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Adrenaline and the Regulation of Acetyl-Coenzyme A Carboxylase in Rat Epididymal Adipose Tissue

INACTIVATION OF THE ENZYME IS ASSOCIATED WITH PHOSPHORYLATION AND CAN BE REVERSED ON DEPHOSPHORYLATION

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1. Exposure of rat epididymal fat-pads or isolated fat-cells to adrenaline results in a decrease in acetyl-CoA carboxylase activity measured both in initial extracts and in extracts incubated with potassium citrate; in addition the concentration of citrate required to give half-maximal activation may also be increased. 2. Incorporation of $^{32}P_i$ into acetyl-CoA carboxylase within intact fat-cells was investigated and evidence is presented that adrenaline increases the extent of phosphorylation of the enzyme. 3. Dephosphorylation of ^{32}P-labelled acetyl-CoA carboxylase was studied in cell extracts. The rate of release of ^{32}P is increased by 5mM-$MgCl_2$ and further enhanced by 5mM-$MgCl_2$ plus 10–100μM-Ca^{2+}, whereas it is inhibited by the presence of bivalent metal ion chelators such as EDTA and citrate. 4. The effects of adrenaline on the kinetic properties of acetyl-CoA carboxylase disappear if pad or cell extracts are treated with Mg^{2+} and Ca^{2+} under conditions that also lead to dephosphorylation of the enzyme. 5. The results of this study represent convincing evidence that adrenaline inactivates acetyl-CoA carboxylase in adipose-tissue preparations by increasing the degree of phosphorylation of the enzyme.

Acetyl-CoA carboxylase (EC 6.4.1.2) appears to be an important site for the hormonal regulation of fatty acid synthesis in rat epididymal adipose tissue. Brief exposure of the tissue to insulin, which greatly increases rates of fatty acid synthesis from glucose, results in a 2–3-fold increase in the initial activity of the enzyme measured in freshly prepared extracts (Halestrap & Denton, 1973, 1974; Stansbie *et al.*, 1976). In contrast, exposure to adrenaline, which decreases rates of fatty acid synthesis, results in a diminution of initial activity in both the presence (Halestrap & Denton, 1974) and absence (Lee & Kim, 1978) of insulin. These effects appear to be, at least in part, the result of alterations in the proportion of the enzyme occurring in its active polymeric form (Halestrap & Denton, 1974). Such alterations could be due to changes in the cytoplasmic concentrations of citrate or long-chain acyl-CoA, since these metabolites promote polymerization and depolymerization of the enzyme respectively (Lane *et al.*, 1974; Ogiwara *et al.*, 1978). Evidence has been presented that indicates that changes in citrate concentrations are unlikely to be important in the regulation of acetyl-CoA carboxylase activity in rat epididymal adipose tissue (Halestrap & Denton, 1974; Brownsey *et al.*, 1977*a*), but it is possible that a lowering of the concentration of long-chain acyl-CoA may be involved in the effects of insulin on the enzyme (Halestrap & Denton, 1974).

It is becoming increasingly evident that the activity of acetyl-CoA carboxylase in mammalian tissue may be regulated by phosphorylation. The rapid phosphorylation of acetyl-CoA carboxylase within intact rat epididymal fat-cells has been demonstrated (Brownsey *et al.*, 1977*b*), but no change in the extent of phosphorylation of the enzyme was detected after exposure of the cells to insulin.

Studies by Kim and his colleagues have shown that phosphorylation and inactivation of the rat liver enzyme may be achieved by incubation of partially purified preparations of the enzyme in the presence of $MgATP^{2-}$ (Carlson & Kim, 1974*a*,*b*; Lee & Kim, 1977).

Subsequent studies have shown that cyclic AMP will stimulate phosphorylation of the liver enzyme and that such phosphorylation is accompanied by depolymerization of the enzyme into less-active intermediate and protomeric forms (Lent *et al.*, 1978). These studies may offer an explanation for the inhibition of fatty acid synthesis observed after treatment of rat liver preparations with glucagon and/or dibutyryl cyclic AMP (Cook *et al.*,

Abbreviations used: SDS, sodium dodecyl sulphate; Mops, 4-morpholinepropanesulphonic acid.

1977; Watkins *et al.*, 1977). Evidence that treatment of rat liver cells with glucagon leads to increased phosphorylation of acetyl-CoA carboxylase has been presented (Witters *et al.*, 1979). However, Pekala *et al.* (1978) were unable to find any effects of dibutyryl cyclic AMP on the degree or rate of phosphorylation of acetyl-CoA carboxylase in chick liver cells. Hardie & Cohen (1978*a*) have found that acetyl-CoA carboxylase purified from rabbit mammary gland may be phosphorylated not only by skeletal-muscle cyclic AMP-dependent protein kinase but also by other protein kinases that remain associated with the enzyme during purification.

Direct evidence is presented in this paper that the inactivation of acetyl-CoA carboxylase that follows treatment of adipose tissue with adrenaline is associated with an increase in the phosphorylation of the enzyme in intact fat-cells. It is also shown that the effects of adrenaline on enzyme activity may be reversed by the action of bivalent-metal-ion-dependent phosphoprotein phosphatase activity present in epididymal adipose-tissue extracts.

Experimental

Chemicals and materials

Sources of chemicals, biochemicals and rats and the preparation of incubation media and isolated fat-cells were as described by Severson *et al.* (1976). 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.). Adrenaline was obtained as the bitartrate salt from BDH (Poole, Dorset, U.K.). Collagenase (lot number 504-19) was obtained from P.-L. Biochemicals, Milwaukee, WI, U.S.A. Cellophane (grade 325-PV-39) was a gift from British Cellophane, Bridgwater, Somerset, U.K. Stock solutions (adjusted to pH 7.4 with KOH) of EGTA and EGTA containing equimolar $CaCl_2$ were prepared and checked as explained by Denton *et al.* (1978).

Incubation of fat-pads and isolated fat-cells

All preincubations and incubations were carried out at 37°C in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.25mM-$CaCl_2$ with the stated additions of glucose and hormones, and gassed with O_2/CO_2 (19:1). Albumin (10mg/ml) was also present in media used for the incubation of isolated fat-cells. At the end of the incubation period, fat-pads were briefly blotted and then frozen by plunging into liquid N_2, and isolated fat-cells were separated from the incubation medium by brief centrifugation (1000*g* for 15s) before also being frozen with liquid N_2.

Extraction and assay of acetyl-CoA carboxylase

Frozen fat-cells and fat-pads were extracted in medium, pH 7.4, containing sucrose (0.25M), Tris (20mM), Mops (20mM), dithiothreitol (1mM) and EGTA (2mM). Extractions were carried out in a Polytron (PT20) homogenizer at setting number 3 for 5–10s at 0°C. Defatted bovine albumin (10mg/ml) was added and the extracts were centrifuged at 10000*g* for 30s in an Eppendorf 3200 minifuge. Acetyl-CoA carboxylase was assayed in samples of the infranatant below the floating fat essentially as described by Halestrap & Denton (1973), except that the assay time was decreased to 2min. The assays were initiated by adding a sample (50μl) of infranatant to 0.45ml of 100mM-Tris/HCl, pH 7.4, containing EDTA (0.5mM), $MgSO_4$ (10mM), ATP (2.5mM), acetyl-CoA (150μM), dithiothreitol (1mM), albumin (10mg/ml), $KH^{14}CO_3$ (15mM and 0.5μCi/μmol) and with additions of citrate or magnesium citrate as indicated.

Incorporation of $^{32}P_i$ into acetyl-CoA carboxylase and other proteins in fat-cells

The techniques for incubation and extraction of fat-cells, SDS/polyacrylamide-gel electrophoresis, radioautography and densitometric scanning of radioautographs were very similar to those described by Brownsey *et al.* (1977*b*). Essential details were as follows. Fat-cells were incubated in bicarbonate buffered medium (Krebs & Henseleit, 1932) containing $CaCl_2$ (1.25mM), albumin (10mg/ml), glucose (1mM) and $^{32}P_i$ (0.2mM and 500–1500d.p.m./pmol) for 75min at 37°C. Where appropriate, adrenaline was added after 60min. Fat-cells were then separated from the medium and extracted in medium, pH 7.4, containing sucrose (0.25M), Tris (20mM), Mops (20mM), reduced glutathione (7.5mM) and EGTA (2mM).

Separation of proteins was carried out by SDS/polyacrylamide-gel electrophoresis in 1cm tracks on 7.5% (w/v) slab gels (14cm × 14cm) using solutions described by Laemmli (1970). Samples for electrophoresis were prepared by precipitation with 10% (w/v) trichloroacetic acid and dissolving the protein pellets in stacking-gel buffer containing 40mg of SDS/ml, 200mg of sucrose/ml, 0.2mg of Bromophenol Blue/ml and 100mM-2-mercaptoethanol by heating at 100°C for 5min and further at 60°C if necessary. After electrophoresis, the protein bands were made visible by staining with Coomassie Blue, and the gels laid on Cellophane and dried on boards under vacuum. The dried gels were exposed to Kodak Kodirex KT X-ray film for 2–7 days and the radioautographs scanned at 630nm. Exposure of the film was such that peak heights did not exceed an absorbance of 1.0. The stained protein bands in the

dried gels were also scanned (at 600nm). Coinciding with band 1 of the separated ^{32}P-labelled phosphoproteins (see Fig. 1) there is a major protein band that includes the subunits of both acetyl-CoA carboxylase and fatty acid synthetase (Brownsey *et al.*, 1977*b*; Hardie & Cohen, 1978*b*). The intensity of staining of this band with Coomassie Blue was used as the basis for making small corrections (less than 10%) for differences in loading of samples from control and adrenaline-treated cells applied to the same slab gel.

Immunoprecipitation of acetyl-CoA carboxylase was carried out by using high-speed supernatants of cell extracts centrifuged for 60min at 105000*g* at 4°C. No acetyl-CoA carboxylase is spun down under these conditions (Brownsey *et al.*, 1977*b*). The supernatants were incubated for 60min at 30°C in the presence of antiserum to acetyl-CoA carboxylase (15μl of antiserum/ml of supernatant) and the immune precipitates collected by centrifugation at 80000*g* for 30min at 4°C. For these experiments EDTA (2mM) and NaF (20mM) were added immediately after preparation of the cell extracts.

Expression of results

Activities of acetyl-CoA carboxylase are expressed as munits/g wet wt. (fat-pads) or munits/g dry wt. (fat-cells), where 1 unit transforms 1μmol of substrate/min at 30°C. Results throughout are given as means±S.E.M. The indicated number of observations refers in all cases to observations made on separate preparations of fat-cells or groups of fat-pads.

Results and Discussion

Effects of adrenaline on acetyl-CoA carboxylase activity in rat epididymal adipose-tissue preparations

In our past studies, we have either assayed acetyl-CoA carboxylase activity immediately after preparation of the extract or after incubation of extracts with potassium citrate (20mM) for 20–30min. We have referred to these activities as 'initial' and 'total' respectively. Insulin was found to greatly increase the former without altering the latter (Halestrap & Denton, 1974; Stansbie *et al.*, 1976). Similar effects of insulin were found in the studies reported in Table 1. In contrast, adrenaline decreased the activity both in initial extracts and in extracts after incubation with potassium citrate (20mM). Significant decreases were found with adrenaline both with intact fat-pads incubated in the presence or absence of insulin and in isolated fat-cells (Table 1). The results shown in Table 1 were obtained after 15min exposure to hormones. Very similar effects of adrenaline were observed after 30 and 60min of incubation; inhibitory effects of adrenaline were also apparent after incubating fat-pads with adrenaline for 7min, but the effects were smaller than those found at 15min and thereafter.

Studies were also carried out to ensure that the effect of adrenaline on activity was still present after longer periods of exposure to potassium citrate. In fact, time courses showed that the increase in acetyl-CoA carboxylase activity after incubation with potassium citrate was maximal in extracts from both control and adrenaline-treated tissue after 10–15min, and the activity then remained essentially constant

Table 1. *Effects of adrenaline on the activity of acetyl-CoA carboxylase in rat epididymal adipose-tissue preparations*
Fat-pads were preincubated for 30min in bicarbonate-buffered medium containing glucose (11mM), and then transferred to fresh medium containing additions of insulin (0.1μM) and adrenaline (5μM), as indicated, and incubated for a further 15min. Isolated fat-cells were preincubated for 15–30min in bicarbonate-buffered medium containing glucose (1mM) and albumin (10mg/ml) and then incubated in fresh medium with or without adrenaline (5μM) for 15min. Pads and cells were then frozen with liquid N_2 and extracts prepared. Acetyl-CoA carboxylase activity was assayed immediately after preparation of the extracts or after incubation of the extracts at 30°C with added potassium citrate (20mM) for 20min. For further details see the Experimental section. Results are given as means±S.E.M. for the numbers of determinations in parentheses. * Effect of adrenaline, $P<0.01$; † effect of insulin, $P<0.01$.

		Acetyl-CoA carboxylase activity (munits/g)	
Tissue preparation	Additions to incubation medium	Initial extract assayed in the absence of citrate	Extract incubated with potassium citrate
Fat-pads	None	17.6±4.7 (6)	110±2.6 (6)
	Adrenaline	5.1±2.2* (6)	79±8.7* (6)
	Insulin	39.8±6.0† (6)	120±3.8 (6)
	Insulin+adrenaline	12.4±6.0* (3)	95±5.6* (3)
Isolated fat-cells	None	16.2±0.6 (3)	135±4.8 (5)
	Adrenaline	10.0±0.6* (3)	91±3.2* (5)

for at least a further 40min of incubation with potassium citrate.

The finding that adrenaline treatment results in a less active form of acetyl-CoA carboxylase even after extensive incubation of extracts with high concentrations of potassium citrate is in general agreement with the results of Lee & Kim (1978), which were published after the completion of the above studies.

Effects of adrenaline on incorporation of $^{32}P_i$ into acetyl-CoA carboxylase in isolated fat-cells

When fat-cells are incubated with $^{32}P_i$, label is incorporated into a number of phosphoproteins which can be resolved by SDS/polyacrylamide-gel electrophoresis (Benjamin & Singer, 1975; Avruch *et al.*, 1976*a*,*b*; Forn & Greengard, 1976; Hughes *et al.*, 1977; Brownsey *et al.*, 1977*b*; Benjamin & Clayton, 1978). Fig. 1 shows densitometric traces of radioautographs obtained in a typical experiment in which the effects of adrenaline on the incorporation of ^{32}P into fat-cell phosphoproteins were investigated. Altogether eight such experiments were conducted and the combined results are summarized in Table 2. Adrenaline treatment caused significant increases in incorporation of ^{32}P into the bands that we have designated 1, 2 and 4A (approx. mol.wts. of 230000, 130000 and 65000) by about 40, 80 and 200% respectively. In these experiments, where necessary, a correction was used to allow for small differences in the amount of protein applied to the gel. Without these corrections the increase in incorporation into

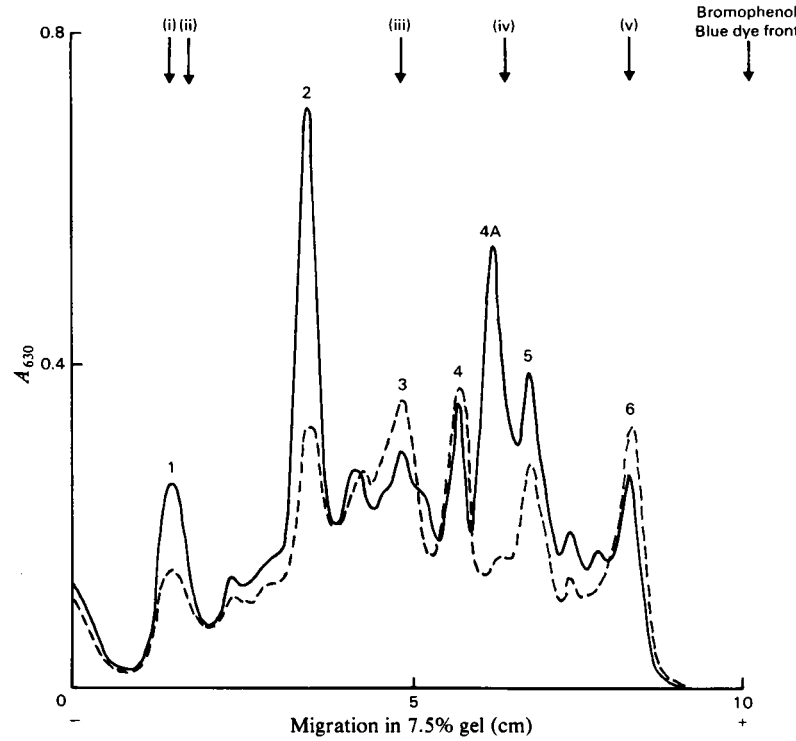

Fig. 1. *Densitometric traces of radioautographs obtained in a typical experiment in which the effects of adrenaline on the incorporation of ^{32}P from medium P_i into acetyl-CoA carboxylase and other phosphoproteins was studied*

Isolated fat-cells were incubated for 1 h in bicarbonate-buffered medium containing glucose (1 mM), albumin (10mg/ml) and $^{32}P_i$ (0.2mM and 1000 d.p.m./pmol) and then for a further 15min in the same medium with or without adrenaline (5μM). Preparation of whole-cell extracts, separation of proteins in the extracts by SDS/polyacrylamide-slab-gel electrophoresis and location of labelled phosphoproteins by radioautography was as described in the Experimental section. Traces of radioautographs from adrenaline-treated (——) and control (– – – –) cells are shown. Protein associated with band 1 was the same in each case. The numbering of the labelled protein bands used in the text and Table 2 is also shown together with the migration of marker proteins: (i) purified rat epididymal adipose-tissue acetyl-CoA carboxylase; (ii) rabbit skeletal-muscle myosin (mol.wt.210000); (iii) rabbit skeletal-muscle phosphorylase (mol.wt.98000); (iv) bovine serum albumin (mol.wt.68000); (v) α-subunit of pig heart pyruvate dehydrogenase (mol.wt.41000).

Table 2. *Effect of adrenaline on incorporation of ^{32}P into acetyl-CoA carboxylase and other phosphoproteins in isolated fat-cells*

Results are taken from eight separate experiments carried out as described in the legend to Fig. 1. Peak heights from the densitometric traces of radioautographs of phosphoproteins separated by SDS/polyacrylamide-gel electrophoresis from fat-cells incubated with adrenaline are expressed as % of control values. As described in the Experimental section, a correction (less than 10% in all cases) was applied to allow for small differences in protein associated with band 1. Results are shown as means ± S.E.M. for eight paired observations: *effect of adrenaline, $P<0.01$.

Band no.	R_f	Effect of adrenaline on peak height (% of control)
1 (Acetyl-CoA carboxylase)	0.15	141 ± 7.5*
2	0.35	181 ± 17.4*
3	0.48	100 ± 7.8
4	0.56	103 ± 5.9
4A	0.60	306 ± 53*
5	0.66	121 ± 9.7
6	0.80	95 ± 10.8

band 1 was 149±11% (mean±S.E.M. for eight observations) and that into band 2 was 190±18%. Adrenaline had little or no effect on incorporation into the other major bands (3, 4 and 5). The extents of incorporation observed in these experiments represent steady-state labelling of the phosphoproteins, since no further changes in the amounts of radioactivity associated with any of the major bands was observed in incubations of fat-cells with $^{32}P_i$ continued for a further 45min (see also Brownsey *et al.*, 1977*b*).

Our previous studies showed that addition of antiserum raised against acetyl-CoA carboxylase led to complete and specific precipitation of ^{32}P associated with band 1 (Brownsey *et al.*, 1977*b*). Thus the results in Table 2 indicate that adrenaline increases the phosphorylation of acetyl-CoA carboxylase in fat-cells. However, it seemed important to ensure that the increased ^{32}P associated with band 1 in cells exposed to adrenaline was also completely precipitated by antiserum raised against acetyl-CoA carboxylase. Our earlier studies had shown this to be the case only after incubation of cells in the absence of hormones and in the presence of insulin. Results

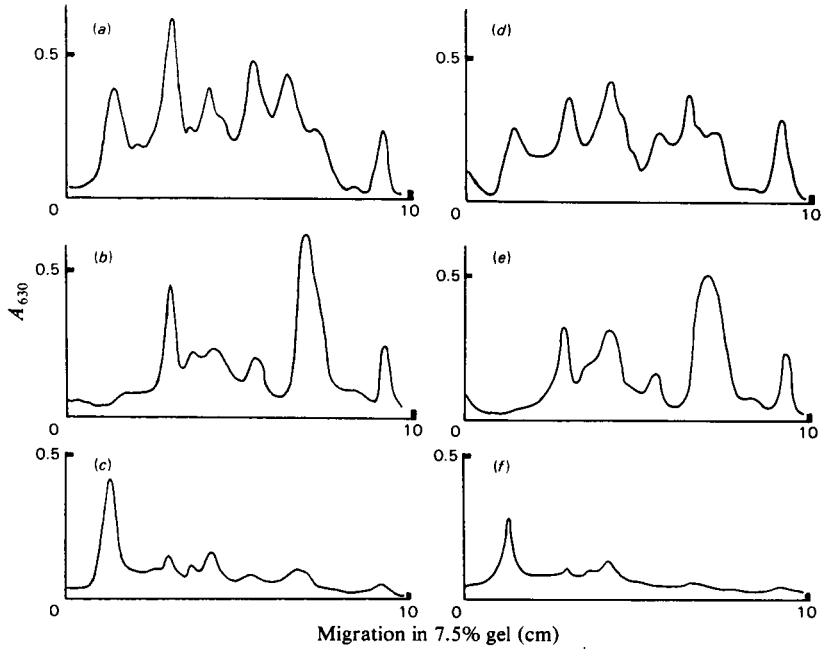

Fig. 2. *Densitometric traces of radioautographs demonstrating the specific and complete precipitation by antiserum to acetyl-CoA carboxylase of ^{32}P-labelled protein from band 1 of a 100000g supernatant of fat-cells incubated with or without adrenaline*

Fat-cells were incubated with (*a–c*) or without (*d–f*) adrenaline as described in legend to Fig. 1. Cell extracts were centrifuged at 105000*g* for 60min at 4°C, and supernatants incubated for 60min at 30°C with antiserum to acetyl-CoA carboxylase (15μl/ml). Samples were then centrifuged at 80000*g* for 30min at 4°C, and the protein from equivalent samples of cell extracts (*a*, *d*), antibody supernatants (*b*, *e*) and antibody pellets (*c*, *f*) was separated on adjacent tracks by SDS/polyacrylamide-slab-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10cm.

from one typical experiment demonstrating the complete and specific immunoprecipitation in cells incubated with or without adrenaline is shown in Fig. 2. Overall, in five experiments conducted as described in Fig. 2, 91±1% (mean±S.E.M.) of the radioactivity associated with band 1 appeared in the pelleted immunocomplex after exposure of cells to adrenaline compared with 88±1% for samples from control cells. The corresponding values for band 2 were 10.4±1.4% and 9.6±0.5% respectively.

The increased incorporation of $^{32}P_i$ into phosphoproteins of mol.wt. about 130000 and 65000 (bands 2 and 4A) in cells exposed to adrenaline has been described previously (Benjamin & Singer, 1975; Avruch *et al.*, 1976*b*; Forn & Greengard, 1976; Benjamin & Clayton, 1978). The role of these phosphoproteins is unknown; insulin also causes increased phosphorylation of band 2, but appears to have no discernible effect on the phosphorylation of band 4A (Benjamin & Singer, 1975; Avruch *et al.*, 1976*b*; Forn & Greengard, 1976; Hughes *et al.*, 1977; Brownsey *et al.*, 1977*b*). The results of Avruch *et al.* (1976*a*,*b*) and Forn & Greengard (1976) also suggest that adrenaline may increase the phosphorylation of a phosphoprotein of mol.wt. about 230000 (corresponding to acetyl-CoA carboxylase). The increases were not remarked upon by the authors at the time presumably because the identity of the phosphoprotein was not realized and the changes were less evident than those in phosphoproteins of mol.wt. about 130000 and 65000.

So far, the results presented in this paper show that the effects of adrenaline on acetyl-CoA carboxylase activity are associated with an increase in phosphorylation of the enzyme in fat-cells. In order to substantiate further the link between phosphorylation and activity, we have investigated the conditions required for the dephosphorylation of prelabelled acetyl-CoA carboxylase in cell extracts. Our aim was then to show that treatment of extracts under conditions optimal for dephosphorylation led to the reversal of the effects of adrenaline on acetyl-CoA carboxylase activity.

Dephosphorylation of acetyl-CoA carboxylase in cell extracts

In these experiments, fat-cells were first incubated in the presence of $^{32}P_i$ for 75min to achieve maximum incorporation of ^{32}P into acetyl-CoA carboxylase. Cell extracts were then prepared in the sucrose-based medium (which contains 2mM-EGTA) and the extracts centrifuged at 10000*g*-min. Samples were then incubated at 30°C with various additions, and changes in the phosphorylation of acetyl-CoA carboxylase determined after separation of the labelled phosphoproteins by SDS/polyacrylamide-gel electrophoresis (Fig. 3).

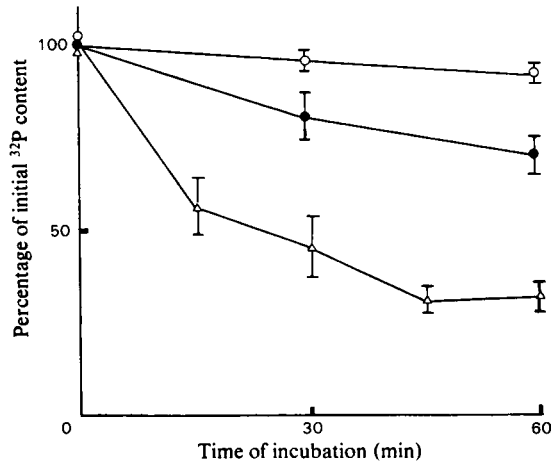

Fig. 3. *Dephosphorylation of acetyl-CoA carboxylase on incubation of fat-cell extracts*

Fat-cells were incubated with $^{32}P_i$ for 75min in the absence of added hormones as described in the legend to Fig. 1. Cell extracts prepared in sucrose-based medium containing 2mM-EGTA were centrifuged at 10000*g* for 1min and the supernatants treated with 20mM-potassium citrate (○), no additions (●) or 5mM-$MgCl_2$ and 10mM-EGTA plus 12mM-$CaCl_2$ (△) (giving calculated free [Ca^{2+}] of 20–50μM) for various times at 30°C. Radioactivity in acetyl-CoA carboxylase was determined as the peak height of band 1 in densitometric traces obtained from radioautographs of the phosphoproteins separated by SDS/polyacrylamide-gel electrophoresis as described in Fig. 1. Results are given as percentages of the peak height of band 1 in unincubated initial extracts (means±S.E.M. for five to ten observations in each case).

If no additions were made, 29.7±5.1% (mean± S.E.M. for ten observations) of the original ^{32}P in acetyl-CoA carboxylase was released during 60min of incubation at 30°C (Fig. 3). However, this rate of dephosphorylation was increased in the presence of added $MgCl_2$ (5mM) and further enhanced on the addition of both $MgCl_2$ (5mM) and 10mM-EGTA plus 12mM-$CaCl_2$. Under these latter conditions, after allowance is made for the 2mM-EGTA already present in the extracts, the free Ca^{2+} can be estimated to be in the range 10–100μM [for details of estimation see Denton *et al.* (1978)]. The percentage loss of radioactivity from acetyl-CoA carboxylase after incubation for 60min at 30°C in the presence of 5mM-$MgCl_2$ was 48.1±3.0 (five observations). This was increased on the further addition of EGTA plus $CaCl_2$ to 63.5±3.3% (five observations; mean± S.E.M.; effect of Ca^{2+}, $P<0.01$). No significant effects of EGTA plus $CaCl_2$ or $CaCl_2$ (3mM) or $MnCl_2$ (3mM) on the rate of dephosphorylation were observed in the absence of $MgCl_2$. On the other

hand, as indicated in Fig. 3, the release of ^{32}P was almost completely inhibited if extracts were incubated in the presence of potassium citrate (20mM). Citrate binds Mg^{2+} more strongly than does EGTA and thus it is possible that the inhibitory effect of citrate results from binding of the small amounts of Mg^{2+} likely to be present in the cell extracts. This view is supported by the observation that EDTA also markedly inhibits the rate of dephosphorylation (mean ± S.E.M. from four observations was 13.2 ± 5.9% of initial ^{32}P released in 1 h).

The release of ^{32}P from acetyl-CoA carboxylase could be the result of either phosphoprotein phosphatase or proteinase activities in the cell extracts. Although we cannot completely rule out the possibility that some ^{32}P is released as a phosphopeptide cleaved from acetyl-CoA carboxylase by the action of proteinase activity, it seems very unlikely for the following reasons: (*a*) addition of the proteinase inhibitor phenylmethanesulphonyl fluoride (0.1 mM) had no appreciable effects on the release of ^{32}P; (*b*) there was no apparent loss of protein associated with band 1 or any other separated protein band and no new bands of protein become evident; (*c*) there was no loss of acetyl-CoA carboxylase activity (see the following section) and, indeed, in extracts of cells previously exposed to adrenaline the activity of acetyl-CoA carboxylase increased; (*d*) the metal ion requirements would be most unusual for a proteinase. It should be noted that Ca^{2+} had no effect on the loss of radioactivity in the absence of added $MgCl_2$.

Rapid release of ^{32}P from acetyl-CoA carboxylase was also observed when extracts of cells previously incubated with $^{32}P_i$ in the presence of adrenaline were subsequently treated with added 5mM-$MgCl_2$ and 10mM-EGTA plus 12mM-$CaCl_2$. In six experiments, the mean release (± S.E.M.) was 68 ± 3.8% after 1 h and the residual phosphorylation of acetyl-CoA carboxylase was no longer significantly different from that of acetyl-CoA carboxylase in control extracts after the same treatment. These conditions were therefore employed to explore the effects of dephosphorylation on the activity of acetyl-CoA carboxylase in tissue extracts.

Effects of dephosphorylation by treatment with Mg^{2+} plus Ca^{2+} on acetyl-CoA carboxylase activity in extracts of fat-pads previously incubated in the absence or presence of adrenaline

Figs. 4 and 5 show the effects of various concentrations of citrate on acetyl-CoA carboxylase activity in extracts of fat-pads before and after treatment with Mg^{2+} and Ca^{2+} for 1 h. Acetyl-CoA carboxylase in the extracts was either exposed to magnesium citrate

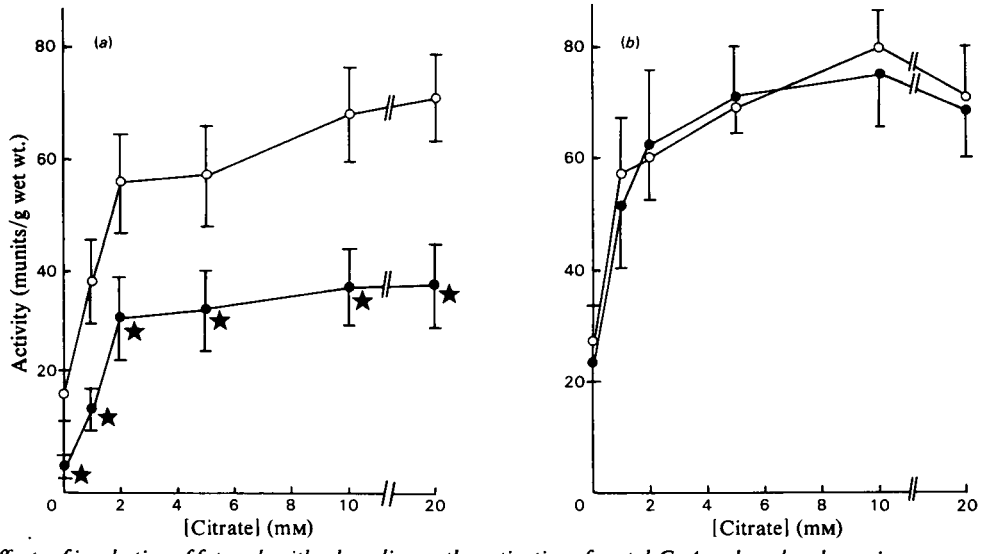

Fig. 4. *Effects of incubation of fat-pads with adrenaline on the activation of acetyl-CoA carboxylase by various concentrations of magnesium citrate in the assay (a) before and (b) after treatment of tissue extracts with Mg^{2+} and Ca^{2+}*

Fat-pads were incubated with (●) or without (○) adrenaline (5 μM) as described in Table 1. Extracts were prepared and assayed in the presence of the indicated concentrations of magnesium citrate either (*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. Results are given as means ± S.E.M. for four to eight observations in each case. * Effect of adrenaline: $P < 0.05$.

only during the assay of enzyme activity (Fig. 4) or the extracts were incubated with potassium citrate for 20min at 30°C before the assay of acetyl-CoA carboxylase activity (Fig. 5). Kinetic constants calculated from these experiments are given in Table 3. As expected from earlier studies, higher values of V are obtained if extracts are incubated with citrate before the assay of activity (Halestrap & Denton, 1973, 1974).

Before treatment of extracts with Mg^{2+} and Ca^{2+}, the activity of acetyl-CoA carboxylase in extracts of cells exposed to adrenaline was less than control values at all concentrations of citrate (Figs. 4 and 5; see also Table 1). Overall, in addition to marked differences in the calculated values for V, there was also evidence that the concentration of citrate required to give half-maximal activation during incubation of extracts was higher in extracts from adrenaline-treated cells (Table 3).

After treatment with Mg^{2+} and Ca^{2+} these differences in the kinetic properties of acetyl-CoA carboxylase in extracts of cells exposed to adrenaline were no longer apparent (Figs. 4 and 5 and Table 3). The treatment had no effect on the calculated values of V for the enzyme from control tissues, but it resulted in significant increases in the values for the

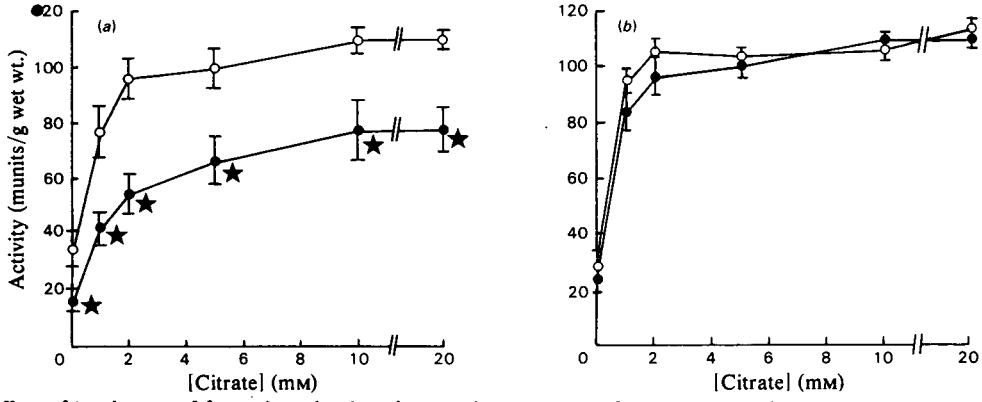

Fig. 5. *Effect of incubation of fat-pads with adrenaline on the activation of acetyl-CoA carboxylase by incubation of tissue extracts with various concentrations of potassium citrate (a) before and (b) after treatment of tissue extracts with Mg^{2+} and Ca^{2+}*

Details are as for Fig. 4, except that the additions of the indicated concentrations of citrate were made to tissue extracts, and the extracts were incubated for 20min at 30°C before assay. Results are given as means ± S.E.M. for five observations in each case. * Effect of adrenaline: $P<0.05$.

Table 3. *Kinetic constants for the effects of citrate on acetyl-CoA carboxylase activity*

Data were taken from (*a*) Fig. 4 and (*b*) Fig. 5 and fitted to $v=[(V-A)/(1+K_{cit.}/c)]+A$ [where A is the activity (v) in the absence of citrate, c is the concentration of citrate in (*a*) assay or (*b*) extract incubation and $K_{cit.}$ is the concentration of citrate giving half-maximal activation] by using a non-linear least-squares regression program. Means ± S.E.M. (for the indicated numbers of observations) are given in each case. Effect of adrenaline: * $P<0.02$; ** $P<0.001$. Effect of treatment with Mg^{2+} and Ca^{2+}: † $P<0.02$; †† $P<0.001$.

	V (munits/g wet wt.)		$K_{cit.}$ (mM)		Number of observations	
	No hormone	Adrenaline	No hormone	Adrenaline	No hormone	Adrenaline
(*a*) Effect of citrate in assay:						
Before treatment of extracts with Mg^{2+} and Ca^{2+}	73 ± 4.4	40 ± 4.3**	1.40 ± 0.45	1.36 ± 0.69	42	42
After treatment of extracts with Mg^{2+} and Ca^{2+}	78 ± 4.1	80 ± 1.7††	0.70 ± 0.30	1.14 ± 0.17	33	33
(*b*) Effect of citrate in extract incubation:						
Before treatment of extracts with Mg^{2+} and Ca^{2+}	114 ± 3.0	85 ± 1.9**	0.77 ± 0.15	1.53 ± 0.17*	30	30
After treatment of extracts with Mg^{2+} and Ca^{2+}	116 ± 2.6	113 ± 2.0††	0.32 ± 0.09†	0.43 ± 0.06††	30	30

enzyme from tissue exposed to adrenaline, so that they were restored to values close to those found in extracts of control tissues. The value of $K_{cit.}$ appeared to be diminished after treatment with Mg^{2+} and Ca^{2+} in extracts from both control and adrenaline-treated tissue.

Similar effects of treatment with Mg^{2+} and Ca^{2+} were also found with extracts of isolated fat-cells rather than extracts of fat-pads as used in Figs. 4 and 5. For example, after incubation with 20mM-potassium citrate for 20min at 30°C, the activity in extracts of cells exposed to adrenaline for 15min was 90.6±3.2munits/g dry wt., and this was significantly ($P<0.001$) less than that in control cells (136.6± 7.8munits/g dry wt.). However, if the extracts were pretreated with added $MgCl_2$ (5mM) and 10mM-EGTA plus 12mM-$CaCl_2$ for 60min at 30°C, the effects of adrenaline were no longer present. The actual values found were 124.4±5.3 and 138.8± 14.3munits/g dry wt. for extracts from adrenaline-exposed and control cells respectively. However, there was no appreciable decrease in the effect of adrenaline, if extracts were pretreated for 60min at 30°C without the additions of Mg^{2+} and Ca^{2+}; values obtained under these conditions were 90.2±5.1 and 125.2±6.9munits/g dry wt. respectively. (Values given are means±S.E.M. for six observations in each case.)

General conclusions

The results of this paper strengthen the view that the activity of acetyl-CoA carboxylase in mammalian tissues may be regulated by phosphorylation. Certainly it seems reasonable to conclude that inactivation of acetyl-CoA carboxylase in adipose tissue with adrenaline involves an increase in phosphorylation of the enzyme. Not only has increased phosphorylation been directly demonstrated in intact fat-cells but it has also been shown that conditions that promote dephosphorylation of the enzyme in cell extracts reverse the effects of adrenaline on enzyme activity. However, there remain a number of important aspects that will require extensive investigation in the future.

At present, we can only presume that the effects of adrenaline on acetyl-CoA carboxylase activity are mediated by stimulation of cyclic AMP-dependent protein kinase. Although it has been shown that rabbit skeletal-muscle cyclic AMP-dependent protein kinase is capable of catalysing the phosphorylation of the enzyme purified from rabbit mammary gland (Hardie & Cohen, 1978*a*) and rat adipose tissue (R. W. Brownsey & R. M. Denton, unpublished observations), the effects of phosphorylation on activity and polymerization of purified enzyme have not been clearly established. Under certain conditions, cyclic AMP has been shown to increase the rate of both phosphorylation and inactivation of partially purified preparation of the rat liver enzyme incubated with $MgATP^{2-}$ (Lent *et al.*, 1978). However, it seems likely that the phosphorylation of acetyl-CoA carboxylase in cells occurs at more than one class of sites (Pekala *et al.*, 1978) and is catalysed by both cyclic AMP-dependent and cyclic AMP-independent kinases (Hardie & Cohen, 1978*a*). The fairly modest increases in overall phosphorylation of the enzyme observed in the present study with adrenaline may reflect the fact that adrenaline does not affect the phosphorylation of all the sites.

Extracts of fat-cells appear to contain phosphoprotein phosphatase activity, which is able to catalyse the release of phosphate from acetyl-CoA carboxylase. This activity appears to be dependent on the presence of Mg^{2+} and to be further stimulated by micromolar concentrations of Ca^{2+}. This sensitivity to Ca^{2+} does raise the important possibility that changes in the concentration of Ca^{2+} in the cytoplasm may be important in the regulation of phosphorylation and thus the activity of acetyl-CoA carboxylase.

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