pig heart enzyme (Sugden & Randle, 1978; Sugden *et al.*, 1979). In particular, the amino acid sequences of the tryptic phosphopeptides were shown to be nearly identical with those of the bovine kidney enzyme, the difference being that the asparagine residue in peptides T-1 and T-2 appeared to be replaced by an aspartic acid residue (Sugden *et al.*, 1979). The physiological roles of phosphorylation at sites 2 and 3 are not fully established, although it has been suggested that phosphorylation at these sites may lead to some loss of catalytic activity (Teague *et al.*, 1979). Evidence has also been presented with the pig heart complex that indicated that phosphorylation of sites 2 and 3 may lead to a decrease in the rate of re-activation of the complex catalysed by the phosphatase (Sugden *et al.*, 1978; Kerbey & Randle, 1979), although this was apparently not found with the bovine kidney complex (Teague *et al.*, 1979).

To date, estimates of the proportion of pyruvate dehydrogenase complex in its inactive, phosphorylated, form in the various intact cell preparations have been made solely on the basis of measurements of enzyme activity in cell extracts. The 'initial'

Abbreviation used: SDS, sodium dodecyl sulphate.

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activity has been taken as a measure of the concentration of the non-phosphorylated enzyme in the preparation. The extract has then been incubated for 10–30 min with exogenous pyruvate dehydrogenase phosphate phosphatase in the presence of Mg^{2+} and Ca^{2+} and the activity of pyruvate dehydrogenase reassayed to obtain a value for the 'total' activity of the enzyme. The difference in initial and total values has been assumed to be a measure of the extent of the phosphorylation of the complex in the cell (see Denton *et al.*, 1975). By using such techniques, it has been found that, within a few minutes of exposure of rat epididymal adipose tissue or isolated fat-cells to physiological concentrations of insulin, the initial activity of pyruvate dehydrogenase is increased 2–3-fold but the total activity remains unaltered (Jungas, 1971; Coore *et al.*, 1971; Weiss *et al.*, 1971; Denton *et al.*, 1975, 1978*a*; Denton & Hughes, 1978). The assumption has been made that this increase in initial activity is associated with a decrease in the phosphorylation of the complex.

One objective of the present studies was to demonstrate directly the phosphorylation of the α-subunits of pyruvate dehydrogenase in intact fat-cells and to investigate the effects of insulin on the extent of phosphorylation. This we have accomplished by incubating fat-cells in the presence of $^{32}P_i$ until steady-state labelling of the intracellular phosphoproteins was achieved. The α-subunits have then been separated by a combination of subcellular fractionation, immunoprecipitation and SDS/polyacrylamide-gel electrophoresis. We have used similar techniques before to investigate the phosphorylation of acetyl-CoA carboxylase in fat-cells (Brownsey *et al.*, 1977, 1979). The other objective was to explore the extent to which multi-site phosphorylation of the α-subunits occurred in whole fat-cells. Previously we have reported evidence that multiple phosphorylation of the α-subunits occurs when mitochondria isolated from rat adipose tissue and heart muscle are incubated in medium containing $^{32}P_i$ (Hughes & Denton, 1978).

Experimental

Materials

Sources of biochemicals, rats and fat-cells were as described by Severson *et al.* (1976) and Belsham *et al.* (1980*b*). Two batches of collagenase were used throughout; one was obtained from International Enzymes, Windsor, Berks., U.K. (lot no. SD4-22), and the other from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. (lot no. 54C/0363). Each batch acted on fat-pads to release cells with similar sensitivities to insulin as measured by changes in pyruvate dehydrogenase activity. Ampholines were obtained from L.K.B. Instruments, South Croydon, Surrey CR2 8YD, U.K., and trypsin (bovine, type X1-T1005) was from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964) as modified by England (1979).

Preparation of pig heart pyruvate dehydrogenase and pyruvate dehydrogenase [^{32}P]phosphate

Pyruvate dehydrogenase complex (dephosphorylated) was prepared from pig heart as described by Cooper *et al.* (1974) with the following modifications: (*a*) replacement of 5 mM-2-mercaptoethanol by 1 mM-dithiothreitol throughout the preparation, (*b*) no NaCl was added in the first poly(ethylene glycol) fractionation and (*c*) use of 50 mM-Mops (4-morpholinepropanesulphonic acid) rather than phosphate-based buffers after the second poly(ethylene glycol) fractionation. In five separate preparations over the period of this study, the specific activity of the purified pyruvate dehydrogenase ranged from 5 to 10 units/mg of protein. The separation of the complex on SDS/polyacrylamide-gel electrophoresis revealed that over 80% of material that stained with Coomassie Blue could be accounted for by the four major peaks of the component enzymes in the complex (Barrera *et al.*, 1972).

Pyruvate dehydrogenase from pig heart was phosphorylated with [γ-^{32}P]ATP by the method of Sugden *et al.* (1978). Essential details were as follows. Pyruvate dehydrogenase complex (about 5 units/ml) was incubated at 30°C in 20 mM-potassium phosphate/1 mM-dithiothreitol/0.5 mM-EGTA/1 mM-$MgCl_2$ medium. To prepare 'partially' phosphorylated pyruvate dehydrogenase, additions of 2 μM-[γ-^{32}P]ATP (1000–3000 d.p.m./pmol) were made at 4 min intervals. The activity of the complex was followed after each addition until it had decreased to between 50% and 10% of the original value. 'Fully' phosphorylated complex was prepared under the same conditions except that 0.5 mM-[γ-^{32}P]ATP (1000–3000 d.p.m./pmol) was added at the start of the incubation. The reaction was allowed to continue for 30 min, at which time the enzyme activity was decreased by over 99%.

In each of these regimes the progress of phosphorylation and inactivation was followed by removing samples at intervals to determine protein-bound ^{32}P (Corbin & Reimann, 1974) and pyruvate dehydrogenase activity (measured by following the production of NADH as described in Cooper *et al.*, 1974). When preparations were used as standards in electrophoretic systems, pyruvate dehydrogenase [^{32}P]phosphate was precipitated by the addition of 10% (w/v) trichloroacetic acid at the end of the incubation.

The extents of $^{32}P_i$ incorporation were 0.31 ± 0.05

and 0.64 ± 0.05 nmol/unit of pyruvate dehydrogenase for 'partially' and 'fully' phosphorylated forms respectively. (Values are means ± S.E.M for nine preparations.)

Preparation of antiserum to pig heart pyruvate dehydrogenase complex and its characteristics

Antiserum was raised in a sheep against purified pig heart pyruvate dehydrogenase complex (specific activity 5 units/mg) by Dr. J. Mayer, University of Nottingham, Nottingham, U.K., as described by Sinnett-Smith *et al.* (1980).

The antiserum to pyruvate dehydrogenase was able to form an immuno-complex with pyruvate dehydrogenase after incubation for 30 min at 30°C. A typical immunoprecipitation of pyruvate dehydrogenase [^{32}P]phosphate from ^{32}P-labelled fat-cells was carried out as follows. After an initial solubilization of the cell fraction with 1% (w/v) Triton X-100, the insoluble proteins were pelleted by centrifugation at 10000 **g** for 5 min at 20°C. The supernatant was incubated for 30 min at 30°C in the presence of antiserum (50 μl/ml of supernatant), and the immunoprecipitate was then collected by centrifugation at 10000 **g** for 5 min at 20°C.

Under the conditions described above, incubation with antiserum led to the precipitation of both pig heart pyruvate dehydrogenase and pig heart pyruvate dehydrogenase [^{32}P]phosphate with a titre of 15 units or equivalent/ml of antiserum. Similar behaviour was observed for the reaction of the antiserum to fat-cell pyruvate dehydrogenase and pyruvate dehydrogenase [^{32}P]phosphate labelled in intact rat epididymal fat-pad mitochondria (Hughes & Denton, 1976) and also labelled in intact mitochondria from rat heart and rat interscapular brown adipose tissue. The presence of 1% (w/v) Triton X-100 did not appreciably affect the titre of the antiserum. The specificity of the antiserum was illustrated by the lack of reaction with both pig and rat heart oxoglutarate dehydrogenase and also rat heart branched-chain 2-oxo acid dehydrogenase activity. These enzymes are known to be closely related to the pyruvate dehydrogenase complex.

Preparation, incubation and fractionation of isolated fat-cells

For the study of the incorporation of $^{32}P_i$ into fat-cell proteins the procedure of Hughes *et al.* (1977) and Brownsey *et al.* (1977) was followed. Isolated fat-cells were prepared by collagenase digestion and incubated for 75 min at 37°C in 2 ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing albumin (10 mg/ml), glucose (0.2 mg/ml), $CaCl_2$ (1.25 mM) and potassium [^{32}P]-phosphate (0.2 mM, approx. 1000 d.p.m./pmol). When indicated, insulin (0.1 μM) was added for the last 15 min of incubation. Cells were separated from the incubation medium by centrifugation (1000 **g** for 30 s) through 2 ml of dinonyl phthalate and subsequently broken by Vortex-mixing vigorously with ice-cold medium (0.25 M-sucrose/20 mM-Tris/HCl/7.5 mM-reduced glutathione/2 mM-EGTA, pH 7.4). After removal of the fat by centrifugation at 1000 **g** for 15 s, the defatted whole-cell homogenate was either treated with 10% (w/v) trichloroacetic acid for analysis on the appropriate gel system, incubated with antiserum in the presence of 1% (w/v) Triton X-100, 2 mM-EDTA and 20 mM-NaF (for details see below), or further subfractionated. A particulate fraction was prepared by centrifugation of a 1 ml portion of extract for 1 min at 10000 **g** at 20°C in an Eppendorf 3200 Minifuge and the pellet was immediately frozen in liquid N_2. The pellet was resuspended in 100 mM-potassium phosphate/2 mM-EDTA/5 mM-mercaptoethanol/1% (w/v) Triton X-100, pH 7.4. Purified mitochondrial fractions were prepared by G. Belsham from fat-cells with the use of centrifugation in Percoll as described in the preceding paper (Belsham *et al.*, 1980*b*).

Preparation and incubation of mitochondrial fractions from rat epididymal fat-pads

Mitochondria were prepared from rat epididymal fat-pads by the method described by Severson *et al.* (1976). Mitochondria were incubated at 30°C in KCl-based medium (125 mM-KCl/2 mM-EGTA/25 mM-Tris/HCl, pH 7.4) containing 2-oxoglutarate (5 mM), malate (0.5 mM) and potassium phosphate (0.2 mM). After 3 min preincubation, $^{32}P_i$ was added (final specific radioactivity 1000 d.p.m./pmol), and incubation was continued for various times. Incubations were terminated by either precipitation of proteins by 10% (w/v) trichloroacetic acid or sedimentation of proteins by centrifugation at 10000 g for 30 s, and the resultant pellet was frozen as indicated in the Figure legends.

Separation and detection of [^{32}P]phosphoproteins

Separation of proteins was routinely carried out by SDS/polyacrylamide-gel electrophoresis in 1 cm tracks on 10% (w/v) slab gels (14 cm × 14 cm) with the solutions described by Laemmli (1970). Samples for analysis, whether acid-precipitated proteins or antibody pellets, were dissolved in stacking-gel buffer, containing SDS (40 mg/ml), sucrose (200 mg/ml), Bromophenol Blue (0.2 mg/ml) and 100 mM-2-mercaptoethanol, by heating at 100°C for 5 min. Electrophoresis was carried out at 20°C for 2–3 h at 40 mA/slab.

Separation of protein samples by using a two-dimensional system [isoelectric focusing in the first dimension, followed by SDS/polyacrylamide-gel electrophoresis (10%, w/v) in the second dimension] was essentially as described by O'Farrell (1975). The ratio of Ampholines in the focusing gel

was range pH 5–7/range pH 3.5–10 (4:1, v/v), which gave a linear pH gradient over the range pH 5.0–6.5.

After electrophoresis each type of gel was washed for 1 h in 1 M-trichloroacetic acid in 50% (v/v) methanol to precipitate proteins in the gel. Protein was detected by staining with Coomassie Blue, and the gels were laid on cellophan and dried on boards under vacuum. The dried gels were exposed to Kodak Kodirex KT X-ray film for 2–7 days. The radioautographs were scanned at 630 nm. Exposure of the film was such that peak heights did not exceed an absorbance of 1.0. The peak height is measured from a base-line, which is taken as the lowest reading of absorbance on that track.

Extraction and assay of initial and total activities of pyruvate dehydrogenase

At the end of the incubation period fat-cells were frozen in liquid N_2 after careful removal of the medium with a syringe. Frozen fat-cells (approx. 200 mg dry wt.) were extracted in 2 ml of a solution containing 100 mM-potassium phosphate, pH 7.4, 2 mM-EDTA, 5 mM-2-mercaptoethanol and rat serum (50 μl/ml). Extractions were carried out in a Polytron (PT20) homogenizer at setting number 3 for 30 s at 0°C. After addition of Triton X-100 (final concn. 1%, w/v) to the suspension, samples were frozen and thawed, then centrifuged at 10000 *g* for 1 min in an Eppendorf 3200 Minifuge. Initial and total activities of pyruvate dehydrogenase were determined in the infranatant as described by Stansbie *et al.* (1976), where 1 unit of enzyme activity transforms 1 μmol of substrate/min at 30°C.

Digestion of α-subunits of pyruvate dehydrogenase [^{32}P]phosphate by trypsin and separation of the phosphopeptides

Labelled α-subunits were separated from other [^{32}P]phosphoproteins in defatted whole fat-cell homogenates by a combination of immunoprecipitation and SDS/polyacrylamide-gel electrophoresis as detailed in the Figure and Table legends. Samples of labelled α-subunits from pig heart pyruvate dehydrogenase [^{32}P]phosphate and from fat-cell pyruvate dehydrogenase [^{32}P]phosphate obtained by incubating epididymal fat-pad mitochondria with $^{32}P_i$ were separated by SDS/polyacrylamide-gel electrophoresis alone. In all cases the ^{32}P-labelled α-subunits were eluted from the appropriate gel region (R_F 0.45–0.55) as follows. The slice was dispersed in 5 ml of a solution containing SDS (0.1% w/v), 1 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride, 5 mM-NH_4HCO_3 and 2.5 mg of bovine serum albumin with a Polytron homogenizer at setting number 4 for 30 s. The mixture was stirred until the radioactivity appearing in the solution remained constant (usually overnight at 4°C), and then spun at 5000 *g* to separate the gel phase from the medium. A second brief (1 h) washing at 4°C with 2 ml of the above solution with the gel phase allowed an overall total recovery of over 90% of the radioactivity associated with the gel slice. The supernatant was then dialysed against 100 vol. of water, then against 40% (v/v) methanol and finally against water (4–6 h in each case).

After being freeze-dried, the protein samples were dissolved in 100 μl of 2 M-urea/0.2 M-NH_4HCO_3 and then digested with trypsin (50 μg) for 3–5 h at 37°C. The extent of reaction was monitored by the disappearance of [^{32}P]phosphoprotein measured by the method of Corbin & Reimann (1974). Incubation was stopped by boiling samples for 5 min, and the digest was then freeze-dried. No change in the subsequent pattern of radioactive tryptic peptides (see below) was seen on further (up to 24 h) incubation with trypsin.

The tryptic peptides released were separated by high-voltage electrophoresis (Davis *et al.*, 1977). Samples were resuspended in 1 M-HCl, applied to Whatman 3MM chromatography paper and then subjected to electrophoresis at pH 1.7 [6.7% (v/v) formic acid] at 2000 V for 40 min. Typically N^6-dinitrophenyl-lysine marker migrated 10 cm from the origin. The paper was dried overnight and the radioactive peptides were located by radioautography (with Kodak Kodirex KT X-ray film), or, if an increased sensitivity was required, intensifier screens were used in combination with Kodak X-Omat X-ray film (Laskey & Mills, 1977).

The radioautograms were scanned at 630 nm and the area under each peak was determined by excision and weighing. In parallel experiments, it was found that peak area was proportional to the radioactivity in each peak determined directly.

Results and discussion

Demonstration of the phosphorylation of the α-subunit of pyruvate dehydrogenase in intact fat-cells

Among the several bands of fat-cell ^{32}P-labelled phosphoproteins that can be separated by SDS/polyacrylamide-gel electrophoresis after the incubation of fat-cells with $^{32}P_i$ is one that has the same R_F (about 0.45) as the phosphorylated α-subunit of the pig heart pyruvate dehydrogenase phosphate (Figs. 1*a* and 1*c*). In our other studies we assigned numbers to the major bands, and this band was designated band 6 (Hughes *et al.*, 1977; Brownsey *et al.*, 1977, 1979; Belsham *et al.*, 1980*a*,*b*). Evidence that 35–45% of the peak height of band 6 on the densitometric traces of radioautographs represents incorporation into the α-subunit of fat-cell pyruvate dehydrogenase was obtained by a combination of subcellular fractionation, immunoprecipitation and both one- and two-dimensional gel electrophoresis.

Results of typical experiments are given in Figs. 1–4; in all cases the experiments were repeated two to ten times. In the following account we have included overall mean values where possible, and these are expressed as means ± S.E.M. for the numbers of independent observations given in parentheses.

A crude particulate fraction containing mitochondria was obtained by centrifuging the defatted whole-cell extract at 10000g for 1 min. This fraction contained more than 90% of the total pyruvate dehydrogenase activity in the whole-cell extract but only 60 ± 6% (4) of the ^{32}P in band 6 (Fig. 1*b*). On the other hand, if 1% Triton was added to lyse the mitochondria before centrifugation of the whole-cell fraction, more than 90% of the pyruvate dehydrogenase activity remained in the supernatant fraction and there was no appreciable activity in the particulate fraction. Under these conditions, all the ^{32}P in band 6 also remained in the supernatant fraction (Fig. 1*d*). The Triton-treated supernatant was incubated for 30 min with the antiserum raised against pyruvate dehydrogenase, and the mixture was centrifuged. This procedure resulted in the specific precipitation of labelled phosphoprotein with the same subunit molecular weight as the α-subunit of pyruvate dehydrogenase. However, although 85 ± 5% (4) of the total pyruvate dehydrogenase activity was precipitated, only 35 ± 5% (10) of the ^{32}P associated with band 6 was present in the immunoprecipitate (Figs. 1*e* and 1*f*). In parallel experiments with control serum, less than 5% (four experiments) of either total pyruvate dehydrogenase activity or ^{32}P associated with band 6 was precipitated. No evidence was found for any dephosphorylation of the phosphoproteins associated with band 6 during incubation with the antiserum or control serum.

The particulate fraction obtained by centrifugation of the defatted whole-fat-cell extracts at 10000g for 1 min contains plasma membranes as well as mitochondria, and thus the fraction contains labelled phosphoproteins associated with bands 4 and 5 as well as band 6 (Fig. 2*a*; Belsham *et al.*, 1980*b*). Also present is a band that we have

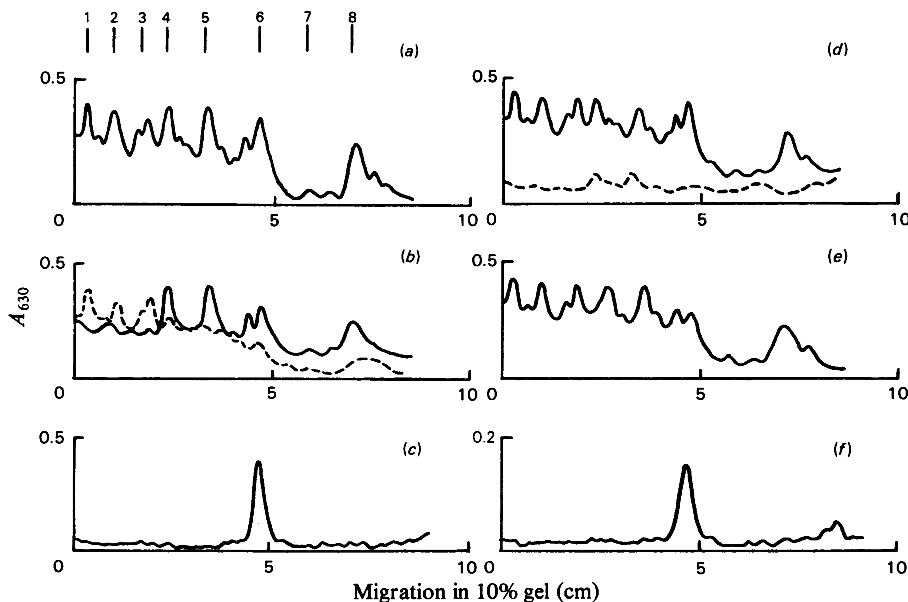

Fig. 1. *Densitometric traces of radioautographs locating the labelled proteins separated by SDS/polyacrylamide-gel electrophoresis in intact fat-cells previously incubated with* $^{32}P_i$

(*a*) Defatted whole-cell fraction of fat-cells after incubation of the cells with ^{32}P$_i$ for 75 min in medium containing no added hormone. (The numbering of the labelled phosphoprotein bands used in the text is also shown). (*b*) Particulate fraction (——) and soluble fraction (----) after centrifugation of the same whole-cell extract at 10000g for 5 min. (*c*) Sample of pig heart pyruvate dehydrogenase [^{32}P]phosphate. (*d*) Soluble fraction (——) and particulate fraction (----) derived from the defatted whole-cell fraction after treatment with Triton X-100 (1%, w/v) and centrifugation at 10000g for 5 min. (*e*) and (*f*) A sample of the soluble fraction (*d*) was incubated at 30°C for 30 min with 50 µl of anti-(pyruvate dehydrogenase) serum/ml, and, after centrifugation at 10000g for 5 min, the supernatant (*e*) and pellet (*f*) fractions were resolved by electrophoresis. In all cases proteins from equivalent samples (derived from approx. 20 mg dry wt. of fat-cells) were separated on adjacent tracks by SDS/polyacrylamide-slab-gel elecrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10 cm. For further details see the Experimental section.

designated band 5a, which has a molecular weight only slightly greater than that of band 6 and is therefore often poorly resolved from it (Figs. 1*b* and 2*a*). Indeed, band 5a cannot always be clearly discerned when the phosphoproteins in the defatted whole-cell fraction are separated by SDS/polyacrylamide-gel electrophoresis (Fig. 1*a*). Treatment of an extract of the particulate fraction with pyruvate dehydrogenase phosphate phosphatase in the presence of Mg^{2+} and Ca^{2+} under conditions that lead to expression of the maximum pyruvate dehydrogenase activity was found to result in the disappearance of band 6 (Fig. 2*c*). In five experiments, the complete loss of any discernible band 6 was associated with diminutions of only 17 ± 5% and 19 ± 2% respectively in the ^{32}P in bands 4 and 5 respectively and no obvious decrease in band 5a. Fat-cells were incubated with dichloroacetate, which is an inhibitor of pyruvate dehydrogenase kinase (Whitehouse & Randle, 1973), as an alternative means of converting all fat-cell pyruvate dehydrogenase into its active form. Again, as illustrated in Fig. 2(*b*), the phosphorylation of the band 6 in the particulate fraction could no longer be discerned. In seven experiments, there was a complete disappearance of any separate band 6, whereas the ^{32}P in bands 4 and 5 remained unaltered (115 ± 13% and 128 ± 14% of control respectively). It was also evident that much of band 5a remained.

Treatment of the particulate fraction with 1% Triton led to the solubilization of the ^{32}P associated with band 6 (95 ± 2% in three experiments) and

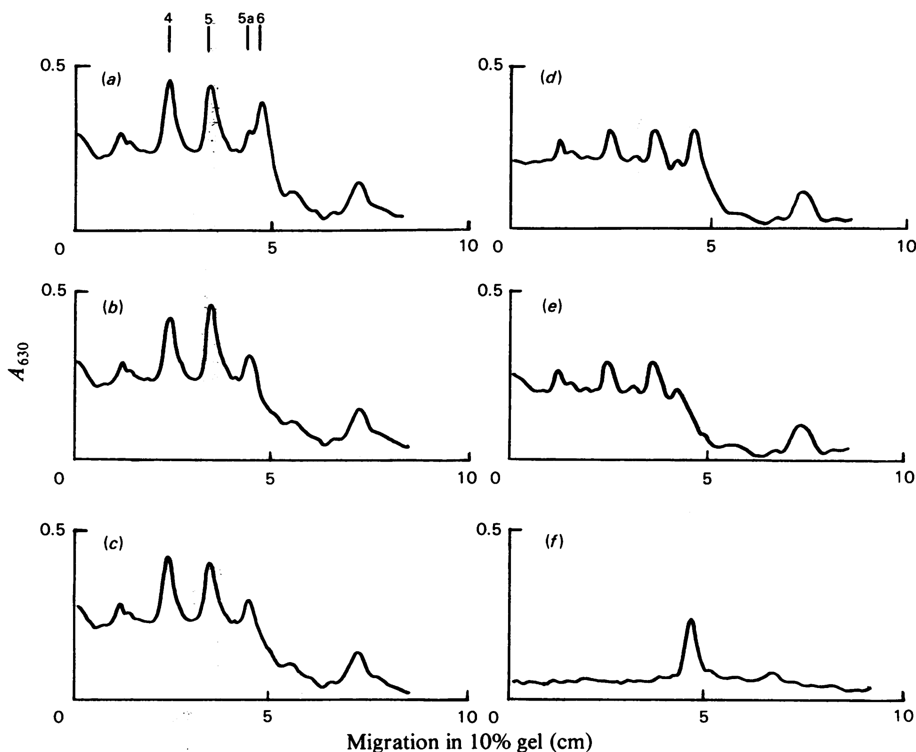

Fig. 2. *Densitometric traces of the radioautographs locating the labelled proteins of a particulate fraction derived from fat-cells previously incubated with* $^{32}P_i$

(*a*) and (*b*) After incubation of fat-cells with $^{32}P_i$ for 75 min in the absence (*a*) or presence (*b*) of 2 mM-dichloroacetate, a particulate fraction was prepared by centrifugation of the defatted whole-cell fraction at 10000 *g* for 5 min. (*c*) Particulate fraction, derived from (*a*), after 20 min incubation at 30°C with 25 mM-$MgCl_2$, 1 mM-$CaCl_2$ and pyruvate dehydrogenase phosphatase (1 munit/ml). (*d*)–(*f*) Supernatant derived from the particulate fraction (*a*) after treatment with Triton X-100 (1%, w/v) and centrifugation at 10000 *g* for 5 min; a sample of this supernatant (*d*) was incubated at 30°C for 30 min with 50 μl of anti-(pyruvate dehydrogenase) serum/ml, and, after centrifugation at 10000 *g* for 5 min, the supernatant (*e*) and pellet (*f*) fractions were resolved by electrophoresis. In all cases proteins from equivalent samples (derived from approx. 50 mg dry wt. of fat-cells) were separated on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10 cm. For further details see the Experimental section.

band 5a, but only about half of that associated with bands 4 and 5 (Fig. 2*d*). Incubation of the Triton-treated particulate fraction with antiserum raised against pyruvate dehydrogenase resulted in the immunoprecipitation of more than 90% of band 6, whereas less than 10% of the ^{32}P associated with bands 4, 5 and 5a was present in the immunoprecipitate (Figs. 2*d–f*, which are typical of three separate experiments).

Plasma membranes and mitochondria in a fat-cell particulate fraction may be conveniently separated by centrifugation with Percoll (Belsham *et al.*, 1980*b*). As reported in that paper, only two bands of [^{32}P]phosphoproteins are evident in the purified mitochondrial fraction, namely bands 5a and 6 (Figs. 3*a–c*). Treatment of this fraction with antiserum to pyruvate dehydrogenase in the presence of 1% Triton led to the complete and specific immunoprecipitation of phosphoprotein band 6 without any discernible precipitation from band 5a. Separate studies (Hughes & Halestrap, 1980) have indicated that band 5a, which has a subunit mol.wt. of 48000, may represent the phosphorylation of a subunit of the branched-chain 2-oxo acid dehydrogenase complex. In particular, these studies have shown that incorporation of ^{32}P into this phosphoprotein band is specifically diminished in the presence of one of its substrates, 4-methyl-2-oxopentanoate (Hughes & Halestrap, 1980). In our earlier studies on isolated mitochondria from rat epididymal fat-pads or fat-cells, we found that $^{32}P_i$ was incorporated into a single phosphoprotein, which appeared to be the α-subunit of the pyruvate dehydrogenase complex (Hughes & Denton, 1976; Hughes *et al.*, 1977). This view was confirmed in the present studies by the use of the antiserum to pyruvate dehydrogenase (Figs. 3*d–f*). Treatment of an extract of mitochondria previously incubated with $^{32}P_i$ for 10min in the presence of an oxidizable substrate such as oxoglutarate with malate leads to the complete immunoprecipitation of the sole ^{32}P-labelled phosphoprotein, which has the same R_F as the α-subunit of the pyruvate dehydrogenase complex. There is little or no evidence for any phosphoprotein corresponding to band 5a, and thus, under these conditions, the rate of incorporation of $^{32}P_i$ into the α-subunit of the pyruvate dehydrogenase complex was apparently much faster than into the corresponding subunit of the branched-chain 2-oxo acid dehydrogenase (see also Hughes & Halestrap, 1980).

In summary, a number of different [^{32}P]phosphoproteins appeared to contribute to the peak height of band 6 in densitometric traces of radioautographs of the separated ^{32}P-labelled phosphoproteins in the defatted whole-cell extract. From the above evidence we estimate that the α-subunit of the pyruvate dehydrogenase complex accounted for

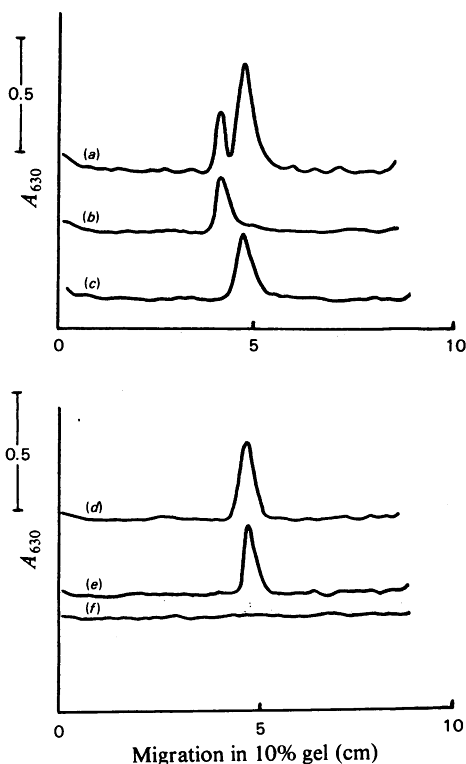

Fig. 3. *Densitometric traces of radioautographs locating mitochondrial [^{32}P]phosphoproteins labelled in either intact fat-cells (a–c) or isolated fat-cell mitochondria (d–f) incubated with $^{32}P_i$*

(*a*)–(*c*) Fat-cells were incubated with $^{32}P_i$ for 75min, and the mitochondrial fraction was prepared by centrifugation in the presence of Percoll, treated with Triton X-100 (1%, w/v) and centrifuged at 10000**g** for 5min. The supernatant (*a*) was then incubated at 30°C for 30min with 50μl of anti-(pyruvate dehydrogenase) serum/ml. After centrifugation at 10000**g** for 5min, proteins in equivalent samples of the supernatant (*b*) and pellet (*c*) fractions were resolved on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis. (*d*)–(*f*) Isolated fat-cell mitochondria were incubated with $^{32}P_i$ for 10min as described in the Experimental section, and then centrifuged at 10000**g** for 30s. The sedimented mitochondria were immediately frozen and then extracted in 100mM-potassium phosphate, pH7.4, containing 2mM-EDTA, 1mM-dithiothreitol and Triton X-100 (1% w/v), and the extract was centrifuged at 10000**g** for 5min. The supernatant (*d*) was incubated at 30°C for 30min with 50μl of anti-(pyruvate dehydrogenase) serum/ml, and then centrifuged at 10000**g** for 5min. The proteins in equivalent samples of antibody supernatant (*e*) and pellet (*f*) were resolved on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10cm.

35–45%. Other phosphoproteins that contributed include a mitochondrial phosphoprotein that may be a subunit of the branched-chain 2-oxo acid dehydrogenase (band 5a) and a phosphoprotein or phosphoproteins associated with the soluble fraction of the fat-cell.

This view was substantiated by the two-dimensional separation of the [^{32}P]phosphoproteins by using the system of O'Farrell (1975). Fig. 4(*d*) shows that 'fully phosphorylated' pig heart pyruvate dehydrogenase [^{32}P]phosphate gave a single major spot of mol.wt. about 42000 and an isoelectric point of approx. pH 5.0. Also present were two minor spots (combined peak area less than 20% of peak area of major spot) of the same molecular weight but with slightly higher isoelectric points (Fig. 4*d*). The relative intensities of these minor spots were increased in equivalent samples of 'partially phosphorylated' pig heart pyruvate dehydrogenase [^{32}P]phosphate (results not shown). It is possible the three spots represent α-subunits with different amounts of phosphate. In the array of [^{32}P]phosphoproteins in the defatted whole fat-cell and particulate extract it was possible to distinguish the spots (see single-headed arrow) representing incorporation into pyruvate dehydrogenase (Figs. 4*a* and 4*b*). Treatment with antiserum to pyruvate dehydrogenase resulted in the specific precipitation of these phosphoproteins (Fig. 4*c*). As with the 'fully phosphorylated' form of purified pig heart enzyme, a major component and two minor, less basic, components were found to be labelled. More-

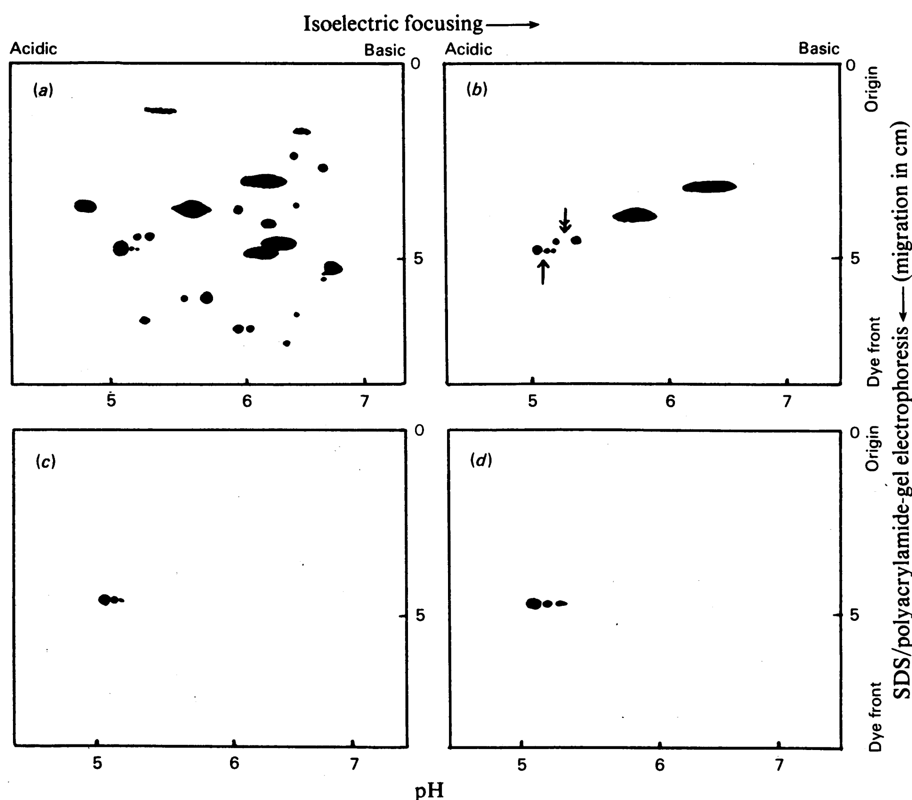

Fig. 4. *Diagrammatic representation of typical radioautographs of two-dimensional separation of* [^{32}P]*phosphoproteins from* (*a–c*) *defatted whole-cell extract derived from cells previously incubated with* $^{32}P_i$ *and from* (*d*) *pig heart pyruvate dehydrogenase* [^{32}P]*phosphate*

(*a*) Defatted whole-cell extract of fat-cells after incubation of the cells with $^{32}P_i$ for 75 min in medium containing no added hormone. (*b*) Particulate fraction after centrifugation of the same whole-cell extract at 10000 **g** for 5 min. The single-headed and double-headed arrows refer to the phosphorylated subunits of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes respectively. (*c*) Immunoprecipitate with antiserum to pyruvate dehydrogenase derived from the defatted whole-cell extract as described for Fig. 1(*f*). (*d*) 'Fully' phosphorylated pig heart pyruvate dehydrogenase: samples were applied to the acidic end of the gel (top left-hand corner of diagram). For further details see the Experimental section.

over, it was clear that these were not the only labelled phosphoproteins in the defatted whole-cell extract with mol.wt. close to 42000 (Fig. 4*a*). At least one other phosphoprotein of isoelectric point close to pH 6 was evident; this phosphoprotein was associated with the soluble fraction of fat-cells and did not appear in the particulate fraction (Fig. 4*b*). It is worthy of note that peak 5a, the phosphorylated subunit of branched-chain 2-oxo acid dehydrogenase, also appeared as a doublet in the same region of the gel (see double-headed arrow in Fig. 4*b*).

Effect of insulin on the phosphorylation of the α-subunit of the pyruvate dehydrogenase complex and other phosphoproteins within isolated fat-cells

In these studies fat-cells were incubated for 75 min with $^{32}P_i$. Previously incorporation of ^{32}P into the major labelled phosphoproteins had been shown to reach a maximum steady-state extent by this time (Brownsey *et al.*, 1977, 1979). The effects of insulin were investigated by exposing cells to the hormone for the final 15 min of the 75 min incubation period. A number of changes in the incorporation of ^{32}P into the major phosphoprotein bands in the defatted whole-cell extract were apparent (Fig. 5 and Table 1). The extent of these changes was not altered if the period of exposure to insulin was increased to 1 h; thus we have taken the changes observed after 15 min exposure to represent steady-state changes in the phosphorylation of the various phosphoproteins.

The most obvious effect of insulin was the increased phosphorylation of bands 2 and 7, which have approximate subunit mol.wts. of 130000 and 35000 (see also Benjamin & Singer, 1975; Avruch *et al.*, 1976*a*,*b*; Hughes *et al.*, 1977; Brownsey *et al.*, 1977; Belsham *et al.*, 1980*a*,*b*). Both these phosphoproteins remained in the soluble fraction after centrifugation of the whole-cell extract at 10000 *g* for 1 min (Fig. 1*b*), but band 7 was sedimented when centrifugation was carried out at 100000 *g* for 1 h (W. A. Hughes, unpublished work; Hughes & Denton, 1978). Incorporation into band-2 phosphoprotein probably represents the phosphorylation

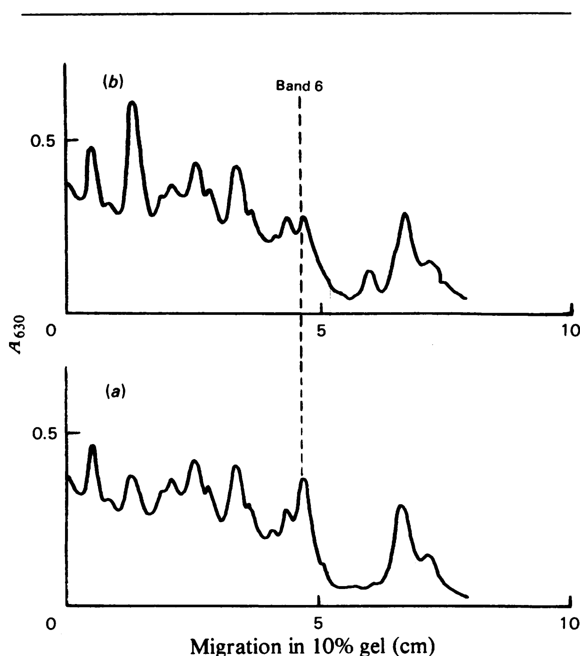

Fig. 5. *Densitometric traces of the radioautographs obtained in a typical experiment in which the effects of insulin on the incorporation of ^{32}P from P_i in the medium into phosphoproteins of isolated fat-cells was studied*
Defatted whole-cell fraction of fat-cells was prepared after prior incubation of the cells with $^{32}P_i$ for 75 min in the (*a*) absence or (*b*) presence of insulin (0.1 μM) for the last 15 min. Proteins from equivalent samples (derived from approx. 20 mg dry wt. of fat-cells) were separated on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis. There was no measurable difference between the protein staining of the two samples. The dye front (Bromophenol Blue) was allowed to migrate 10 cm. For further details see the Experimental section.

Table 1. *Effect of insulin on incorporation of $^{32}P_i$ into fat-cell-homogenate phosphoproteins*

Results are taken from 12 separate experiments performed as described in the legend to Fig. 1. Peak heights from the densitometric traces of radioautographs of phosphoproteins separated by SDS/polyacrylamide-gel electrophoresis (10%, w/v) from fat-cells incubated with insulin are expressed as percentages of control values, as means ± S.E.M. with the numbers of observations in parentheses. In five of these experiments band 5a was not resolved, and in one band 1 was poorly resolved from the origin. In all experiments there was no measurable difference between the protein staining on adjacent lanes of the samples from control and insulin-treated cells. Significance of effect of insulin: *$P < 0.01$ versus control.

Band no.	Approx. mol.wt.	Effect of insulin on peak height (% of control)
1	220000	107 ± 8 (11)
2	130000	192 ± 10* (12)
3	85000	104 ± 4 (12)
4	67000	106 ± 4 (12)
5	61000	110 ± 5 (12)
5a	48000	112 ± 8 (7)
6	42000	82 ± 4* (12)
7	35000	380 ± 55* (12)
8	26000	100 ± 8 (11)

of the cytoplasmic enzyme ATP–citrate lyase (Linn & Srere, 1979; Ramakrishna & Benjamin, 1979; Alexander *et al.*, 1979). It has been reported that insulin results in the increased phosphorylation of a phosphoprotein with a subunit molecular weight similar to that of band 7 in cultures of differentiated 3T3-L1 pre-adipocytes, and evidence was presented that it is the ribosomal S6 protein (Smith *et al.*, 1979). With one exception, insulin did not alter significantly the incorporation of ^{32}P into the other major phosphoprotein bands, including band 1, which is largely acetyl-CoA carboxylase (Brownsey *et al.*, 1977, 1979). The exception was band 6, which exhibited a 20% decrease in the peak height of the densitometric traces in the presence of insulin (Fig. 5 and Table 1). In a parallel series of six separate experiments, fat-cells were incubated under identical conditions but in the absence of $^{32}P_i$. Exposure of the fat-cells to insulin increased the initial activity of pyruvate dehydrogenase from $30 \pm 6\%$ of total activity to $52 \pm 7\%$ of total activity (means $\pm$ S.E.M. for six observations given in both cases). There was no effect of insulin on the total activity, which was 228 ± 49 munits/g dry wt. in the control cells and 258 ± 36 munits/g dry wt. in the cells exposed to insulin.

The studies described in the preceding section demonstrated that the phosphorylated α-subunit of the fat-cell pyruvate dehydrogenase complex is a major component of band 6. The decrease in peak height of band 6 therefore probably represented the dephosphorylation of the α-subunit, resulting in the observed increase in initial pyruvate dehydrogenase activity.

To demonstrate this, in four separate experiments we determined the effect of insulin on the ^{32}P in the phosphoprotein of band 6 that was precipitated after incubation with the antisera raised to pyruvate dehydrogenase. The experiments were carried out as described in Fig. 5 and Table 1, but samples of the defatted whole-cell extract were treated with antiserum as described for Fig. 1. In these experiments the effect of insulin was to diminish the peak height of band 6 of the labelled phosphoproteins in the defatted whole-cell extract to $80 \pm 6\%$ of the control value. This diminution could be accounted for entirely by the change in extent of phosphorylation of the α-subunit of pyruvate dehydrogenase in the immunoprecipitate. The mean decrease in the four experiments was to $55 \pm 13\%$ of control values. If the activity of pyruvate dehydrogenase in fat-cells were strictly in inverse proportion to the extent of phosphorylation of the α-subunits, the expected decrease in phosphorylation from the observed change in activity would be to 69% of control values.

Further supportive evidence for the view that the decrease in the peak height of peak 6 in defatted whole cell homogenates was due to dephosphorylation of α-subunits was obtained by studying the effect of insulin in the presence of dichloroacetate. As explained above, the initial activity of pyruvate dehydrogenase closely approaches the total activity in fat-cells incubated in the presence of 2mM-dichloroacetate (Whitehouse & Randle, 1973), and, in consequence, exposure to insulin in the presence of dichloroacetate does not result in any change in pyruvate dehydrogenase activity. It follows that insulin should have no effect on the peak height of band 6 when the cells were incubated with 2mM-dichloroacetate. In four experiments performed as described in Table 1, but with the presence of 2mM-dichloroacetate throughout the incubation, the mean ($\pm$ S.E.M.) effect of insulin on the peak height of band 6 in defatted whole cell homogenates was $97 \pm 5\%$ of control. In the four parallel experiments performed in the absence of dichloroacetate, insulin resulted in an $18 \pm 7\%$ diminution in the peak height of band 6.

Investigation into the extent of multiple phosphorylation of the α-subunits of the pyruvate dehydrogenase complex within fat-cells

The studies described above indicated that ^{32}P-labelled α-subunits of pyruvate dehydrogenase complex could be separated from all other labelled phosphoproteins in the defatted whole-cell extract by a combination of immunoprecipitation and SDS/polyacrylamide-gel electrophoresis. The extent of multiple phosphorylation was thus explored by digesting the isolated ^{32}P-labelled α-subunits with trypsin and separating the [^{32}P]phosphopeptides by paper electrophoresis at pH 1.7, with [^{32}P]phosphopeptides derived from the parallel trypsin digestion of 'partially' and 'fully' phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate as markers (Fig. 6 and Table 2).

The pattern of [^{32}P]phosphopeptides derived from the preparations of 'partially' and 'fully' phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate was in close agreement with that reported in other studies with the kidney or pig heart enzyme complexes (Davis *et al.*, 1977; Sugden *et al.*, 1979), namely that a total of three different phosphopeptides (T-1, T-2 and T-3) were evident, all with an overall net positive charge at pH 1.7. In 'partially' phosphorylated preparations incorporation of ^{32}P into phosphopeptide T-1 was greater than into phosphopeptides T-2 and T-3, whereas it was much less in the 'fully' phosphorylated preparations of the complex (Fig. 6). The phosphopeptides were all retarded on a Sephadex G-25 column ($K_{av.}$ about 0.3) suggesting mol.wts. of 1000–2000, which are compatible with the published amino acid sequences (Davis *et al.*, 1977; Sugden *et al.*, 1979).

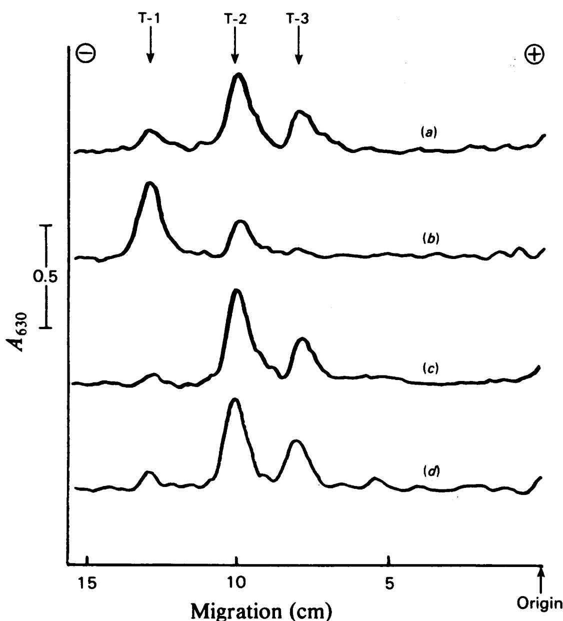

Fig. 6. *Densitometric traces of the radioautographs locating the tryptic phosphopeptides of the α-subunits of pyruvate dehydrogenase [^{32}P]phosphate phosphorylated with (a and b) purified complex, (c) isolated mitochondria and (d) intact fat-cells*

(*a*) and (*b*) Phosphopeptides derived from the α-subunits of (*a*) 'fully' or (*b*) 'partially' phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate (the extents of ^{32}P incorporation were 0.53 and 0.28 nmol/unit respectively; the pattern of phosphopeptides shown was typical of seven separate preparations of each form). The α-subunits were separated by SDS/polyacrylamide-gel electrophoresis as in Fig. 1(*c*). (*c*) Phosphopeptides derived from α-subunits of pyruvate dehydrogenase [^{32}P]phosphate formed by incubating mitochondria isolated from epididymal fat-pads with $^{32}P_i$ for 5 min in the presence of oxoglutarate and malate. The α-subunits were separated by SDS/polyacrylamide-gel electrophoresis as described in Fig. 3(*d*). (*d*) Phosphopeptides derived from α-subunits of pyruvate dehydrogenase [^{32}P]phosphate formed by incubating fat-cells with $^{32}P_i$ for 75 min in the absence of added hormones. The α-subunits were separated by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis as described for Fig. 1(*f*). Further details of elution of the α-subunits from the electrophoresis gels and tryptic digestion are given in the Experimental section. The phosphopeptides (labelled T-1, T-2 and T-3; see the text for details) were separated by paper electrophoresis at pH 1.7 and 2000 V for 40 min.

The pattern of [^{32}P]phosphopeptides derived from α-subunits phosphorylated both within isolated mitochondria and intact fat-cells closely matched those derived from 'fully' phosphorylated pig heart pyruvate dehydrogenase phosphate (Fig. 6 and Table 2). The incorporation into phosphopeptide T-1 was much less than that into phosphopeptides T-2 and T-3, even in the isolated mitochondria exposed to $^{32}P_i$ for only 2 min. Moreover, the incorporation into phosphopeptide T-2 was approx. twice that into phosphopeptide T-3.

Exposure of fat-cells to insulin, although resulting in an increase in the initial activity of pyruvate dehydrogenase and an overall decrease in the extent of phosphorylation of α-subunits in the immunoprecipitates, did not have any significant effect on the pattern of ^{32}P incorporation into the three phosphopeptides (Table 2).

General conclusions

The present studies show clearly that $^{32}P_i$ is incorporated into the α-subunits of the pyruvate dehydrogenase complex within the mitochondria of intact fat-cells. This appears to represent the first direct demonstration of the phosphorylation of the α-subunits in a whole-cell preparation. The observed incorporation almost certainly would necessitate both pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase to be active simultaneously within the cells, and that together the two activities catalyse the constant turnover of phosphate. Any changes in the extent of phosphorylation of the α-subunits observed within fat-cells could therefore be brought about by either alterations in phosphatase or kinase activity or both.

The well-established increase in the initial activity of pyruvate dehydrogenase in fat-cells exposed to insulin has been demonstrated to be associated with a decrease in the overall steady-state incorporation of ^{32}P into the α-subunits. The extent of the decrease is of a magnitude similar to that predicted if there were a strict inverse relationship between phosphorylation and the initial activity of pyruvate dehydrogenase. However, precise measurement of the incorporation of ^{32}P is made difficult by the presence of other phosphoproteins in fat-cells with subunit molecular weights on SDS/polyacrylamide-gel electrophoresis very close to that of the α-subunits. One of these phosphoproteins is mitochondrial in origin, and may be a subunit of the branched-chain 2-oxo acid dehydrogenase (Hughes & Halestrap, 1980); the other phosphoproteins are soluble. Insulin appears to have little or no effect on the incorporation of $^{32}P_i$ into these phosphoproteins.

With the recognition that the regulation of the activity of a number of enzymes in mammalian cells may involve changes in the phosphorylation of more than one site (Nimmo & Cohen, 1977; Krebs & Beavo, 1979), it has become important to develop methods whereby changes in the relative phosphorylation of multiple sites on a single enzyme

Table 2. *Distribution of ^{32}P in the tryptic peptides T-1, T-2 and T-3 of α-subunits of pyruvate dehydrogenase [^{32}P]-phosphate obtained from isolated mitochondria or fat-cells incubated in the presence or absence of insulin*
Details of incubation of epididymal fat-pad mitochondria and isolated fat-cells are as given in the legend to Fig. 6 and in the Experimental section. Radioactivity not associated with phosphopeptides running as T-1, T-2 and T-3 (including radioactivity at origin and associated with P_i) was less than 20% of the total in phosphopeptides T-1 + T-2 + T-3.

Source of ^{32}P-labelled α-subunits	^{32}P in phosphopeptides (% of total in T1 + T2 + T3)		
Phosphopeptide ...	T-1	T-2	T-3
Mitochondria incubated with $^{32}P_i$ for:			
2 min	14.5 ± 1.4	54.1 ± 5.7	31.4 ± 4.5 (3)
5 min	5.0 ± 2.0	57.1 ± 5.2	37.6 ± 5.9 (4)
Fat-cells incubated with $^{32}P_i$ for 75 min plus:			
No additions	12.0 ± 5.1	56.3 ± 4.2	31.0 ± 1.9 (4)
Insulin for last 15 min	13.7 ± 5.2	58.4 ± 5.2	27.9 ± 2.3 (4)

subunit can be explored. The technique used in the present study was to compare the pattern of phosphopeptides released from α-subunits separated from cell and mitochondrial extracts by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis with those obtained from purified pig heart pyruvate dehydrogenase [^{32}P]phosphate. The separation of the α-subunits was rapid, specific and could be executed without any apparent dephosphorylation. Approximate values for the relative incorporation of ^{32}P into the labelled phosphoproteins released by trypsin could be obtained, but the specific radioactivity of the phosphoproteins could not be determined because of the minute amounts available.

Our findings are compatible with the view that all three sites of the α-subunit are phosphorylated in both isolated mitochondria and intact fat-cells. Moreover the extent of phosphorylation at each site appears to be similar. Theoretically it is possible that the observed incorporation into phosphopeptide T-2 was restricted to site 1 combined with the presence of non-exchangeable phosphate at site 2. This appears to be unlikely, as the overall incorporation into phosphopeptide T-2 was consistently found to be double that into phosphopeptide T-3. It follows that sites 1 and 2 must be phosphorylated to a large extent on the same α-subunit. The behaviour of the ^{32}P-labelled subunits on the two-dimensional electrophoresis (Fig. 5) was also compatible with the extent of phosphorylation of all three sites being similar. It is possible that changes in isoelectric point may prove to be a useful technique for detecting changes in multiple phosphorylation of phosphoproteins within cells.

The effect of insulin on pyruvate dehydrogenase in fat-cells may involve activation of the phosphatase rather than inhibition of the kinase (Hughes & Denton, 1976). We could find no evidence in the present studies that this activation was associated with a change in the phosphorylation of sites 2 and 3 relative to that of site 1. Rather, it would appear that there is a parallel decrease in the phosphorylation of all three sites in cells exposed to insulin, this presumably being the result of the increase in phosphatase activity.

The use of cell-free systems to explore the mechanism of action of insulin offers the possibility of exploring the second messenger(s) involved (Seals *et al.*, 1979; Jarett & Seals, 1979). A still plausible hypothesis is that insulin results in an increase in the intramitochondrial concentration of Ca^{2+} (Denton *et al.*, 1978*a*; Denton & Hughes, 1978). Recent studies in this laboratory on the regulation of NAD-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase suggest that the intramitochondrial concentration of Ca^{2+} in mammalian mitochondria probably varies within the range in which the phosphatase is sensitive (Denton *et al.*, 1978*b*, 1980; McCormack & Denton, 1979, 1980).

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