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Demonstration of the Phosphorylation of Acetyl-Coenzyme A Carboxylase within Intact Rat Epididymal Fat-Cells

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Intact rat epididymal fat-cells were incubated with $^{32}P_i$ and the intracellular proteins separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. One of the phosphorylated proteins has the same R_F value as [^{14}C]biotin-labelled acetyl-CoA carboxylase purified from fat-cells and is specifically precipitated after incubation with antiserum raised against acetyl-CoA carboxylase. No significant changes in the extent of phosphorylation of acetyl-CoA carboxylase were detected after exposure of the cells to insulin.

The initial activity of acetyl-CoA carboxylase (EC 6.4.1.2) measured in extracts of rat epididymal tissue is increased 2–3-fold if the tissue is briefly exposed to insulin before extraction (Halestrap & Denton, 1973). This effect of insulin appears to be the result of a greater proportion of the enzyme occurring in its active polymeric form (Halestrap & Denton, 1974). The alteration could be due to changes in the cytoplasmic concentrations of citrate or fatty acyl-CoA, and some evidence has been presented that a lowering of the concentration of fatty acyl-CoA may be involved in the action of insulin on the enzyme (Halestrap & Denton, 1974; Brownsey *et al.*, 1977). Another possibility, which is explored in the present study, is that the extent of phosphorylation of the enzyme is altered after exposure of the tissue to insulin. The enzyme from rat liver has been shown to contain phosphate (Inoue & Lowenstein, 1972), and, moreover, evidence has been presented (Carlson & Kim, 1974*a*,*b*; Lee & Kim, 1977) that the phosphorylation of the rat liver enzyme in tissue extracts in the presence of $MgATP^{2-}$ results in inhibition of enzyme activity. In the present paper, we have used either immunoisolation or binding to Sepharose–avidin combined with SDS*/polyacrylamide-slab-gel electrophoresis to demonstrate that the enzyme within intact fat-cells is phosphorylated. It has also been suggested (Qureshi *et al.*, 1975) that pigeon liver fatty acid synthetase may be regulated by phosphorylation, but we have been unable to find any evidence for phosphorylation of the fat-cell enzyme in the present study.

* Abbreviations: SDS, sodium dodecyl sulphate; GSH, reduced glutathione.

Methods

Sources of chemicals, biochemicals and rats and the preparation of incubation media and isolated fat-cells were as described by Severson *et al.* (1976), except as follows. Monospecific antiserum to acetyl-CoA carboxylase and control serum were prepared as described by Walker *et al.* (1976). Antiserum to fatty acid synthetase was prepared as described by Speake *et al.* (1975). Immunoglobulin G was prepared from each serum essentially as described by Walker & Mayer (1977).

Sepharose–avidin was prepared by activating Sepharose 4B [Pharmacia (G.B.) Ltd., London W.5, U.K.] with CNBr (Syska *et al.*, 1974) and then immediately incubating with avidin (Sigma, Kingston-upon-Thames, Surrey, U.K.) and subsequently with excess bovine serum albumin (Landman & Dakshinamurti, 1973).

[^{14}C]Biotin-labelled acetyl-CoA carboxylase was prepared by depriving rats of food for 48–72h, injecting each rat intraperitoneally with 15–20μCi of [*carbonyl*-^{14}C]biotin (The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.5ml of NaCl (90mg/ml) and then re-feeding them for 48h on a diet of meringues made from sucrose and egg white. The rats were stunned and decapitated, and the liver and epididymal fat-pads were removed and homogenized in sucrose medium (0.25M-sucrose, 100mM-Tris, 5mM-2-mercaptoethanol, 2mM-EDTA,

pH7.5). The homogenates were centrifuged at 150000*g* for 60min at 4°C and the supernatants incubated for 45min at 30°C with 10mM-potassium citrate. The polymerized acetyl-CoA carboxylase was then sedimented by centrifugation at 200000*g* for 60min at 20°C. Pellets were taken up in the sucrose medium. Preparations from both tissues had a specific activity of approx. 100munits/mg of protein (where 1 unit transforms 1 μmol of substrate/min at 30°C), were free of pyruvate carboxylase activity and gave a single ^{14}C-labelled band on SDS/polyacrylamide-slab-gel electrophoresis (Fig. 1). Purification of unlabelled fat-cell acetyl-CoA carboxylase to homogeneity as assessed by SDS/polyacrylamide-gel electrophoresis was accomplished by further chromatography on DEAE-cellulose and Sepharose 4B. The specific activity of such preparations varied between 2 and 3 units/mg of protein.

Acetyl-CoA carboxylase and fatty acid synthetase were assayed as described by Halestrap & Denton (1973). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

For the study of the incorporation of $^{32}P_i$ into fat-cell proteins the procedure of Hughes *et al.* (1977) was followed. Isolated fat-cells (approx. 200mg dry wt.) were incubated for 75 or 120min at 37°C in 2ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing albumin (10mg/ml), glucose (0.2mg/ml), $CaCl_2$ (1.25mM) and potassium [^{32}P]phosphate (0.2mM and approx. 1000d.p.m./pmol). When indicated, insulin (10m-i.u./ml) was added after 60min incubation. Cells were separated from the incubation medium by centrifugation through 2ml of dinonyl phthalate and subsequently broken by vortexing vigorously with ice-cold medium (0.25M-sucrose, 20mM-Tris, 7.5mM-GSH, 2mM-EGTA, pH7.4). After removal of fat by centrifugation at 1000*g* for 15s, the whole-cell extract was centrifuged at 10000*g* for 1min, after which EDTA (2mM) and NaF (20mM) were added to the supernatant. In some experiments, the supernatant was further centrifuged at 105000*g* for 60min at 4°C. Samples of the supernatants were incubated with sera or applied to Sepharose–avidin as described in the text and Figure legends.

SDS/polyacrylamide-gel electrophoresis of proteins in the various fractions was carried out in 1cm tracks on 5 or 7.5% (w/v) slab gels (14cm×14cm) as described by Hughes *et al.* (1977). Samples for electrophoresis were prepared by precipitation with 10% (w/v) trichloroacetic acid and dissolving the protein pellets by incubation for 5min at 100°C (and if necessary a further period of up to 60min at 60°C) in 200mM-sodium phosphate, pH7.0, containing SDS (40mg/ml), sucrose (200mg/ml), Bromophenol Blue (0.2mg/ml) and 2-mercaptoethanol (100mM). This procedure precluded the possibility of partial proteolysis (see Mackall & Lane, 1977). Dried slab gels were exposed to Kodak Kodirex KT X-ray film for 2–7 days and the radioautographs scanned at 625nm in a Gilford scanning spectrophotometer. (Exposure of the film was such that the peak heights did not exceed an absorbance of 1.0.)

Results and Discussion

Characterization of rat fat-cell acetyl-CoA carboxylase activity and its interaction with antiserum raised against the rabbit mammary-gland enzyme

Acetyl-CoA carboxylase purified from rat adipose tissue gave a single band on SDS/polyacrylamide-gel electrophoresis. The mobility of the band on both 7.5 and 5% gels was equivalent to mol.wt. about 230000, which is similar to that for the enzyme from rabbit and rat mammary glands (Mackall & Lane, 1977; R. Manning, R. Dils & R. J. Mayer, unpublished work). Whole-tissue extracts of adipose tissue from [*carbonyl*-^{14}C]biotin-treated rats contained two [^{14}C]biotin-labelled protein bands which were separable by electrophoresis and had mol.wts. of about 230000 and 150000. The lower-molecular-weight band was largely associated with the mitochondrial fraction and was assumed to be pyruvate carboxylase. After purification as described in the Methods section, the preparation contained only a single ^{14}C-labelled band corresponding to the molecular weight of acetyl-CoA carboxylase (Fig. 1*a*).

The antiserum raised against rabbit mammary-gland acetyl-CoA carboxylase reacted with the rat fat-cell enzyme. Incubation with antisera (20μl/ml of cell extract) for 30min at 30°C led to the loss of over 90% of the acetyl-CoA carboxylase activity without any appreciable loss of pyruvate carboxylase or fatty acid synthetase activity. Centrifugation (80000*g* for 30min) of cell high-speed supernatants after incubation with antiserum under those conditions led to precipitation of immuno-complexes containing protein of mol.wt. 230000. Moreover, if purified [^{14}C]-biotin-labelled acetyl-CoA carboxylase was added to such supernatants before incubation with antibody, 82.8±8.5% (mean±S.E.M. for four observations) of the ^{14}C-labelled protein was precipitated. Thus near-complete precipitation of the fat-cell enzyme could be achieved after only a short period of interaction with the antiserum, as long as high-speed centrifugation was used to sediment the immuno-complex. No precipitation of [^{14}C]biotin-labelled acetyl-CoA carboxylase was observed in the absence of antiserum.

Phosphorylation of fat-cell proteins

After incubation of fat-cells with $^{32}P_i$ for 75min, the whole-cell extract contained at least eight proteins labelled with ^{32}P, which could be clearly and reproducibly separated by SDS/polyacrylamide-gel electrophoresis (Hughes *et al.*, 1977; Fig. 1*b*). If insulin was added to the cell incubation medium for

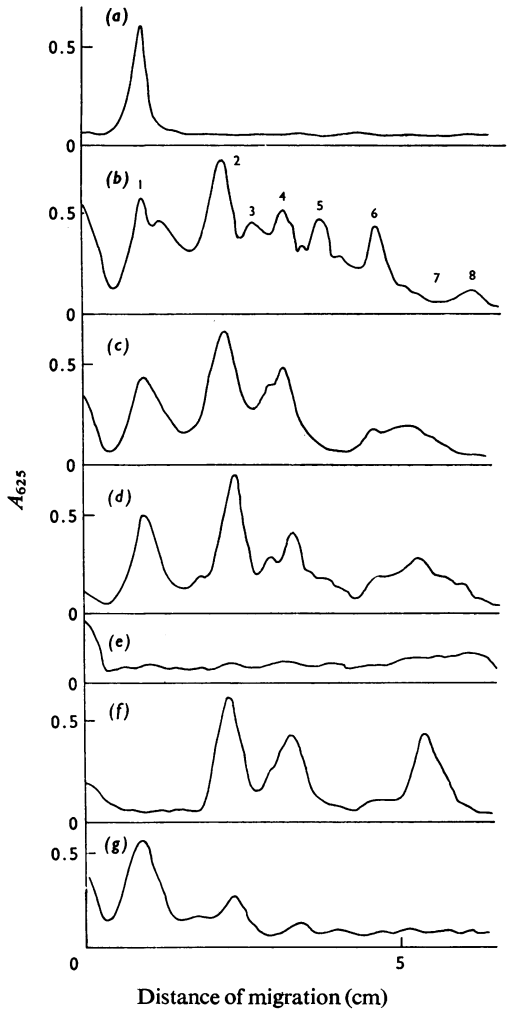

Fig. 1. *Densitometric traces of radioautographs locating the labelled proteins separated by SDS/polyacrylamide-gel electrophoresis (7.5% gel)*

(*a*) Purified [^{14}C]biotin-labelled acetyl-CoA carboxylase; (*b*) whole-cell extract of fat-cells after incubation of the cells with $^{32}P_i$ for 75min in the absence of insulin (the numbering of the labelled protein bands used in the text is also shown); (*c*) supernatant after centrifugation of the same whole-cell extract for 60min at 100000*g*; (*d*)–(*g*) specific precipitation of the ^{32}P-labelled protein from band 1 of the 100000*g* supernatant after interaction with antiserum to acetyl-CoA carboxylase. Samples of the supernatants were incubated at 30°C for 30min with 20μl/ml of either antiserum to acetyl-CoA carboxylase (*f*, *g*) or control serum (*d*, *e*). After centrifugation at 80000*g* for 30min at 4°C, proteins in equivalent samples of the pellet (*e*, *g*) and supernatant (*d*, *f*) fractions were separated on adjacent tracks by SDS/polyacrylamide-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10cm.

the last 15min of incubation, the extent of phosphorylation of bands 2 and 7 was increased (2–3-fold in each case), whereas, in contrast, that of band 6 (which corresponds to the α-subunit of pyruvate dehydrogenase) was significantly decreased (Hughes *et al.*, 1977). The apparent molecular weight of band 1 was close to that of fat-cell acetyl-CoA carboxylase. Moreover, both acetyl-CoA carboxylase activity and radioactivity associated with band 1 were exclusively in the supernatant after centrifugation of the whole-cell extract at 100000*g* for 60min (Fig. 1*c*). No further phosphorylation of band 1 was seen if incubation of the cells with $^{32}P_i$ was continued for a further 45min, and also the labelling of the band was unchanged by treatment of the protein pellet with 0.1M-NaOH for 15min at 20°C before electrophoresis.

Identity of band 1 with acetyl-CoA carboxylase

Addition of antiserum raised against acetyl-CoA carboxylase to 100000*g* supernatants prepared from fat-cells incubated with $^{32}P_i$ led to specific and near-complete precipitation of the $^{32}P_i$ associated with band 1. Little or no precipitation was found if control serum was added instead of antiserum (Figs. 1*d*–1*g*).

In some experiments, antiserum was added to 10000*g* supernatants prepared from fat-cells incubated in $^{32}P_i$ (Fig. 2). Again near-complete precipitation of ^{32}P in band 1 was seen after treatment with antiserum, but no precipitation occurred with control serum. The advantage of using the 10000*g* supernatant was that the fraction could be prepared within a few minutes of breaking the cells, whereas the 100000*g* supernatant took about 90min to prepare. On the other hand, labelled proteins in particulate components of the cell remaining in the 10000*g* supernatant were sedimented by the high-speed centrifugation at the end of the period of treatment with antiserum or control serum. However, it is evident that there are no differences in the amounts of ^{32}P-labelled proteins other than band 1 being sedimented in the presence of antiserum or control serum. Overall, in seven separate experiments conducted as described in either Fig. 1 or Fig. 2 with the use of both 7.5% and 5% gels, 82.9±6.2% (mean±S.E.M.) of the radioactivity associated with band 1 appeared in the pelleted immuno-complex after treatment with antiserum, compared with only 11.8±2.2% (five observations) after treatment with control serum and 6.0±4.7% (four observations) when no serum was added. The corresponding values for band 2 were 14.9±1.9% (seven observations), 14.8±3.3% (five observations) and 10.3±1.6% (four observations).

Further evidence that band 1 represented ^{32}P bound to acetyl-CoA carboxylase was obtained through the use of Sepharose–avidin. Samples (0.5ml) of 10000*g* supernatant of fat-cells incubated with $^{32}P_i$ were placed on a 1ml column of Sepharose–avidin and allowed to enter the gel. After 30min at

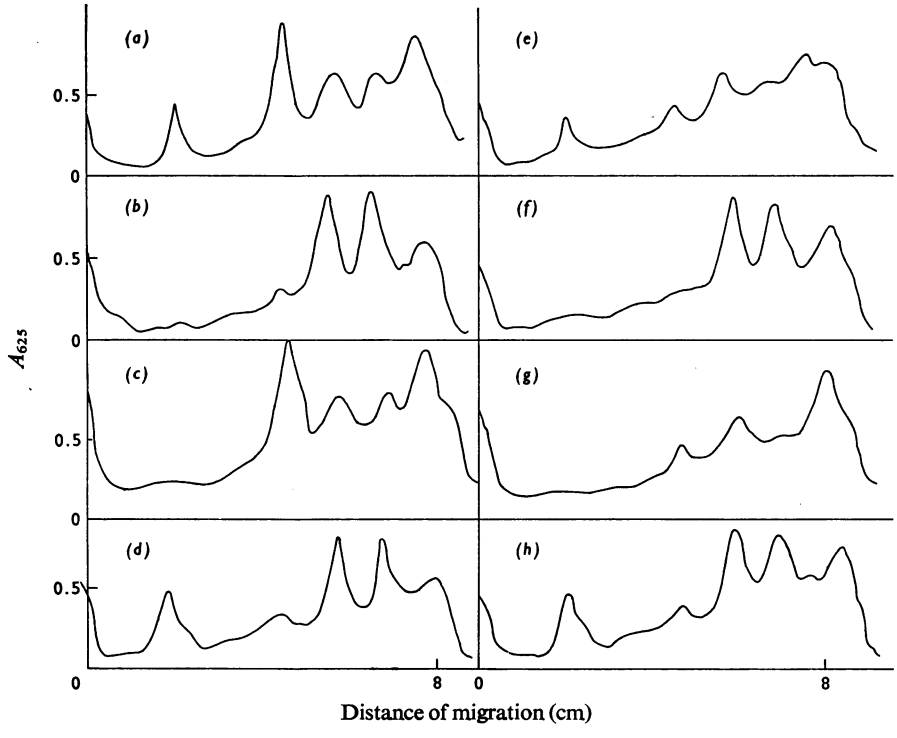

Fig. 2. *Densitometric traces of radioautographs demonstrating the specific precipitation of ^{32}P-labelled protein from band 1 of a 10000g supernatant of fat-cells after incubation with antiserum to acetyl-CoA carboxylase*

Fat-cells were incubated for 75min with $^{32}P_i$ with (*a–d*) or without (*e–h*) insulin (10m-i.u./ml) added for the last 15min. After centrifugation of the whole-cell extracts at 10000*g* for 1min, the supernatants were incubated for 30min at 30°C with either antiserum to acetyl-CoA carboxylase (*c*, *d*, *g* and *h*) or control serum (*a*, *b*, *e* and *f*) (20μl/ml in each case). Samples were then centrifuged at 80000*g* for 30min at 4°C, and the proteins from the supernatant (*a*, *c*, *e* and *g*) and pellet (*b*, *d*, *f* and *h*) fractions separated on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis (5% gel). The dye front (Bromophenol Blue) was allowed to migrate 10cm.

20°C, the column was washed with 50ml of sucrose extraction medium. The column was then treated with 8M-urea, and ^{32}P-labelled protein was eluted. This protein migrated as a single band on electrophoresis with the same R_F as band 1.

The molecular weights of subunits of fatty acid synthetase are likely to be close to 250000 (Stoops *et al.*, 1975; Lornitzo *et al.*, 1975) and thus migrate close to band 1. However, treatment of fractions of fat-cells with antiserum raised against fatty acid synthetase resulted in little or no sedimentation of ^{32}P-labelled components. The mean sedimentation of ^{32}P associated with band 1 was 12.0±4.2% (mean ±S.E.M. for three separate experiments); this is no higher than with control serum. Under these conditions, the fatty acid synthetase activity was decreased by 78±4% (mean±S.E.M. for five observations), but no decrease in acetyl-CoA carboxylase activity was observed.

Role of phosphorylation in the regulation of acetyl-CoA carboxylase

The above results show that acetyl-CoA carboxylase is phosphorylated within fat-cells and that the rate of phosphorylation is quite rapid, since ^{32}P labelling of the enzyme reached a plateau within 75min of incubation of cells with $^{32}P_i$. However, the relationship between phosphorylation and the regulation of acetyl-CoA carboxylase is much less clear-cut. The inhibition of acetyl-CoA carboxylase observed in extracts of rat adipose tissue on addition of ATP and Mg^{2+} may be the result of carboxylation rather than phosphorylation (Halestrap & Denton, 1974), and the same might be true for the inhibition observed by Carlson & Kim (1974*a*,*b*) in liver extracts.

In fat-cells the extent of phosphorylation of acetyl-CoA carboxylase does not appear to be significantly lowered when insulin is added to the medium for the

final 15min of incubation with $^{32}P_i$, yet under these conditions there is a doubling of the initial activity of acetyl-CoA carboxylase. There are also marked increases in the extent of phosphorylation of bands 2 and 7 and a decrease in that of the α-subunit of pyruvate dehydrogenase (band 6) (Hughes *et al.*, 1977). Combining the results of 14 different cell preparations, the effects of insulin addition on the peak heights of bands 1, 2 and 7 in densitometric traces of the protein of whole-cell extracts were 117 ± 10, 202 ± 15 and $450\pm60\%$ of control values respectively (results given as means$\pm$S.E.M. for 14 observations). With six of these cell preparations, high-speed supernatants were treated with antibody to acetyl-CoA carboxylase as described in Figs. 1 and 2. In fractions from insulin-treated cells, the peak height of band 1 sedimented with antiserum was $106\pm11\%$ of the control values. We have therefore not found any association between the change in the activity of acetyl-CoA carboxylase observed in the presence of insulin with changes in the $^{32}P_i$ content of the enzyme. The possibility of there being more than one class of phosphorylated sites remains to be explored.

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