Biochem. J. (1985) **226**, 139–145
Printed in Great Britain

Evidence that glucagon-mediated inhibition of acetyl-CoA carboxylase in isolated adipocytes involves increased phosphorylation of the enzyme by cyclic AMP-dependent protein kinase

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(*Received 30 July 1984/Accepted 22 October 1984*)

1. The kinetic parameters and phosphorylation state of acetyl-CoA carboxylase were analysed after purification of the enzyme by avidin–Sepharose chromatography from extracts of isolated adipocytes treated with glucagon or adrenaline. 2. The results provide evidence that the mechanism of inhibition of acetyl-CoA carboxylase in adipocytes treated with glucagon [Zammit & Corstorphine (1982) *Biochem. J.* **208**, 783–788] involves increased phosphorylation of the enzyme. 3. Hormone treatment had effects on the kinetic parameters of the enzyme similar to those of phosphorylation of the enzyme *in vitro* by cyclic AMP-dependent protein kinase. 4. Glucagon treatment of adipocytes led to increased phosphorylation of acetyl-CoA carboxylase in the same chymotryptic peptide as that containing the major site phosphorylated on the enzyme by purified cyclic AMP-dependent protein kinase *in vitro* [Munday & Hardie (1984) *Eur. J. Biochem.* **141**, 617–627]. 5. The dose–response curves for inhibition of enzyme activity and increased phosphorylation of the enzyme were very similar, with half-maximal effects occurring at concentrations of glucagon (0.5–1 nM) which are close to the physiological range. 6. In general, the patterns of increased ^{32}P-labelling of chymotryptic peptides induced by glucagon or adrenaline were similar, although there were quantitative differences between the effects of the two hormones on individual peptides. 7. The results are discussed in terms of the possible roles of cyclic AMP-dependent and -independent protein kinases in the regulation of acetyl-CoA carboxylase activity and of lipogenesis in white adipose tissue.

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the first step committed to fatty acid synthesis, and there is general agreement that it is a key enzyme in the regulation of the pathway from cytosolic acetyl-CoA. The activity of acetyl-CoA carboxylase is controlled by (i) allosteric regulation (e.g. activation by citrate and inhibition by long-chain acyl-CoA: see, e.g., Denton *et al.*, 1977; Hardie, 1980) and (ii) reversible phosphorylation. The latter mechanism has been demonstrated in isolated cells (Brownsey *et al.*, 1979; Witters *et al.*, 1979; Holland *et al.*, 1984), tissue homogenates (McNeillie *et al.*, 1981; Zammit & Corstorphine, 1982*a*) and the purified enzyme. Thus acetyl-CoA carboxylase purified from rat mammary gland (Hardie & Cohen, 1978; Hardie & Guy, 1980; Munday & Hardie, 1984) and rat liver (Tipper & Witters, 1982) is phosphorylated by cyclic AMP-dependent protein kinase, producing an inactivation of the enzyme, which can be reversed by dephosphorylation. In addition, a number of cyclic AMP-independent protein kinases have been shown to phosphorylate the enzyme purified from a variety of tissues (Brownsey, 1981; Shiao *et al.*, 1981; Song & Kim, 1981; Munday & Hardie, 1984): some of these phosphorylations also result in a change in its activity.

Acetyl-CoA carboxylase activity is inhibited in isolated adipocytes incubated with glucagon (Zammit & Corstorphine, 1982*b*). This may partly account for the effect of glucagon on lipogenesis in adipocytes (Robson *et al.*, 1984). Adrenaline also inhibits acetyl-CoA carboxylase activity in adipocytes and is thought to exert its effect on the enzyme by elevating intracellular concentrations of cyclic AMP, which results in the activation

of cyclic AMP-dependent protein kinase and increased phosphorylation of the enzyme (Lee & Kim, 1978; Brownsey *et al.*, 1979). The increased phosphorylation of acetyl-CoA carboxylase in adrenaline-treated adipocytes occurs at the same site as that phosphorylated on purified acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase *in vitro* (Brownsey & Hardie, 1980). Inhibition of acetyl-CoA carboxylase activity in adipocytes incubated with adenosine deaminase has also been observed (Zammit & Corstrophine, 1982*b*). This too was probably mediated by changes in the intracellular concentration of cyclic AMP and, consequently, in the activity of cyclic AMP-dependent protein kinase, since the addition of adenosine deaminase would have resulted in a lowering of the extracellular concentration of adenosine and hence a lessening of its inhibitory effect on adipocyte adenylate cyclase (Trost & Stock, 1979; Fain & Malbon, 1979).

The technique of avidin–Sepharose chromatography, which allows a single-step purification of acetyl-CoA carboxylase from cell extracts (Tipper & Witters, 1982), has been used to study the effects of glucagon on the phosphorylation state and kinetic parameters of the enzyme in isolated hepatocytes (Holland *et al.*, 1984). The results suggested that phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase could entirely account for the inhibition of its activity by glucagon in this cell preparation.

In order to examine the mechanism whereby glucagon elicits inhibition of acetyl-CoA carboxylase in adipocytes, we have studied the effects of glucagon treatment of isolated adipocytes on the kinetic parameters and the phosphorylation of acetyl-CoA carboxylase purified by avidin–Sepharose chromatography from cell extracts. We have also re-examined the effects of adrenaline on the enzyme in isolated adipocytes, since, unlike the immunoprecipitation methods used previously (Brownsey *et al.*, 1979; Brownsey & Hardie, 1980), the avidin–Sepharose technique allows detailed analysis of the kinetic parameters and specific radioactivity of the purified enzyme.

Materials and methods

Animals

Male Wistar rats (130–150g body wt.) were used; they were allowed unrestricted access to food and water. Details of the source and care of animals have been given previously (Zammit, 1980, 1981).

Preparation of adipocytes

For a typical experiment, the epididymal fat-pads from 30 rats were used. Adipocytes were prepared as described previously (Zammit & Corstorphine, 1982*b*), and after a final wash in low-phosphate Krebs buffer (0.24mM-P_i), they were dispensed into stoppered silicone-treated glass conical flasks for incubation (see below).

Incubation of adipocytes in medium containing $[^{32}P]P_i$

Adipocytes were suspended in a buffered Krebs–Henseleit medium (1.5ml per fat-pad equivalent) containing 1.25mM-$CaCl_2$ and 0.24mM-P_i, i.e. respectively one-half and one-fifth the originally recommended concentrations (Krebs & Henseleit, 1932). The medium also contained fatty acid-free bovine albumin (40mg/ml), glucose (5mM) and $[^{32}P]P_i$ (40–65μCi/ml). Incubations were performed under an atmosphere of O_2/CO_2 (19:1) in flasks (see above) which were shaken at 120 excursions/min in a 37°C water bath. Incubation was allowed to proceed for 1h (sufficient to ensure steady-state labelling of phosphoproteins, including acetyl-CoA carboxylase, under these conditions; Brownsey *et al.*, 1977), after which time solutions of adrenaline or glucagon were added to the adipocyte suspension in a volume not exceeding 0.2% of the total. Flasks were returned to the water bath and incubated as before for a further 10min.

Preparation of ^{32}P*-labelled acetyl-CoA carboxylase*

After incubation with $[^{32}P]P_i$ as described above, adipocytes were rapidly harvested by centrifugation; the subnatant medium was aspirated off, and the packed adipocytes (equivalent to 10 fat-pads in each tube) were transferred to an ice-cold glass centrifuge tube containing 15ml of the following homogenizing medium: 250mM-sucrose, 20mM-Tris/HCl, 2mM-EGTA, 2mM-EDTA, 100mM-KF, 7.5mM-reduced glutathione, 2mM-phenylmethanesulphonyl fluoride, and leupeptin, pepstatin and antipain each at 100μg/ml. The final pH was 7.4 at 0°C. The adipocytes were immediately disrupted by vigorous shaking and vortex-mixing over a 2min period in the stoppered tube. Brief centrifugation (1000***g***, 2min) was then used to separate and consolidate the floating fat layer. The subnatant was removed, strained through glass wool and centrifuged at 0°C for 40min at 105000***g***. A fraction of cytosolic proteins, including acetyl-CoA carboxylase, was then precipitated from the resulting supernatant by the addition of $(NH_4)_2SO_4$ to 35% saturation. The precipitate was collected by centrifugation at 25000***g*** for 20min and the supernatant discarded. Acetyl-CoA carboxylase was purified by avidin–Sepharose affinity chromatography from this 35%-satd.-$(NH_4)_2SO_4$ pellet as described by Holland *et al.* (1984).

Analysis by reversed-phase h.p.l.c. of phosphopeptides resulting from chymotryptic digestion of ^{32}P-labelled acetyl-CoA carboxylase, assay of acetyl-CoA carboxylase activity and details of other analytical methods have also been previously described (Holland *et al.*, 1984).

Specific radioactivity of adenine nucleotides

At the same time as cells were harvested for enzyme isolation, $HClO_4$ was added to a 1 ml portion of the adipocyte suspension to a final concentration of 5% (w/v). Specific radioactivities of adenine nucleotides were then determined as described by Holland *et al.* (1984).

Materials

The sources of materials were as given in Zammit & Corstorphine (1982*a*,*b*) and Holland *et al.* (1984).

Results

Isolation of acetyl-CoA carboxylase from adipocyte extracts

The purification of acetyl-CoA carboxylase from isolated ^{32}P-labelled adipocytes was carried out in the presence of EDTA and fluoride ions to prevent changes in the phosphorylation status of the enzyme during these procedures (Holland *et al.*, 1984). Fig. 1 shows Coomassie Blue-stained gels and autoradiograms typical of those obtained routinely of the purified preparations. The major polypeptide present had a mobility corresponding to a molecular mass of 240000 Da, and co-migrated with acetyl-CoA carboxylase purified by avidin–Sepharose chromatography from mammary gland or liver (results not shown). There was a minor (10–20%) contamination of this major polypeptide by another polypeptide, with molecular mass approx. 130000 Da, which may represent pyruvate carboxylase (cf. Tipper & Witters, 1982). However, ^{32}P-labelling was confined exclusively to the 240000-Da subunit of acetyl-CoA carboxylase.

Kinetic parameters of purified acetyl-CoA carboxylase

The activity of the purified acetyl-CoA carboxylase was assayed at two different concentrations of citrate (0.5 and 5 mM). Glucagon or adrenaline treatment of the cells resulted in inhibition of enzyme activity measured at either citrate concentration (Table 1). In another series of experiments the enzyme was assayed in the presence of a wider range of citrate concentrations in order to obtain estimates of $V_{max.}$, K_a for citrate and the Hill coefficient of the velocity-versus-[citrate] curve. The results indicated that glucagon and adrenaline both decreased the $V_{max.}$ and increased the K_a for citrate. Although the number of replicate experimental results was too small to demonstrate statistical significance for the increases in K_a, the fact that inhibition was greater at 0.5 mM- than at 5 mM-citrate (63% versus 32% for glucagon, $P<0.01$; 83% versus 63% for adrenaline; $P<0.02$) clearly demonstrates that the citrate-dependence of acetyl-CoA carboxylase is increased by treatment of cells with either hormone.

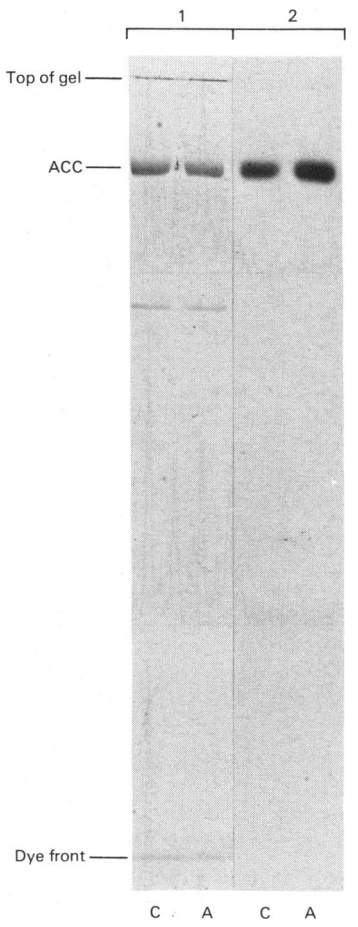

Fig. 1. *Electrophoretic analysis of acetyl-CoA carboxylase purified from ^{32}P-labelled isolated adipocytes*

Adipocytes were incubated without hormone (C) or with 1 μM-adrenaline (A). (1) Coomassie Blue-stained gel; (2) autoradiogram. Electrophoresis was in 7%-polyacrylamide gels in the presence of sodium dodecyl sulphate; 1.3 μg of protein was loaded in each track. 'ACC' represents the migration position of acetyl-CoA carboxylase purified from hepatocytes and run in the same slab gel.

^{32}P-labelling of acetyl-CoA carboxylase

Treatment of isolated adipocytes with glucagon or adrenaline in the presence of [^{32}P]P_i led to an

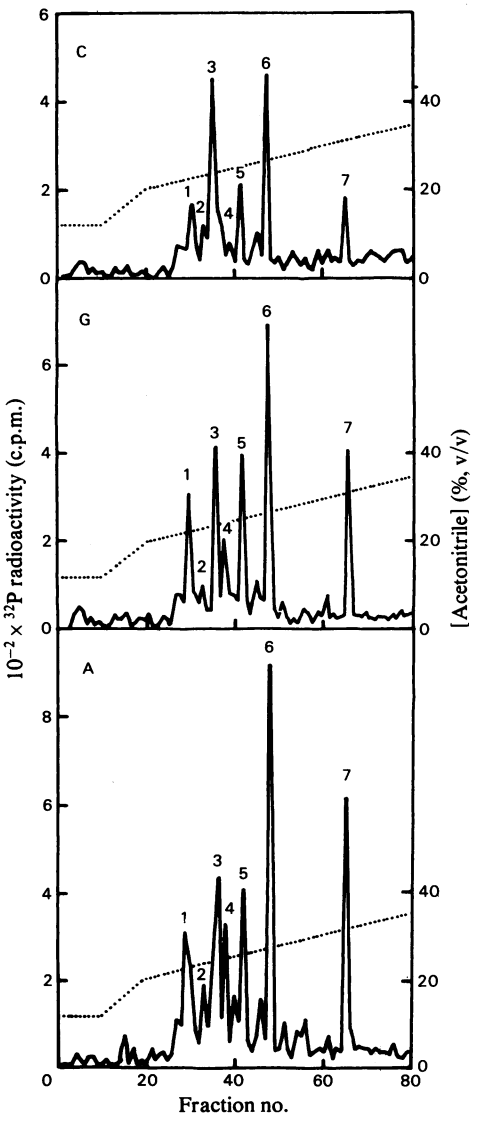

Fig. 2. *Reversed-phase-h.p.l.c. analysis of chymotryptic peptides derived from acetyl-CoA carboxylase purified from ^{32}P-labelled adipocytes*

Adipocytes were incubated without hormone (C), with 0.1 μM-glucagon (G) or with 1 μM-adrenaline (A). In preliminary experiments these concentrations of hormones were shown to have optimal effects. The continuous line represents radioactivity determined by Čerenkov counting of 1 ml fractions. The dotted line represents the concentration of acetonitrile (%) in the eluent. The data have been corrected to compensate for slight (±10%) variation in recovery from the h.p.l.c. column, so that the total radioactivities (c.p.m.) after subtraction of background are in the same ratio as the specific radioactivities of the protein digests loaded. Varying the digestion time from 3h to 72h, or doubling the concentration of chymotrypsin, did not significantly affect the pattern of peptides produced.

increase, relative to untreated adipocytes, in the specific radioactivity of the subsequently isolated acetyl-CoA carboxylase. Thus the ^{32}P content per mg of enzyme protein was 109±3% ($n=5$, $P<0.02$, paired t test) and 144±8% ($n=3$, $P<0.01$) relative to controls for the enzyme from adipocytes treated with glucagon and adrenaline respectively; in the experiment illustrated in Fig. 2, the absolute values of incorporation calculated from the specific radioactivity of the γ-phosphate of intracellular ATP (28700c.p.m./nmol) were 3.3, 3.7 and 4.7mol of ^{32}P per 240000-Da subunit respectively.

Seven major ^{32}P-labelled peptides were obtained when reversed-phase h.p.l.c. analysis of chymotryptic peptides of acetyl-CoA carboxylase was performed (Fig. 2). Except for peptide 6, they co-migrated with peptides phosphorylated on the enzyme purified from rat mammary tissue by cyclic AMP-dependent protein kinase (whether analysed by h.p.l.c. or isoelectric focusing; Munday & Hardie, 1984). They also had retention times similar to those of the seven chymotryptic peptides obtained from the enzyme phosphorylated in isolated hepatocytes (Holland *et al.*, 1984). Glucagon and adrenaline both caused large and highly significant increases in the labelling of the earliest-eluted labelled peptide (peptide 1, Figs. 2 and 3), which was also the major peptide phosphorylated on the purified enzyme by cyclic AMP-dependent protein kinase (Munday & Hardie, 1984). In addition, both hormones also caused increased phosphorylation of several other peptides, although to a lesser degree than for peptide 1 (Figs. 2 and 3).

Effect of different concentrations of glucagon on activity and phosphorylation of acetyl-CoA carboxylase

The activity and phosphorylation state of acetyl-CoA carboxylase were analysed in enzyme samples purified from the same preparation of adipocytes, incubated with different concentrations of glucagon. The dose–response curves for the effects of glucagon on enzyme activity (measured at 0.5mM-citrate) and for the increase in ^{32}P labelling of peptide 1 were reciprocally related (Fig. 4); half-maximal effect of the hormone was obtained at 0.5–1.0nM-glucagon for both parameters.

Discussion

The results reported in the present paper provide strong evidence that inhibition of acetyl-CoA carboxylase in adipocytes incubated with glucagon (Zammit & Corstorphine, 1982*b*) involves increased phosphorylation of the enzyme. In addition to the directly observed increased ^{32}P labelling

Table 1. *Kinetic parameters of acetyl-CoA carboxylase purified from isolated adipocytes by avidin–Sepharose chromatography*

Values are means ± S.E.M. for the numbers of separate cell preparations shown in parentheses. Values expressed per mg of protein have not been corrected for contamination by the M_r-130000 peptide and may therefore represent slight underestimates of the true values per mg of acetyl-CoA carboxylase protein. Kinetic parameters were estimated by fitting to the Hill equation $v/V_{max.} = [C]^h/(K_a + [C]^h)$, where [C] is the citrate concentration and h is the Hill coefficient (Holland *et al.*, 1984). Asterisks indicate where the value obtained after hormonal treatment was significantly different from control by the paired t test (*$P<0.02$; **$P<0.01$).

Parameter	Treatment of cells		
	Control	Adrenaline (1 μM)	Glucagon (0.1 μM)
Activity at 0.5 mM-citrate (nmol/min per mg)	192 ± 43 (5)	**29 ± 5 (3)	**69 ± 18 (4)
Activity at 5 mM-citrate (nmol/min per mg)	1250 ± 166 (6)	*344 ± 61 (3)	*847 ± 179 (5)
$V_{max.}$ (nmol/min per mg)	1822 ± 106 (3)	**613 ± 159 (3)	1004 ± 7 (2)
K_a for citrate (mM)	4.8 ± 0.63 (3)	6.8 ± 1.2 (3)	7.1 ± 0.3 (2)
Hill coefficient, h	1.07 ± 0.11 (3)	1.39 ± 0.05 (3)	1.28 ± 0.14 (2)

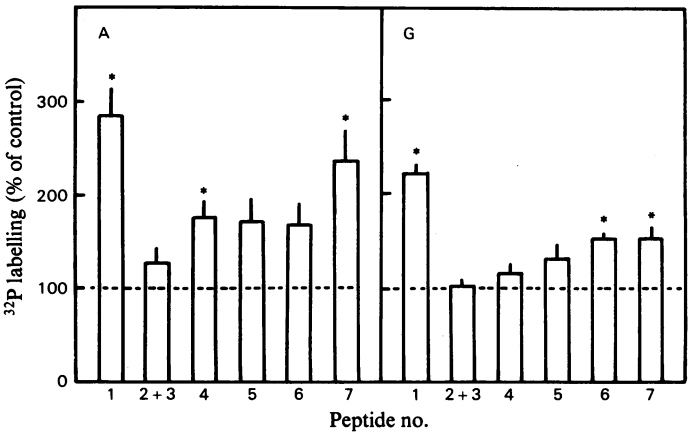

Fig. 3. *Changes in the labelling of individual phosphopeptides in acetyl-CoA carboxylase purified from glucagon- or adrenaline-treated adipocytes*

The radioactivity in the peaks as shown in Fig. 2 was determined for the enzyme isolated from cells treated with either 0.1 μM-glucagon (G) or 1 μM-adrenaline (A). Data were corrected for slight variation in recoveries as in Fig. 2. The results are expressed as mean percentages of control values (i.e. from cells incubated in the absence of hormones) ± S.E.M. ($n = 4$ and 3 separate adipocyte preparations respectively for G and A). Peaks 2 and 3 were not clearly resolved in every analysis, and they have been considered as a single peak. Asterisks indicate statistically significant changes ($P<0.05$; paired t test).

of the enzyme, the persistence of the inhibitory effect of the hormone on the enzyme during its subsequent purification indicated that it was caused by a covalent modification rather than by an allosteric effect (e.g. a change in cellular citrate concentrations). In the present study, maximally effective doses of glucagon inhibited activity of the enzyme (assayed after purification) by 60–70%; this inhibition was very similar to that observed previously in crude cell extracts (70%; Zammit & Corstorphine, 1982*b*). Moreover, the dose–response curves for glucagon effects on enzyme activity and phosphorylation of peptide 1 were identical (but reciprocal).

The inhibition of acetyl-CoA carboxylase activity measured at 0.5 mM- and 5 mM-citrate after glucagon or adrenaline treatment of cells appeared to result from both a decrease in $V_{max.}$ and an increase in K_a for citrate. The changes in these parameters for the enzyme isolated from adrena-

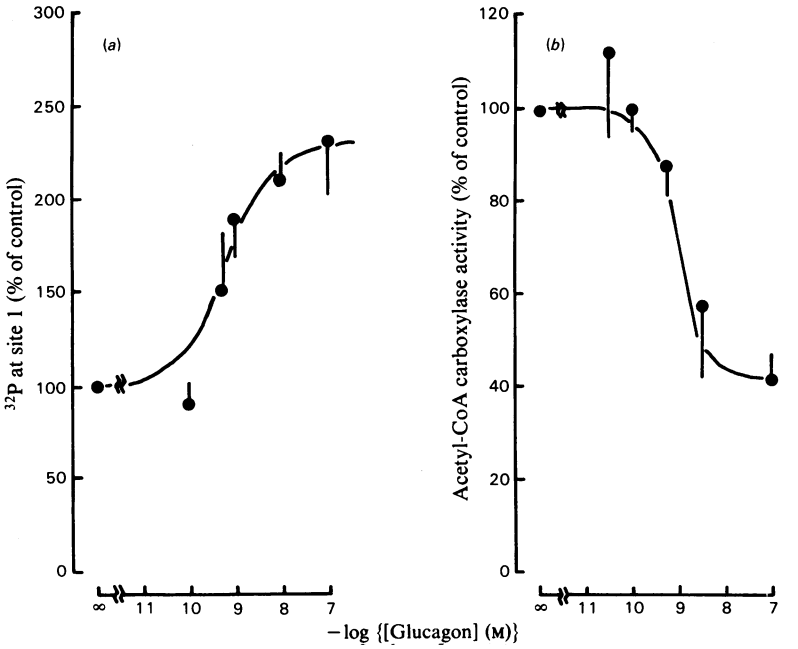

Fig. 4. *Dose–response curves for the effects of glucagon on increased ^{32}P labelling of peptide 1 (a) and inhibition of acetyl-CoA carboxylase activity (b)*

The activity of acetyl-CoA carboxylase purified from adipocytes treated with various concentrations of glucagon was measured in the presence of 0.5mM-citrate as described in the Materials and methods section. Results are expressed as mean ± S.E.M. for three separate cell preparations.

line-treated cells confirmed previous results obtained on crude extracts of similarly treated adipocytes (Brownsey *et al.*, 1979). The present data also provide strong evidence that both hormones act via the stimulation of cyclic AMP-dependent protein kinase activity. This evidence may be summarized as follows: (i) the changes in $V_{max.}$ of the enzyme and K_a for citrate (see above) were very similar to the effects of phosphorylation of purified rat mammary acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase (Hardie & Guy, 1980; Munday & Hardie, 1984); (ii) the major site of phosphorylation on the enzyme in glucagon- and adrenaline-treated cells occurred in the same chymotryptic peptide (peptide 1) as the major site of phosphorylation by cyclic AMP-dependent protein kinase. Amino acid-sequence studies (D. G. Hardie, P. S. Guy, A. Aitken & L. Witters, unpublished work) have confirmed that this chymotryptic peptide contains the same phosphorylated serine residue as the tryptic peptide (pI 7) previously shown to be phosphorylated in response to adrenaline in adipocytes (Brownsey & Hardie, 1980).

Although the present results obtained with adipocytes were qualitatively similar to those obtained previously with glucagon-treated hepatocytes (Holland *et al.*, 1984), there were some noteworthy differences. In particular: (i) hormone treatment of adipocytes produced increased phosphorylation of peptides 4–7 as well as peptide 1, whereas changes were restricted to peptide 1 in hepatocytes: (ii) treatment of adipocytes, particularly with adrenaline, produced larger decreases in $V_{max.}$ of acetyl-CoA carboxylase than were observed in hepatocytes. Although phosphorylation at peptide 1 is known to affect mainly the K_a for citrate of this enzyme (Munday & Hardie, 1984), it seems likely that the large decrease in $V_{max.}$ is brought about by phosphorylation at one or more of peptides 4–7. It is possible that these effects were less evident in isolated hepatocytes because the enzyme was already phosphorylated at these additional sites under the basal conditions used. Thus, enzyme isolated from control hepatocytes (Holland *et al.*, 1984) had a higher phosphate content (4.4mol/subunit, determined from the specific radioactivity) and lower $V_{max.}$ (0.5μmol/min per mg of protein) compared with corresponding values of 3.3 and 1.8 respectively for the enzyme isolated from control adipocytes in the present study.

At present it is not clear whether increased phosphorylation at the sites located in peptides 4–7

is catalysed by cyclic AMP-dependent or -independent protein kinase(s). One possibility is that cyclic AMP-dependent protein kinase modulates phosphorylation of these sites indirectly via phosphorylation and activation of protein phosphatase inhibitor proteins (Cohen, 1982; Nemenoff *et al.*, 1983).

The finding that glucagon and adrenaline caused increased phosphorylation of acetyl-CoA carboxylase at the same site(s) in isolated adipocytes is consistent with previous observations that the effects of saturating concentrations of these two hormones on acetyl-CoA carboxylase activities in adipocytes were not additive (Zammit & Corstorphine, 1982*b*). By contrast, previous studies on changes in enzyme activity have suggested that insulin activates acetyl-CoA carboxylase by an independent mechanism rather than by merely antagonizing the effect of adrenaline or glucagon (Brownsey, 1981; Zammit & Corstorphine, 1982*b*). Consistent with this suggestion, insulin does not cause dephosphorylation of acetyl-CoA carboxylase in isolated adipocytes, but instead increases the phosphorylation at site(s) located in a single tryptic peptide (Brownsey & Denton, 1982), although Witters *et al.* (1983) observed no effects of this increased phosphorylation on the activity of the purified enzyme. Irrespective of the precise mechanism of insulin action, the existence in white adipose tissue of independent mechanisms of activation and inhibition of the enzyme by insulin and glucagon respectively would make acetyl-CoA carboxylase activity, and hence the rate of lipogenesis from cytosolic acetyl-CoA, very sensitive to small changes in the circulating insulin/glucagon concentration ratio (Zammit & Corstorphine, 1982*b*).

R. H. and D. G. H. acknowledge Project Grant and Research Group support from the M.R.C.

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