Biochem. J. (1982) **202**, 77–86
Printed in Great Britain

Evidence that insulin activates fat-cell acetyl-CoA carboxylase by increased phosphorylation at a specific site

Roger W. BROWNSEY and Richard M. DENTON
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(*Received 14 July 1981/Accepted 25 September 1981*)

1. A new rapid method for the purification of fat-cell acetyl-CoA carboxylase is described; the key step is sedimentation after specific polymerization by citrate. 2. Incubation of epididymal fat-pads or isolated fat-cells with insulin or adrenaline leads to a rapid increase or decrease respectively in the activity of acetyl-CoA carboxylase measured in fresh tissue extracts. The persistence of the effect of insulin through high dilution of tissue extracts and through purification involving precipitation with $(NH_4)_2SO_4$ suggests that the enzyme undergoes a covalent modification after exposure of intact tissue to the hormone. The opposed effects of insulin and adrenaline are not adequately explained through modification of a common site on acetyl-CoA carboxylase, since these hormones bring about qualitatively different alterations in the kinetic properties of the enzyme measured in tissue extracts. 3. The state of phosphorylation of acetyl-CoA carboxylase within intact fat-cells exposed to insulin was determined, and results indicate a small but consistent rise in overall phosphorylation of the M_r-230000 subunit after insulin treatment. 4. Acetyl-CoA carboxylase from fat-cells previously incubated in medium containing [^{32}P]phosphate was purified by immunoprecipitation and then digested with performic acid and trypsin before separation of the released phosphopeptides by two-dimensional analysis. Results obtained show that the exposure of fat-cells to insulin leads to a 5-fold increase in incorporation of ^{32}P into a peptide which is different from those most markedly affected after exposure of fat-cells to adrenaline. 5. These studies indicate that the activation of acetyl-CoA carboxylase in cells incubated with insulin is brought about by the increased phosphorylation of a specific site on the enzyme, possibly catalysed by the membrane-associated cyclic AMP-independent protein kinase described by Brownsey, Belsham & Denton [(1981) *FEBS Lett.* **124**, 145–150].

Exposure of rat epididymal adipose tissue to insulin both *in vivo* and *in vitro* results in a 2–3-fold increase in the activity of acetyl-CoA carboxylase (EC 6.4.1.2) in fresh extracts of the tissue prepared in the absence of citrate (Halestrap & Denton, 1973, 1974; Lee *et al.*, 1973; Stansbie *et al.*, 1976; Brownsey & Denton, 1979). Activation of acetyl-CoA carboxylase by insulin has also been reported in brown adipose tissue *in vivo* (McCormack & Denton, 1977) and liver cells *in vitro* (Geelen *et al.*, 1978; Witters *et al.*, 1979*b*) and *in vivo* (Stansbie *et al.*, 1976). In contrast, exposure to hormones which give rise to increases in the cell concentration of cyclic AMP results in inactivation of acetyl-CoA carboxylase. The most extensively studied examples are exposure of fat-cells to adrenaline (Lee *et al.*, 1973; Halestrap & Denton, 1974; Lee & Kim, 1978, 1979; Brownsey *et al.*, 1979) and of liver cells to glucagon (Geelen *et al.*, 1978; Witters *et al.*, 1979*b*).

Cyclic AMP-dependent protein kinase has been shown to catalyse the phosphorylation and parallel inhibition of acetyl-CoA carboxylase purified from rabbit mammary gland (Hardie & Cohen, 1978*a*; Hardie & Guy, 1980), rat liver (Lee & Kim, 1977; Lent *et al.*, 1978) and rat epididymal fat-cells (Brownsey *et al.*, 1981), and treatment with phosphoprotein phosphatases has been shown to reverse these effects (Hardie & Cohen, 1979; Hardie & Guy, 1980; Krakower & Kim, 1980). Rapid incorporation of ^{32}P from phosphate in the medium into the enzyme within intact cells was initially demonstrated with rat epididymal fat-cells

Abbreviation used: SDS, sodium dodecyl sulphate.

0306-3283/82/010077-10$01.50/1 © 1982 The Biochemical Society

(Brownsey *et al.*, 1977) and subsequently in liver cells (Witters *et al.*, 1979*a*). Incubation of the fat-cells with adrenaline or the liver cells with glucagon leads to increases in the steady-state incorporation of ^{32}P into acetyl-CoA carboxylase (Brownsey *et al.*, 1979; Lee & Kim, 1979; Witters *et al.*, 1979*a*). Moreover, analysis of tryptic peptides derived from acetyl-CoA carboxylase suggests that the enzyme is phosphorylated at several sites and that those sites exhibiting increased phosphorylation after exposure of fat-cells to adrenaline are probably the same as sites on purified mammary-gland acetyl-CoA carboxylase that are phosphorylated by cyclic AMP-dependent protein kinase (Brownsey & Hardie, 1980). Thus there is strong evidence that increases in the concentration of cyclic AMP in fat-cells and liver cells can lead to phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase and that this phosphorylation causes inhibition of the enzyme, which can be reversed by dephosphorylation.

The present paper is concerned with establishing the importance of changes in phosphorylation in the activation of rat epididymal fat-cell acetyl-CoA carboxylase by insulin. Evidence is presented (i) that the effects of insulin on enzyme activity persist during purification, as expected if a covalent modification such as phosphorylation is involved, (ii) that the changes in kinetic properties of the enzyme after treatment of cells with insulin are not the strict reverse of the changes observed after exposure of fat-cells to adrenaline, (iii) that insulin causes a small but significant increase in the overall incorporation of ^{32}P from phosphate in the medium into acetyl-CoA carboxylase, and (iv) that this increase reflects the enhanced phosphorylation of possibly a single site on acetyl-CoA carboxylase that is separate from the site or sites that are phosphorylated to a greater extent after exposure of fat-cells to adrenaline. We conclude that the activation of acetyl-CoA carboxylase by insulin may involve the stimulation of a cyclic AMP-independent protein kinase. We have reported that fat-cells contain such a kinase, apparently associated with the plasma membranes, which is able to catalyse the phosphorylation and parallel activation of fat-cell acetyl-CoA carboxylase (Brownsey *et al.*, 1981). In addition, the present paper also describes in detail a new method for the purification of acetyl-CoA carboxylase from rat epididymal adipose tissue. The method is rapid and can be used to purify the enzyme from adipose tissue in good yield on a small scale. The key step is differential centrifugation after the specific polymerization of acetyl-CoA carboxylase in the presence of citrate.

Experimental

Materials

Sources of chemicals, biochemicals and animals (male Wistar rats, which were fed *ad libitum* up to the time of killing and which weighed 180–220g) have been described previously (Brownsey *et al.*, 1977, 1979). Monospecific antiserum to acetyl-CoA carboxylase was prepared as described by Walker *et al.* (1976) and was a gift from Dr. R. J. Mayer (Department of Biochemistry, University Hospital and Medical School, Nottingham, U.K.). Collagenase (lot no. SD4-22) was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A., and proteinase inhibitors were from the Peptide Research Institute, Osaka, Japan. [γ-^{32}P]ATP and [^{32}P]phosphate were from NEN, 6072 Dreieich, W. Germany, or the Radiochemical Centre, Amersham, Bucks., U.K. Stock solutions (adjusted to pH 7.4 with KOH) of EGTA and EGTA containing equimolar $CaCl_2$ were prepared as described by Denton *et al.* (1978). Incubation of fat-pads and isolated fat-cells and the extraction and assay of acetyl-CoA carboxylase have been described previously (Brownsey *et al.*, 1979).

Purification of acetyl-CoA carboxylase

Epididymal fat-pads from 10–20 rats were incubated for 30min at 37°C in bicarbonate-buffered medium containing glucose (11mM) and then for an

Table 1. *Rapid and small-scale purification of acetyl-CoA carboxylase from rat white adipose tissue*
Acetyl-CoA carboxylase was purified from epididymal fat-pads removed from 15 rats weighing 180–220g and fed *ad libitum* up to the time of killing. For details of methods used see the Experimental section.

		Activity of acetyl-CoA carboxylase			
Fraction	Protein total (mg)	Total (munits)	Sp. activity (munits/mg)	Recovery (%)	Purification (fold)
Fat-free infranant	117	767	6.7	100	1
25000*g* supernatant	87	1115	11.6	145	1.7
Resuspended $(NH_4)_2SO_4$ precipitate	20	900	45	117	6.7
160000*g* post-citrate precipitate	0.4	483	1185	63	177

additional 15 min in fresh medium of the same composition with hormone additions as required. Pads were blotted and extracted by using a Polytron (PT 20) homogenizer at setting 3 for 5–10 s at 0°C in medium (pH 7.4) containing sucrose (0.25 M), Tris/HCl (20 mM), EGTA (2 mM) and reduced glutathione (7.5 mM). Pads were extracted at 1 g/4 ml of medium, and a sample of the initial extract was centrifuged at 10000 **g** for 1 min (Eppendorf 3200 centrifuge) and the fat-free infranatant assayed immediately. Floating fat and particulate fractions were removed from the bulk of the initial extract by centrifugation (1000 **g** for 1 min and 25000 **g** for 30 min respectively at 4°C) and the following additions to the supernatant were made: potassium phosphate (20 mM), KF (20 mM), EDTA (10 mM) and proteinase inhibitors (pepstatin, antipain and leupeptin each at 0.5 mg/ml). Acetyl-CoA carboxylase was precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 35% saturation, and after incubation at 0°C for 20 min the precipitate was collected by centrifugation (25000 **g** for 25 min at 4°C), resuspended in 0.5–1 ml of the sucrose medium containing inhibitors of phosphatases and proteinases and dialysed against 100 vol. of the same medium for 1–2 h at 4°C. The concentrated enzyme solution was then centrifuged at 10000 **g** for 1 min to remove the bulk of the undissolved material and then for 10 min at 100000 **g** (20°C; Beckman air-driven ultracentrifuge). After incubation (30 min at 30°C in the presence of 20 mM-sodium citrate) the supernatant was centrifuged to precipitate polymerized acetyl-CoA carboxylase (160000 **g** for 20 min at 20°C). Precipitated acetyl-CoA carboxylase was resuspended and dialysed for a total of 8 h at 4°C against three changes of the sucrose medium to remove traces of citrate before assay. The results obtained with one such purification are shown in Table 1. The final product contained no detectable activity of fatty acid synthetase. Analysis by SDS/polyacrylamide-gel electrophoresis of the enzyme purified in this way indicated that 70–80% of the protein migrated with subunit M_r 230000. For the determination of alkali-labile phosphate content of acetyl-CoA carboxylase, the enzyme was further purified by precipitation with polyethylene glycol as described by Hardie & Cohen (1978*a*,*b*), except that the concentration of the latter used was 5% (w/v). The enzyme specific activity approached 1.5 unit/mg of protein, and SDS/polyacrylamide-gel electrophoresis indicated that more than 90% of the protein migrated with subunit M_r 230000. This final specific activity is very similar to the values reported for enzyme purified from mammary gland (Hardie & Cohen, 1979) and more recently from liver (Witters & Vogt, 1981), and suggests that proteolytic modification is avoided by the procedures described above.

Determination of alkali-labile phosphate in acetyl-CoA carboxylase

Groups of 30–50 pads were preincubated in bicarbonate-buffered medium containing glucose but no hormones for 30 min, and acetyl-CoA carboxylase was extracted and purified as described above, including the final precipitation with polyethylene glycol. After checking that the purity of the enzyme was greater than 90% as judged by SDS/polyacrylamide-gel electrophoresis, the remainder of the preparation (0.4–0.8 mg of protein) was used for the determination of phosphate as described by Nimmo & Cohen (1976). After the alkali digestion a sample was taken for determination of protein by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Determination of the total incorporation of [^{32}P]phosphate into acetyl-CoA carboxylase within intact fat-cells

Fat-cells (approx. 0.8 g dry wt.) were preincubated in bicarbonate-buffered medium (10 ml) containing albumin (10 mg/ml), glucose (1 mM) and [^{32}P]phosphate (0.2 mM; about 1000 d.p.m./pmol) for 60 min, and hormones were added as indicated for a further 15 min. Previous studies have shown that the incorporation of ^{32}P into acetyl-CoA carboxylase and other intracellular phosphoproteins has reached steady-state values at these times (Brownsey *et al.*, 1977, 1979). The determination of stoichiometry of incorporation of ^{32}P into acetyl-CoA carboxylase was carried out after isolation of the M_r-230000 subunit of the enzyme by immunoprecipitation followed by SDS/polyacrylamide-gel electrophoresis. These procedures have previously been shown to give greater than 90% recovery of acetyl-CoA carboxylase and show that the ^{32}P is exclusively associated with the M_r-230000 subunit of acetyl-CoA carboxylase (Brownsey *et al.*, 1977, 1979). The amount of protein (1–5 μg) migrating with M_r 230000 after gel electrophoresis was estimated by densitometric scanning of wet gels after standard staining and destaining procedures, and calculations were based on the absorbance of known amounts (range 1–10 μg) of purified fat-cell acetyl-CoA carboxylase separated on parallel tracks of the same gel. A linear relationship existed between absorbance and amount of purified acetyl-CoA carboxylase applied to the gels. After densitometry, the ^{32}P content of the same protein bands was determined by liquid-scintillation spectrometry, the gel fragments having been digested (3 h at 80°C in 200 μl of H_2O_2) and the dried residues resuspended (in 200 μl of 1 M-HCl plus 10 ml of scintillation liquid). Calculation of stoichiometry of incorporation of ^{32}P into acetyl-CoA carboxylase assumed that the specific radioactivity of the γ-phosphate of cell ATP was equivalent to that

measured for P_i in the medium. Attempts to measure the value directly by the techniques employed previously for fat-cell mitochondria (Hughes & Denton, 1976) have so far been unsuccessful. Given that the labelling of the phosphoproteins has reached a steady state, it seems unlikely that the specific radioactivity of the γ-phosphate of the cell ATP could be appreciably less than that of the phosphate in the medium.

Digestion of acetyl-CoA carboxylase by trypsin and analysis of tryptic peptides

Fat-cells were prepared by collagenase digestion of pads from 12–16 rats and divided equally into three or four batches each representing approx. 0.8 g dry wt. of cells. Each batch was then preincubated and incubated with appropriate additions of hormones, and the ^{32}P-labelled acetyl-CoA carboxylase was isolated by immunoprecipitation as described in the previous section. Immunoprecipitates were washed twice with 0.5 ml of ice-cold trichloroacetic acid (10%, w/v) and finally with chloroform/methanol (2:1, v/v) plus acid. The protein precipitated at the interface of the two layers was recovered by carefully removing both aqueous and organic phases. Polyacrylamide-gel electrophoresis was used to confirm that all ^{32}P was associated with protein of subunit M_r 230000. Recovery of label associated with this band to this step was greater than 80% of that in the original extract. Precipitated protein was suspended in 50 μl of performic acid (2 h at 0°C) and, after freeze-drying three times, dried residues were suspended in 50 mM-NH_4HCO_3 (pH 8.2) containing trypsin (100 μg/ml) and incubated for 3 h at 37°C. Brownsey & Hardie (1980) have shown previously that complete digestion is achieved in this time. The product was then freeze-dried twice before application of the entire digest in a minimal volume to thin-layer cellulose plates (Eastman–Kodak, no. 13255). Two-dimensional separation of peptides involved in the first dimension high-voltage electrophoresis at pH 3.6 (3 h at 400 V) in the solvent system pyridine/acetic acid/water (1:9:189, by vol.) and in the second dimension ascending chromatography with a solvent system of butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.). Dinitrophenyl-lysine was also added to the thin-layer plates as a marker. ^{32}P-labelled peptides were detected by radioautography for 2–7 days. In any one experiment, the chromatograms were exposed to a single piece of film (Kodak X-O-mat). Estimates of the relative amounts of ^{32}P in the peptides were made from determinations of the areas of the peaks in densitometric traces of the radioautographs. Corrections were made for the elliptical shape of some spots by making densitometric traces in two dimensions.

Expression of results

Activities of acetyl-CoA carboxylase are expressed as munits/g wet wt. of tissue (fat-pads) or munits/mg of protein (partially purified enzyme), where 1 unit transforms 1 μmol of substrate/min at the indicated temperature. Results throughout are given as means ± S.E.M. for the indicated numbers of observations made with separate preparations of enzyme, fat-cells or groups of fat-pads.

Results and discussion

Effects of insulin on activity of acetyl-CoA carboxylase

It has been well established in previous studies that incubation of epididymal fat-pads or isolated fat-cells for a few minutes in the presence of insulin leads to an increase in the activity of acetyl-CoA carboxylase measured in fresh tissue extracts (Halestrap & Denton, 1973, 1974; Lee *et al.*, 1973; Brownsey & Denton, 1979). The results given in Table 2 demonstrate that the effects of insulin on activity of acetyl-CoA carboxylase are largely apparent after partial purification involving precipitation with $(NH_4)_2SO_4$. There was still a small but significant effect after the selective sedimentation by centrifugation in the presence of citrate. Precautions were taken to minimize the likelihood of either limited proteolysis or dephosphorylation during the partial purification by the inclusion in all buffers of KF, potassium phosphate, EDTA and a mixture of pepstatin, antipain and leupeptin. The purification could be completed within 6–8 h and the specific activity was increased from 5–7 munits/mg of protein in the initial extract to 50–100 munits/mg after precipitation with $(NH_4)_2SO_4$ and to 500–1000 munits/mg after the citrate fractionation.

These results suggest that the enzyme may undergo some form of covalent modification when intact tissue is incubated with insulin. The partial preservation of the effects of insulin after sedimentation in the presence of citrate and subsequent dialysis also demonstrates that the complete abolition of the effect of insulin which follows the incubation of acetyl-CoA carboxylase in the presence of citrate (Halestrap & Denton, 1974) can be at least partly reversed on subsequent removal of the citrate.

The possibility has to be considered that insulin causes the dephosphorylation of the sites on acetyl-CoA carboxylase including those that exhibit increased phosphorylation in fat-pads exposed to adrenaline (Brownsey *et al.*, 1979; Brownsey & Hardie, 1980). The first evidence that we obtained which indicated that this was not the case came from a detailed examination of the changes in the activity of acetyl-CoA carboxylase in extracts incubated

Table 2. *Persistence of the effect of insulin on the activity of acetyl-CoA carboxylase through partial purification*
Epididymal fat-pads were preincubated in bicarbonate-buffered medium containing glucose (11mM) for 30min at 37°C and then transferred to fresh medium of the same composition and incubated for 15min in the presence and absence of insulin (0.1 μM). Acetyl-CoA carboxylase was then assayed (*a*) in the initial extract (supernatant after centrifugation at 10000*g* for 1min), (*b*) after purification of about 10-fold by $(NH_4)_2SO_4$ precipitation and (*c*) after purification of about 100-fold by a combination of $(NH_4)_2SO_4$ precipitation and citrate fractionation (see the Experimental section for details). Recovery of acetyl-CoA carboxylase activity (after citrate treatment) after the purification steps was 80–100% and 50–80% respectively. In each case the activity of acetyl-CoA carboxylase was assayed (i) before and (ii) after incubation with 20mM-sodium citrate for 20min at 30°C. As shown in previous studies (Halestrap & Denton, 1974), this latter treatment leads to expression of maximum activity and loss of the effect of insulin. Results are given as means ± S.E.M. for at least seven observations in each case. Effect of insulin: *$P<0.05$, **$P<0.01$.

	Activity of acetyl-CoA carboxylase from fat-pads incubated with no hormone	Effect of insulin (% of no-hormone value)
(*a*) Initial extract		
(i) Activity without citrate treatment (munits/g wet wt.)	19.1 ± 5.2	265 ± 38**
(ii) Activity after citrate treatment (munits/g wet wt.)	110 ± 2.6	115 ± 7.6
(i) as % of (ii)	17.6 ± 4.7	189 ± 26**
(*b*) After $(NH_4)_2SO_4$ precipitation (i) as % of (ii)	16.1 ± 2.1	168 ± 20*
(*c*) After $(NH_4)_2SO_4$ precipitation and citrate fractionation (i) as % of (ii)	19.0 ± 4.7	138 ± 14*

Table 3. *Effect of insulin or adrenaline on the activation of acetyl-CoA carboxylase by incubation of tissue extracts with potassium citrate before* (*a*) *or after* (*b*) *treatment of the extracts with Mg^{2+} and Ca^{2+}*
Epididymal fat-pads were incubated in bicarbonate-buffered medium containing glucose (11mM) for 30min at 37°C and for an additional 15min in fresh medium of the same composition in the presence of insulin (0.1 μM) or adrenaline (5 μM) or no hormone. Extracts were prepared and then incubated for 20min at 30°C in the presence of 0, 1mM- or 20mM-potassium citrate before assay of activity of acetyl-CoA carboxylase (*a*) immediately after preparation of the extracts or (*b*) after treatment of extracts with added 5mM-$MgCl_2$ and 10mM-EGTA plus 12mM-$CaCl_2$ (calculated free Ca^{2+} of 20–50 μM) for 60min at 30°C. Similar results were obtained after incubation at the various citrate concentrations for 10min and after treatment with Mg^{2+} and Ca^{2+} for 30min. Results are given as means ± S.E.M. for five separate experiments. Effect of hormone: *$P<0.05$, **$P<0.01$.

	Activity of acetyl-CoA carboxylase (munits/g wet wt.) in extracts of pads incubated with		
Treatment of extract	No hormone	Insulin	Adrenaline
(*a*) Incubation with			
0mM-citrate	34.1 ± 5.9	67.7 ± 6.6**	16.3 ± 2.5*
1mM-citrate	77.1 ± 9.3	101.2 ± 6.9	43.5 ± 5.0*
20mM-citrate	110.4 ± 2.6	116.6 ± 3.0	78.6 ± 8.7*
(*b*) Incubation with Mg^{2+} and Ca^{2+} and then			
0mM-citrate	27.7 ± 6.2	63.2 ± 8.8*	24.1 ± 5.4
1mM-citrate	94.5 ± 4.1	105.6 ± 5.0	84.0 ± 8.1
20mM-citrate	114.4 ± 0.7	120.0 ± 2.6	112.0 ± 2.2

with citrate or a combination of Mg^{2+} and Ca^{2+} (Table 3).

After incubation of tissue extracts with citrate, which results in the polymerization and activation of acetyl-CoA carboxylase, the effects of insulin are no longer apparent, whereas the effects of adrenaline persist (Table 3*a*). This confirms the results of earlier studies (Halestrap & Denton, 1973, 1974; Brownsey *et al.*, 1979). In contrast, incubation of extracts with Mg^{2+} and Ca^{2+} leads to the loss of the effect of adrenaline, but not that of insulin (Table 3*b*). Earlier studies have shown that under these conditions there is extensive but incomplete dephosphorylation of acetyl-CoA carboxylase (Brownsey *et al.*, 1979).

Perhaps the most straighforward interpretation of these results is that insulin and adrenaline bring about their effects on activity of acetyl-CoA carboxylase through covalent modifications of different sites on the enzyme.

Effects of insulin on the phosphorylation of acetyl-CoA carboxylase within intact fat-cells

The effects of insulin on the steady-state incorporation of phosphate into acetyl-CoA carboxylase can be determined by preincubating fat-cells in medium containing [^{32}P]phosphate until a steady-state extent of labelling is achieved after 1 h and then incubating for a further 15 min in the same medium in the presence or absence of insulin (0.1 μM); acetyl-CoA carboxylase can then be completely resolved from other labelled phosphoproteins by SDS/polyacrylamide-gel electrophoresis of the proteins in cell extracts (Brownsey *et al.*, 1977, 1979). In an earlier study, we found that insulin may cause a small increase (10–20%) in the ^{32}P associated with acetyl-CoA carboxylase, but the statistical significance of the difference was not established (Brownsey *et al.*, 1977; Brownsey & Denton, 1979). Further studies have now been performed, and the increase was found to be statistically significant (overall increase with insulin was $15 \pm 6.7\%$ above control value for a total of 22 determinations; $P < 0.05$ for effect of insulin). This increase is considerably less than that observed after exposure of fat-cells to adrenaline ($41 \pm 8\%$; Brownsey *et al.*, 1979).

The number of mol of [^{32}P]phosphate bound to each mol of acetyl-CoA carboxylase subunit has been estimated. Acetyl-CoA carboxylase from fat-cells incubated with medium containing [^{32}P]phosphate as above was separated from other proteins, including fatty acid synthetase (which has a very similar subunit M_r), by specific immunoprecipitation and the proteins in the precipitate were resolved by SDS/polyacrylamide-gel electrophoresis (see the Experimental section). The amount of acetyl-CoA carboxylase (as μg) was determined from the Coomassie Blue staining of the discrete protein band (M_r 230000) representing acetyl-CoA carboxylase, by using purified fat-cell acetyl-CoA carboxylase as standard. The number of mol of ^{32}P incorporated per mol of subunit was then calculated from the ^{32}P associated with the band (measured by liquid-scintillation spectrometry), the specific radioactivity of the extracellular [^{32}P]phosphate and the subunit M_r of 230000. The value obtained for the enzyme from fat-cells incubated in the absence of hormones was 0.6 ± 0.1 mol of ^{32}P/mol of enzyme subunit. The corresponding value for the enzyme from cells incubated with insulin (0.7 ± 0.1) was not significantly different, whereas that from cells incubated with adrenaline (0.85 ± 0.1) was significantly increased ($P < 0.05$). Results are expressed as means ± S.E.M. for six observations in each case and statistics calculated by paired *t* test.

Total alkali-labile phosphate was also determined in acetyl-CoA carboxylase purified from fat-pads incubated in the absence of hormones. The purification involved $(NH_4)_2SO_4$ precipitation, citrate fractionation plus polyethylene glycol precipitation and gave a product of more than 90% purity as assessed by SDS/polyacrylamide-gel electrophoresis (see the Experimental section). The purified enzyme was found to contain 1.8 ± 0.3 mol of phosphate/mol of M_r-230000 subunit (mean ± S.E.M. for six observations on separate preparations). This value is somewhat less than that reported for the enzyme purified from rabbit mammary gland (Hardie & Cohen, 1978*b*) and more recently from rat liver (Witters & Vogt, 1981), but similar to that previously found for the purified rat liver enzyme (Inoue & Lowenstein, 1972). It can be concluded that each subunit of acetyl-CoA carboxylase contains on average between 1 and 2 molecules of covalently bound phosphate which do not become labelled with ^{32}P during the time course of experiments *in vitro*. These phosphate molecules must turn over very slowly in intact fat-cells, since incubation of fat-cells with [^{32}P]phosphate for 120 min does not increase the amount of ^{32}P incorporation above that found after 60–75 min, namely about 0.6 mol/mol of enzyme subunit.

The possibility that insulin might alter the phosphorylation of a specific site on acetyl-CoA carboxylase was explored by the analysis of ^{32}P-labelled phosphopeptides released by digestion with trypsin. Acetyl-CoA carboxylase from cells incubated with [^{32}P]phosphate was isolated by immunoprecipitation, the tryptic peptides were separated by two-dimensional analysis on thin-layer cellulose plates and the ^{32}P-labelled phosphopeptides located by radioautography. Fig. 1 shows a set of radioautographs from a typical experiment in which the pattern of labelled phosphopeptides from acetyl-CoA carboxylase isolated from fat-cells incubated with and without insulin and adrenaline were compared. As found in a previous study (Brownsey & Hardie, 1980), several different labelled phosphopeptides were observed even in the enzyme isolated from cells incubated in the absence of hormones, indicating that ^{32}P is incorporated into several different sites in this enzyme. The major effect of adrenaline was to increase the incorporation of ^{32}P into a group of two to four poorly separated peptides which migrated close to dinitrophenyl-lysine. We have designated these peptides as the A-group. In contast, insulin resulted in a substantial increase in the incorporation of ^{32}P into apparently a single peptide (denoted as the I-group) which

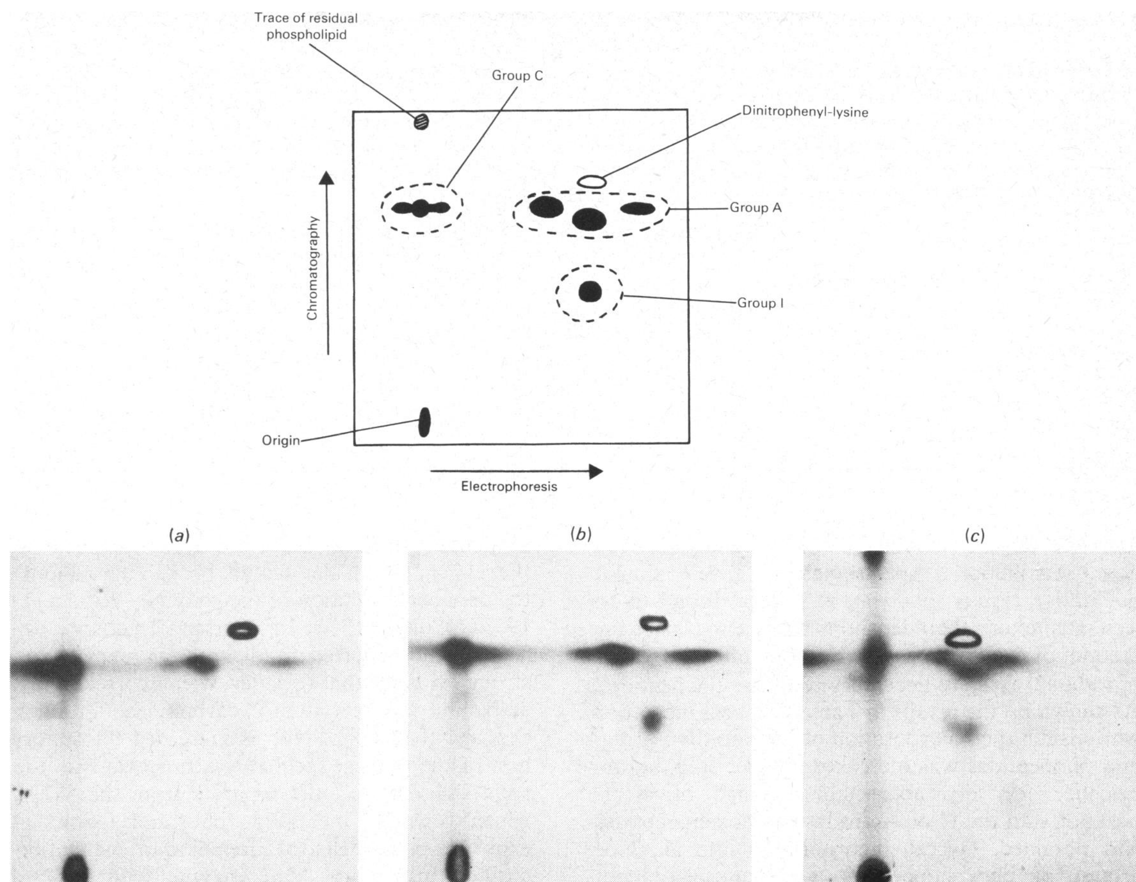

Fig. 1. *Two-dimensional separation of tryptic [^{32}P]phosphopeptides derived from acetyl-CoA carboxylase labelled within intact epididymal fat-cells*

Isolated fat-cells were preincubated in bicarbonate-buffered medium containing glucose (1 mM) and [^{32}P]phosphate for 1 h at 37°C and then for a further 15 min in the same medium with additions of insulin (0.1 μM) or adrenaline (5 μM) as appropriate. Acetyl-CoA carboxylase was isolated by immunoprecipitation and the washed immunoprecipitates were treated with performic acid and then trypsin (see the Experimental section). Peptides were separated on thin-layer cellulose plates by high-voltage electrophoresis in the first dimension and then by chromatography in the second dimension. (*a*)–(*c*) are radioautographs locating the [^{32}P]phosphopeptides derived from acetyl-CoA carboxylase after exposure of fat-cells to (*a*) no hormone, (*b*) insulin or (*c*) adrenaline. A key is shown which designates the groups of [^{32}P]phosphopeptides observed.

exhibited electrophoretic mobility very similar to that of the A-group peptides, but which was clearly resolved by chromatography. The R_F values for the A-group and I-group peptides in the chromatographic dimension were 0.65 ± 0.01 and 0.34 ± 0.03 respectively (means ± S.E.M. for ten observations, with mobility relative to the solvent front). With both insulin and adrenaline there was little change in the incorporation into the third group of two or three labelled peptides (denoted C-group), which migrated essentially only in the chromatographic dimension. Similar results to those shown in Fig. 1 were obtained in nine other separate experiments, and the results of all ten experiments are summarized in Table 4.

The relative incorporation into the three groups of phosphopeptides was estimated from densitometric traces of the radioautographs as described in the Experimental section, and results were expressed as a percentage of the incorporation of ^{32}P into the C-group of peptides derived from the enzyme from cells incubated without hormones in each experiment. It should be emphasized that the values should only be taken as an approximation. Direct deter-

Table 4. *Determination of the ^{32}P content of tryptic peptides derived from acetyl-CoA carboxylase labelled within intact epididymal fat-cells*

Ten separate experiments similar to that described (Fig. 1) were performed and the amounts of radioactivity associated with the groups of peptides designated C, I and A (key of Fig. 1) were determined by densitometric scanning of radioautographs. Values are means ± S.E.M. ($n = 10$) and are expressed as a percentage of the incorporation into group-C phosphopeptides in the absence of hormones. Significance of changes was calculated by paired t test. Effect of hormone versus no hormone: $^{*}P < 0.05$; $^{**}P < 0.01$; effect of both hormones against insulin alone: $^{\dagger}P < 0.05$; effect of both hormones against adrenaline alone: $^{\ddagger}P < 0.05$.

Phosphopeptide group	Relative incorporation of ^{32}P after incubation of fat-cells with			
	No hormone	Insulin	Adrenaline	Insulin plus adrenaline
C	100	98 ± 9	108 ± 16	118 ± 26
I	10 ± 4	51 ± 9**	26 ± 8*	63 ± 14*‡
A	92 ± 27	116 ± 24	228 ± 54*	274 ± 61*†
(C + I + A)	202 ± 28	267 ± 24*	359 ± 45*	445 ± 101*

mination of ^{32}P associated with the peptides by liquid-scintillation spectrometry gave similar qualitative results, but was in general found to be less satisfactory than densitometric tracing, since the amount of radioactivity was so small after the period of radioautography necessary to locate the peptides. As shown by the results in Table 4, in cells incubated with insulin the incorporation of ^{32}P into the I-group phosphopeptides was increased about 5-fold, but no evidence for any appreciable change of incorporation into the C or A groups of phosphopeptides was obtained. Overall incorporation into all three groups of phosphopeptides was increased about 30%, which is in fair agreement with the small increase in overall ^{32}P incorporation into the enzyme noted above. In contrast, treatment of cells with adrenaline alone caused a major increase in the incorporation into the A-group of peptides. This increase accounted for over 87% of the total increases in incorporation in all three groups of phosphopeptides, the rest being accounted for by an increase in incorporation into the I-group phosphopeptide. The lack of effects of insulin or adrenaline on the incorporation of ^{32}P into the C-group peptides indicates that the change in incorporation into the other peptides is most unlikely to be the result of either changes in specific radioactivity of intracellular ATP or differences in recovery of the peptides. The effects of insulin and adrenaline on the overall incorporation of ^{32}P into acetyl-CoA carboxylase appears to be additive, further supporting the action of the two hormones at different sites on the enzyme.

General conclusions

Ever since it became apparent that phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase could lead to a diminution in its activity, it has been expected that the effects of insulin would be brought about by the dephosphorylation of the enzyme (Witters *et al.*, 1979*b*; Cohen, 1980; Ingebritsen & Gibson, 1980). The results reported in the present paper provide strong evidence that this view was incorrect and that activation of acetyl-CoA carboxylase in fat-cells exposed to insulin involves increased phosphorylation of the enzyme, probably at a single site (the 'I' site). This site is quite separate from the 'A' sites, which exhibit increased phosphorylation after exposure of fat-cells to adrenaline or incubation of purified mammary-gland enzyme with ATP and cyclic AMP-dependent protein kinase (Brownsey & Hardie, 1980). We could find no evidence for any decrease in phosphorylation of the 'A' sites with insulin even in the presence of adrenaline. Insulin, in fact, brought about a modest overall increase in the incorporation of ^{32}P from medium in the phosphate into acetyl-CoA carboxylase within fat-cells, and this has also been reported with isolated liver cells (Witters, 1981).

Our results offer a reasonable explanation for the persistence through purification of the change in activity of acetyl-CoA carboxylase observed after treatment of cells with insulin and of the qualitative differences in enzyme properties brought about by insulin and adrenaline. In particular, the inactivation and increased phosphorylation of the enzyme in extracts of fat-cells previously exposed to adrenaline is reversed by incubation with Mg^{2+} and Ca^{2+}, whereas this treatment does not reverse the activation of the enzyme observed after exposure of the cells to insulin. We conclude that different phosphoprotein phosphatases probably catalyse the hydrolysis of phosphate groups on the 'A' and 'I' sites and that for some unknown reason the phosphatase which catalyses the hydrolysis of the 'I' site appears to be inactive in fat-cell extracts.

Many unanswered questions remain about the relationship between phosphorylation of the various sites on acetyl-CoA carboxylase and its catalytic activity. Since insulin has been shown to increase the proportion of the enzyme in fat-cells which is present in a high-molecular-weight form (Halestrap & Denton, 1974), it seems reasonable to suggest that phosphorylation at the 'I' site promotes polymerization and thus activation. This promotion could be brought about directly or through alterations in the response of the enzyme to effectors which can alter the degree of polymerization, including citrate, fatty acyl-CoA esters and free CoA (Lane *et al.*, 1974; Ogiwara *et al.*, 1978; Yeh *et al.*, 1981). The maximum occupancy of the 'I' site observed in the present study represents about 0.15 mol of phosphate/mol of M_r-230000 monomer. The maximum extent of phosphorylation of the 'A' sites was also below 0.5 mol/mol of monomer. These low values raise the interesting possibility that not all the apparently identical monomers may become phosphorylated, at least in the intact cell. This situation would not be unique, as a maximum of only 50% of the α-subunits of pyruvate dehydrogenase are phosphorylated in purified preparations of the pyruvate dehydrogenase complex (Sugden & Randle, 1978; Yeaman *et al.*, 1978).

Progress has been made towards recognition of the protein kinase that catalyses the phosphorylation of the 'I' site (Brownsey *et al.*, 1981). A protein kinase has been found associated with fat-cell plasma membranes which is able to phosphorylate purified fat-cell acetyl-CoA carboxylase, and in parallel with this phosphorylation there is an increase in enzyme activity. The protein kinase is clearly distinct from cyclic AMP-dependent protein kinase and appears to be active in the absence of added Ca^{2+}. Our present working hypothesis is that insulin activates this kinase perhaps by causing its release from the plasma membrane (Denton *et al.*, 1981; Brownsey *et al.*, 1981). Further support for this hypothesis may be offered by experiments which have demonstrated the stimulation of phosphorylation of ribosomal protein S6 (Rosen *et al.*, 1981) and a membrane protein of subunit M_r approx. 16000 (Walaas *et al.*, 1977, 1979) on the addition of insulin to broken cell preparations which contain plasma membranes.

Acetyl-CoA carboxylase is by no means the only protein in fat-cells which exhibits increased phosphorylation after exposure of the cells to insulin. Others include three intracellular proteins: one of M_r approx. 130000, which is probably ATP citrate lyase (Alexander *et al.*, 1979; Linn & Srere, 1979; Ramakrishna & Benjamin, 1979); one of M_r approx. 35000, which may be the ribosomal protein S6 (Hughes *et al.*, 1977, 1980; Smith *et al.*, 1979; Belsham *et al.*, 1980); and one of M_r approx. 22000, which appears to be a protein related to the phosphoprotein phosphatase inhibitor-1 (Belsham *et al.*, 1980; Belsham & Denton, 1980). Insulin also causes the increased phosphorylation of ATP citrate lyase and another protein of M_r 46000 in liver cells (Avruch *et al.*, 1978; Alexander *et al.*, 1979). Increases in the intracellular concentration of cyclic AMP (brought about by adrenaline in fat-cells or by glucagon in liver cells) also result in the increased phosphorylation of these proteins. We have suggested that the kinase responsible for the phosphorylation of the 'I' site of acetyl-CoA carboxylase may also phosphorylate these other phosphoproteins in fat and liver cells, but probably at sites different from those phosphorylated by cyclic AMP-dependent protein kinase (Brownsey *et al.*, 1981). It has been reported that exposure of fat-cells and 3T3-L1 preadipocytes to insulin leads to an increase in the activity of protein kinase in high-speed supernatants which is able to phosphorylate ATP citrate lyase and the ribosomal protein S6 respectively (Benjamin & Singer, 1975; Smith *et al.*, 1980). In both cases, there is evidence that the insulin-stimulated kinase may phosphorylate these proteins at different sites from those phosphorylated by cyclic AMP-dependent protein kinase (Ramakrishna & Benjamin, 1981; Lastick & McConkey, 1981). However, the physiological role of the phosphorylation of ATP citrate lyase, ribosomal protein S6 and the M_r-16000 membrane protein remains obscure, since, in contast with acetyl-CoA carboxylase, no changes in activity or function have yet been reported.

These studies were supported by grants from the Medical Research Council. We thank Ms. Stella Sage for skilled and enthusiastic technical assistance, Dr. A. P. Halestrap of this Department for computer programmes used in the analysis of densitometric traces and Dr. R. J. Mayer (Department of Biochemistry, University of Nottingham, U.K.) for the gift of antiserum to acetyl-CoA carboxylase.

References

Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T. & Avruch, J. (1979) *J. Biol. Chem.* **254**, 8052–8056

Avruch, J., Witters, L. A., Alexander, M. C. & Bush, M. P. (1978) *J. Biol. Chem.* **253**, 4754–4764

Belsham, G. J. & Denton, R. M. (1980) *Biochem. Soc. Trans.* **8**, 382–383

Belsham, G. J., Brownsey, R. W., Hughes, W. A. & Denton, R. M. (1980) *Diabetologia* **18**, 307–312

Benjamin, W. B. & Singer, I. (1975) *Biochemistry* **14**, 3301–3309

Brownsey, R. W. & Denton R. M. (1979) in *Obesity, Cellular and Molecular Aspects* (Ailhaud, G., ed.), pp. 195–212, INSERM, Paris

Brownsey, R. W. & Hardie, D. G. (1980) *FEBS Lett.* **120**, 67–70

Brownsey, R. W., Hughes, W. A., Denton, R. M. & Mayer, R. J. (1977) *Biochem. J.* **168**, 441–445
Brownsey, R. W., Hughes, W. A. & Denton, R. M. (1979) *Biochem. J.* **184**, 23–32
Brownsey, R. W., Belsham, G. J. & Denton, R. M. (1981) *FEBS Lett.* **124**, 145–150
Cohen, P. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 255–268, Elsevier, Amsterdam
Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
Denton, R. M., Brownsey, R. W. & Belsham, G. J. (1981) *Diabetologia* **21**, 347–362
Geelen, M. J. H., Beynen, A. C., Christiansen, R. Z., Lepreau-Jose, M. J. & Gibson, D. M. (1978) *FEBS Lett.* **95**, 326–330
Halestrap, A. P. & Denton, R. M. (1973) *Biochem. J.* **132**, 509–517
Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **142**, 365–377
Hardie, D. G. & Cohen, P. (1978*a*) *FEBS Lett.* **91**, 1–7
Hardie, D. G. & Cohen, P. (1978*b*) *Eur. J. Biochem.* **92**, 25–34
Hardie, D. G. & Cohen, P. (1979) *FEBS Lett.* **103**, 333–338
Hardie, D. G. & Guy, P. S. (1980) *Eur. J. Biochem.* **110**, 167–177
Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473
Hughes, W. A., Brownsey, R. W. & Denton, R. M. (1977) in *Phosphorylated Proteins and Related Enzymes* (Pinna, L., ed.), pp. 17–33, Information Retrieval, London
Hughes, W. A., Brownsey, R. W. & Denton, R. M. (1980) *Biochem. J.* **192**, 469–481
Ingebritsen, T. S. & Gibson, D. M. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 63–94, Elsevier, Amsterdam
Inoue, H. & Lowenstein, J. M. (1972) *J. Biol. Chem.* **247**, 4825–4832
Krakower, G. R. & Kim, K. H. (1980) *Biochem. Biophys. Res. Commun.* **92**, 389–395
Lane, M. D., Moss, J. & Polakis, S. E. (1974) *Curr. Top. Cell. Regul.* **8**, 139–195
Lastick, S. M. & McConkey, E. H. (1981) *J. Biol. Chem.* **256**, 583–585
Lee, K. H. & Kim, K. H. (1977) *J. Biol. Chem.* **252**, 1748–1751
Lee, K. H. & Kim, K. H. (1978) *J. Biol. Chem.* **253**, 8157–8161
Lee, K. H. & Kim, K. H. (1979) *J. Biol. Chem.* **254**, 1450–1453
Lee, K. H., Thrall, T. & Kim, K. H. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1133–1140
Lent, B. W., Lee, K. H. & Kim, K. H. (1978) *J. Biol. Chem.* **253**, 8149–8156
Linn, T. C. & Srere, P. A. (1979) *J. Biol. Chem.* **254**, 1691–1698
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McCormack, J. G. & Denton, R. M. (1977) *Biochem. J.* **166**, 627–630
Nimmo, H. G. & Cohen, P. (1976) *Eur. J. Biochem.* **68**, 21–30
Ogiwara, H., Tanabe, T., Nikawa, J. & Numa, S. (1978) *Eur. J. Biochem.* **89**, 33–41
Ramakrishna, S. & Benjamin, W. B. (1979) *J. Biol. Chem.* **254**, 9232–9236
Ramakrishna, S. & Benjamin, W. B. (1981) *FEBS Lett.* **124**, 140–144
Rosen, O. M., Rubin, C. S., Cobb, M. H. & Smith, C. J. (1981) *J. Biol. Chem.* **256**, 3630–3633
Smith, C. J., Wejksnora, P. J., Warner, J. B., Rubin, C. S. & Rosen, O. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2725–2729
Smith, C. J., Rubin, C. S. & Rosen, O. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2641–2645
Stansbie, D., Brownsey, R. W., Crettaz, M. & Denton, R. M. (1976) *Biochem. J.* **160**, 413–416
Sugden, P. H. & Randle, P. J. (1978) *Biochem. J.* **173**, 659–668
Walaas, O., Walaas, E., Lystad, E., Alertsen, A. L., Horn, R. S. & Fossum, S. (1977) *FEBS Lett.* **80**, 417–422
Walaas, O., Walaas, E., Lystad, E., Alertsen, A. L. & Horn, R. S. (1979) *Mol. Cell. Endocrinol.* **16**, 45–55
Walker, J. H., Betts, S. A., Manning, R. & Mayer, R. J. (1976) *Biochem. J.* **159**, 355–363
Witters, L. A. (1981) *Biochem. Biophys. Res. Commun.* **100**, 872–878
Witters, L. A. & Vogt, B. (1981) *J. Lipid Res.* **22**, 364–369
Witters, L. A., Kowaloff, E. M. & Avruch, J. (1979*a*) *J. Biol. Chem.* **254**, 245–248
Witters, L. A., Moriarty, D. & Martin, D. B. (1979*b*) *J. Biol. Chem.* **254**, 6644–6649
Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C. & Dixon, G. H. (1978) *Biochemistry* **17**, 2364–2370
Yeh, L. A. Song, C. S. & Kim, K. H. (1981) *J. Biol. Chem.* **256**, 2289–2296