

Insulin and the Regulation of Adipose Tissue Acetyl-Coenzyme A Carboxylase

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Rat epididymal fat-pads were incubated for 30 min with glucose (2 mg/ml) in the presence or absence of insulin. A twofold or greater increase in acetyl-CoA carboxylase activity was observed in extracts from insulin-treated tissue provided that assays were performed rapidly after extraction. This effect of insulin was evident whether or not extracts were prepared with albumin, and was not noticeably diminished by the presence of citrate or albumin or both in the assay. Incubation of extracts before assay led to activation of acetyl-CoA carboxylase and a marked diminution in the insulin effect. The enzyme in extracts was very sensitive to reversible inhibition by palmitoyl-CoA even in the presence of albumin (10 mg/ml); inhibition persisted on dilution of enzyme and inhibitor. It is suggested that the observed activation of acetyl-CoA carboxylase by insulin may reflect changes in enzyme activity in the fat-cell resulting from the reduction of long-chain fatty-acyl-CoA that occurs in the presence of insulin. Activation of the enzyme with loss of the insulin effect on incubation of the extracts may be due to the slow dissociation of long-chain fatty acyl-CoA from the enzyme.

Recent studies indicate that the marked increase by insulin of fatty acid biosynthesis from glucose in rat epididymal fat-cells may result not only from a stimulation of glucose transport (Crofford & Renold, 1965), but also from the activation of pyruvate dehydrogenase (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971). These recent studies on pyruvate dehydrogenase explain a number of observations on the control by insulin of fatty acid synthesis from glucose in rat epididymal adipose tissue. However, the possibility exists that insulin may act through a further point of control beyond pyruvate dehydrogenase, since maximum stimulation by insulin increases the rate of fatty acid synthesis from glucose fivefold or more, whereas the whole-tissue concentration of citrate is unaltered and that of acetyl-CoA increases by less than 50% (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). The present studies were initiated as an attempt to find a further enzyme in the pathway of fatty acid synthesis that was activated in the presence of insulin. We report in the present paper the finding that the activity of acetyl-CoA carboxylase is markedly increased in extracts of epididymal fat-pads that have been exposed briefly to insulin in the presence of glucose.

Acetyl-CoA carboxylase from rat adipose tissue, like the enzyme from other mammalian sources, is activated by citrate and inhibited by long-chain fatty acyl-CoA both in crude and purified preparations [see reviews by Moss & Lane (1971) and Vagelos (1971)]. The physiological importance of these effectors has been difficult to assess because there is

little correlation between rates of fatty acid synthesis and whole-tissue concentrations of citrate, and many enzymes are inhibited by long-chain fatty acyl-CoA, in most cases this inhibition being overcome by prior addition of albumin (Taketa & Pogell, 1966). However, the concentration of long-chain fatty acyl-CoA is lowered by insulin in fat-pads incubated in the presence of glucose (Denton & Halperin, 1968) and evidence is presented below that the activation of acetyl-CoA carboxylase by insulin may be related to this.

Experimental

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (150-220 g) allowed free access to water and a stock laboratory diet (modified 41B; Oxoid Ltd., London S.E.1, U.K.). The animals, well matched in age and weight in any one experiment, were killed by decapitation.

Chemicals. Except where stated below, enzymes, substrates, coenzymes and triethanolamine hydrochloride were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

L-Malic acid and bovine serum albumin (fraction V; fatty acid- and citrate-free) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Crystalline insulin, a gift of Boots Pure Drug Co. Ltd., Nottingham, U.K., was dissolved in 3M-HCl to

yield a stock solution of 10 units/ml. Potassium [^{14}C]-bicarbonate was from The Radiochemical Centre, Amersham, Bucks., U.K.

Acetyl-CoA was synthesized by the method of Simon & Shemin (1953); excess of acetic anhydride was removed by two extractions with diethyl ether. Palmitoyl-CoA was synthesized as described by Seubert (1961). Malonyl-CoA was prepared by the method of Lynen (1962), with the slight modification that the reduction of *NN'*-dioctylcysteamine to give *N*-octylcysteamine was performed by refluxing the disulphide (4g) dissolved in 95% (v/v) ethanol (100ml) gently for 2h with NaBH_4 (1.5g) in 95% ethanol (50ml). Refluxing was then continued for 30min more after a further 0.5g of NaBH_4 had been added. The mixture was cooled in ice and an equal volume of 0.25M-NaOH was added slowly to allow excess of disulphide to crystallize out. After removal of the disulphide by filtration, *N*-octylcysteamine was crystallized at 0°C after acidification with 1M-HCl and addition of 2vol. of water. The product (yield about 70%) had a melting point and i.r.-absorption spectrum consistent with its being *N*-octylcysteamine.

Media. Fat-pads were incubated in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with $\text{O}_2 + \text{CO}_2$ (95:5). Additions were made as indicated in the text and tables.

Methods

Extraction of fat-pads for enzyme analysis. After incubation, pads were lightly blotted and rapidly frozen and ground in liquid N_2 . The frozen powders were extracted at 0°C and pH7.3 in an all-glass tissue grinder (about 0.35g of tissue powder/ml of extraction buffer) in either 0.25M-sucrose containing 20mM-Tris-HCl, 2mM-EDTA and 5mM-GSH, or 100mM-potassium phosphate containing 2mM-EDTA and 5mM-GSH, with additions of either albumin or citrate as indicated. After extraction the homogenate was immediately centrifuged at 1500g for 1min to remove fat and cell debris. Enzyme assays were made on samples of the infranatant removed by syringe. Initial activities of acetyl-CoA carboxylase were measured within 5min of extraction.

Enzyme assays. All assays were carried out at 30°C. ATP-citrate lyase (EC 4.1.3.8) and NADP-malate dehydrogenase (EC 1.1.1.40) were assayed as described previously (Martin & Denton, 1970). Fatty acid synthetase was assayed in a split-beam spectrophotometer by following the change in absorption at 340nm under conditions that were found to be optimum. The assay was done in 100mM-potassium phosphate buffer, pH6.5, containing NADPH (0.1mM) and acetyl-CoA (25 μM) and was initiated by addition of malonyl-CoA (60 μM).

Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed by the incorporation of [^{14}C]bicarbonate into malonyl-CoA by a modification of the method of Martin & Vagelos (1962). Assays were initiated by adding a sample (50 μl) of infranatant (see above) to 0.45ml of 100mM-Tris-HCl, pH7.4, containing 5mM-ATP, 10mM-MgCl₂, 0.5mM-EDTA, 1mM-GSH, 15mM-KH¹⁴CO₃ (sp. radioactivity approx. 0.4 $\mu\text{Ci}/\mu\text{mol}$) and 0.15mM-acetyl-CoA, together with citrate and albumin as specified, in a sealed plastic tube. Assays were terminated by addition of 5M-HCl (0.1ml). A sample (0.4ml) was evaporated to dryness in a scintillation vial at 85°C, and the residue was dissolved in water (0.2ml) and assayed for radioactivity in a Packard 3320 Tri-Carb liquid-scintillation counter after addition of scintillator [toluene (600ml), methoxyethanol (400ml), naphthalene (80g) and 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (6g)]. When experiments were done with sucrose-containing buffers, care was necessary at this point to avoid charring and subsequent quenching; because of this, in most experiments phosphate-containing buffers were always used. All assays were performed in duplicate and results corrected for the small amount of ^{14}C found in the absence of added acetyl-CoA. Preincubation of the homogenate was done only when specified. It was confirmed that the concentrations of KHCO₃, MgCl₂ and ATP were optimum, and no further increase in enzyme activity was observed if any acetyl-CoA-regenerating system [acetyl phosphate (2mM) and phosphotransacetylase (EC 2.3.1.8) (1 unit/ml)] was included in the assay mixture. The time-course of this assay is not linear after the first few minutes, especially if excess of extract is added (Figs. 1a and 1b). This is probably the result of the presence of malonyl-CoA decarboxylase activity in crude extracts of adipose tissue (Dakshinamurti & Desjardins, 1969). In the present study, the maximum amount of extract per assay did not exceed the equivalent of 15mg wet wt. of tissue and all assays were terminated after 3min.

Results and Discussion

Effect of insulin on acetyl-CoA carboxylase in rat epididymal fat-pads

Tables 1 and 2 summarize the results of a number of experiments in which the effect of insulin on the activity of fat-pad acetyl-CoA carboxylase was studied. In all experiments the fat-pads were incubated for 30min in the presence of glucose with or without insulin, and then blotted, frozen and powdered under liquid N_2 . Extracts were made from the frozen powder by using media with or without added albumin, and acetyl-CoA carboxylase was assayed immediately, either in the absence (Table 1) or the

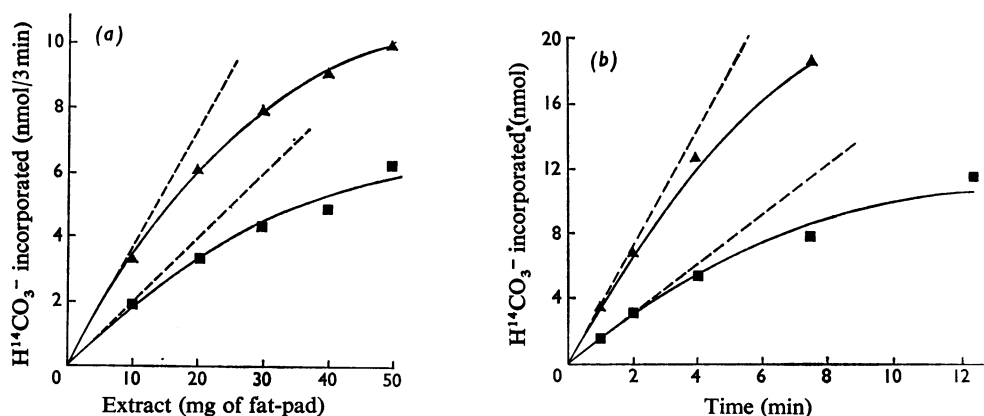


Fig. 1. Non-linearity of the acetyl-CoA carboxylase assay with (a) increasing amounts of fat-pad extract and (b) time

Extracts were prepared from non-incubated fat-pads by using the sucrose buffer as described under 'Methods'. Acetyl-CoA carboxylase activity was assayed in the presence of albumin (10mg/ml) either (a) for 3 min with various amounts of extract or (b) with the equivalent of 50mg of fat-pad for various times. Experiments were carried out before (■) or after (▲) incubation of the extract for 30 min at 30°C. Tangents at the origin are shown as broken lines.

Table 1. Effect of insulin on a number of extramitochondrial enzymes involved in fatty acid synthesis

Paired groups of four fat-pads were preincubated with shaking for 30 min in bicarbonate-buffered medium (10 ml) at 37°C and then transferred to fresh medium (10 ml) containing glucose (2 mg/ml), with or without insulin (10 units/ml), and incubated with shaking for a further 30 min at 37°C. Pads were then extracted with sucrose (0.25 M) containing Tris (20 mM), EDTA (2 mM), GSH (5 mM) and albumin (10 mg/ml), pH 7.3, and enzymes were assayed in extracts as described under 'Methods'. Albumin (10 mg/ml), but not citrate, was present in the assay of acetyl-CoA carboxylase. Results are given as means \pm S.E.M. of four observations on separate groups of fat-pads. * $P < 0.001$ versus control.

Enzyme	Enzyme activity (μ mol/min per g wet wt. of tissue)		
	With insulin	Without insulin (control)	Effect of insulin (% of control)
ATP-citrate lyase	1.06 \pm 0.08	0.99 \pm 0.06	107 \pm 3
Lactate dehydrogenase	10.53 \pm 0.50	10.66 \pm 0.85	99 \pm 8
NADP-malate dehydrogenase	3.58 \pm 0.31	3.48 \pm 0.31	103 \pm 2
Fatty acid synthetase	0.54 \pm 0.05	0.50 \pm 0.03	108 \pm 4
Acetyl-CoA carboxylase	0.024 \pm 0.005	0.006 \pm 0.001*	400 \pm 29*

presence (Table 2) of citrate. Under all assay conditions, exposure of fat-pads to insulin led to a doubling or more of the activity of acetyl-CoA carboxylase (Tables 1 and 2). No change was detected in the activities of a number of other cytoplasmic enzymes involved in fatty acid synthesis, namely ATP-citrate lyase, NADP-malate dehydrogenase and fatty acid synthetase (Table 1). In Table 2, and subsequently in this study, we have expressed activities of acetyl-CoA carboxylase not only in terms of

wet weight of tissue but also in terms of NADP-malate dehydrogenase activity measured in the same extracts. Since NADP-malate dehydrogenase is an exclusively cytoplasmic enzyme (Martin & Denton, 1970), like acetyl-CoA carboxylase, expression of results in terms of NADP-malate dehydrogenase activity should correct for any errors that might arise from differences in extraction efficiency.

Freezing and powdering the tissue with liquid N₂ is a convenient means of arresting metabolism and

Table 2. Effect of insulin on activity of acetyl-CoA carboxylase extracted and assayed in the presence or absence of albumin

The procedure was as described in Table 1 except that fat-pads were extracted in potassium phosphate buffer, pH 7.3 (100mm-potassium phosphate, 2mm-EDTA, 5mm-GSH), with or without albumin (10mg/ml). Citrate (2mm) and, where indicated, albumin (10mg/ml) were added to the acetyl-CoA carboxylase assay medium. In Expts. 3 and 4 the final concentrations of albumin in the assay were 1 and 11mg/ml respectively when correction is made for addition of albumin in the sample of extract. No significant effects of insulin on the activity of NADP-malate dehydrogenase in terms of wet wt. of tissue were observed. Results are given as means \pm S.E.M. of four observations. * $P < 0.02$; ** $P < 0.01$.

Expt. no.	Albumin in extraction medium	Albumin in assay medium	Acetyl-CoA carboxylase activity (nmol/min per g wet wt. of tissue)		$10^3 \times$ Activity ratio (acetyl-CoA carboxylase/NADP-malate dehydrogenase)		Effect of insulin (% of control)
			With insulin	Without insulin (control)	With insulin	Without insulin (control)	
1	-	-	24.6 \pm 4.4	11.8 \pm 1.4*	8.73 \pm 0.93	4.36 \pm 0.20**	200 \pm 17**
2	-	+	137.3 \pm 12.5	68.5 \pm 6.2**	49.37 \pm 1.70	25.62 \pm 0.94**	193 \pm 4**
3	+	-	60.5 \pm 5.4	23.9 \pm 1.2**	19.55 \pm 2.28	8.23 \pm 0.90**	239 \pm 17**
4	+	+	144.7 \pm 8.0	74.9 \pm 5.4**	46.54 \pm 3.07	25.43 \pm 1.84**	182 \pm 4**

aiding speedy extraction. This procedure had been used previously in the study of effects of insulin on pyruvate dehydrogenase (Coore *et al.*, 1971). However, in some experiments not shown in Tables 1 and 2 tissues were extracted without freezing. It was found that, provided that extraction and assay were performed rapidly after termination of incubation, results were obtained similar to those where freezing was employed. The effect of insulin could also be demonstrated by using isolated fat-cells (Rodbell, 1964), extracted by vortex-mixing (Martin & Denton,

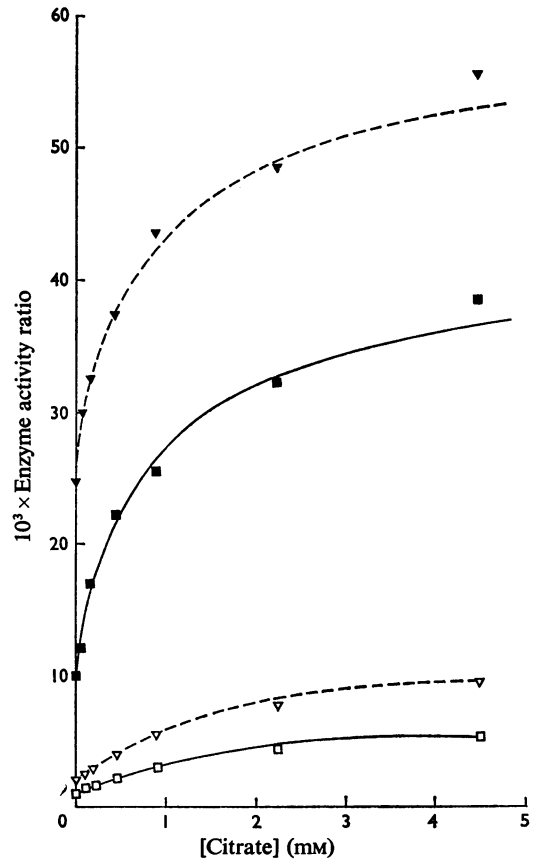


Fig. 2. Effects of citrate and albumin in the assay of acetyl-CoA carboxylase from control (□, ■) or insulin-treated (▽, ▼) fat-pads

The procedure for incubation of fat-pads and preparation of extracts was as given in Table 2. Samples of extract were assayed in the presence (■, ▼) or absence (□, ▽) of albumin (10mg/ml) with increasing concentrations of citrate as indicated. The enzyme activity ratio is given by acetyl-CoA carboxylase/NADP-malate dehydrogenase.

1970), again provided that extraction and assay were performed immediately.

The presence of albumin in the extraction medium and of both albumin and citrate during the assay was necessary to obtain the maximum observed acetyl-CoA carboxylase activity (Table 2; see also Fig. 2). Under these conditions the activity extracted reached approx. 70 nmol/min per g wet wt. from tissue incubated in the absence of insulin, and this was increased to approx. 140 nmol/min per g wet wt. from tissue incubated in the presence of insulin. This latter activity is sufficient to account for the rates of fatty acid synthesis observed in fat-pads incubated with glucose and insulin. This is approx. 160 nmol of acetyl units/min per g wet wt. of tissue at 37°C (Flatt & Ball, 1964; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970), which would be equivalent to approx. 100 nmol of acetyl units/min per g wet wt. of tissue at 30°C.

Relationship between albumin, citrate and the effect of insulin on the activity of acetyl-CoA carboxylase

The activation of acetyl-CoA carboxylase by addition of citrate or albumin or both to the assay is shown in Fig. 2. Increasing citrate concentrations increases the activity of acetyl-CoA carboxylase in both the absence and the presence of albumin, but the effect of insulin is not lost. In the absence of albumin the K_a values for citrate (i.e. the concentrations of citrate required to give 50% activation),

calculated from the results in Fig. 1 by least-squares fit, are 1.73 ± 0.21 and 1.75 ± 0.24 mM for the enzyme from insulin-treated and control fat-pads respectively; with albumin added to the assay medium the values are 0.66 ± 0.10 and 0.76 ± 0.12 mM respectively. It would appear that insulin treatment does not alter the K_a citrate but that this value is decreased in the presence of albumin.

The marked stimulation of acetyl-CoA carboxylase activity in crude extracts of tissues in the presence of albumin has been noted previously in a number of laboratories. The suggestion has been made that the activation stems from the removal of some hydrophobic inhibitor such as long-chain fatty acids (Marquis *et al.*, 1968; Moss & Lane, 1971); long-chain fatty acids have been reported to inhibit the enzyme from rat liver and mammary gland competitively with respect to citrate (Levy, 1963; Moss & Lane, 1971). Table 3 shows the inhibition of fat-pad acetyl-CoA carboxylase by palmitate and triolein (added as a freshly prepared emulsion in water) in the presence of a low concentration of albumin (0.5 or 1 mg/ml) in the assay. Increasing the concentration of palmitate or triolein emulsion increases the inhibition and the enzyme activity approaches that observed in the absence of albumin; increasing the albumin concentration lessens the inhibition by both substances. The amount of triglyceride and fatty acids carried over from fat-pads into extracts is difficult to assess, but it is likely to be sufficient to account for the activation by albumin. Thus it has been reported that free fatty

Table 3. *Effects of palmitate and triolein emulsion on the assay of acetyl-CoA carboxylase*

Acetyl-CoA carboxylase activity in extracts of fat-pads was assayed in the presence of 2 mM-citrate with the addition of palmitate, triolein (emulsified in water) and albumin as indicated. The extracts were prepared from frozen powders of non-incubated fat-pads with phosphate buffer as described under 'Methods'.

Additions to acetyl-CoA carboxylase assay medium			Acetyl-CoA carboxylase activity (% of control)
Palmitate (mM)	Triolein emulsion (mM)	Albumin (mg/ml)	
0	—	1.0	100 (control)
0.05	—	1.0	73
0.10	—	1.0	63
0.20	—	1.0	54
0.50	—	1.0	52
0	—	0	33
0.50	—	10.0	71
—	0	0.5	100 (control)
—	0.05	0.5	77
—	0.10	0.5	72
—	0.20	0.5	56
—	0.50	0.5	38
—	1.0	0.5	37
—	0	0	33
—	0.50	10.0	89

acids in intact pads may reach 2–3 $\mu\text{mol/g}$ wet wt. of tissue (White & Engel, 1958; Vaughan, 1961; Steele *et al.*, 1970), which, from the procedure used in this study, would result in a concentration of 0.1 mM-fatty acids in the assay. The effect of insulin does not appear to stem directly from any changes in tissue fatty acids, since there is no loss of the effect when extraction and assay of acetyl-CoA carboxylase is made in the presence of high concentrations of albumin despite the marked increases in enzyme activities observed.

Effects of incubation of extracts of rat epididymal fat-pads on the activity of acetyl-CoA carboxylase

If fat-pad extracts were incubated at 30°C before assay of acetyl-CoA carboxylase it was found that in both the presence and the absence of albumin the activity of the enzyme was increased. Maximum activity was usually observed after 20–30 min. Differences in acetyl-CoA carboxylase activity between extracts from control and insulin-treated fat-pads diminished and in many cases were abolished (Figs. 3a and 3b; see also Fig. 5). In 32 separate experiments, under a variety of conditions of extraction and assay, the insulin effect decreased from $246 \pm 15\%$ before incubation to $130 \pm 7\%$ of the control value after incubation at 30°C for 30 min.

Activation of acetyl-CoA carboxylase by pre-incubation has been observed in extracts of many tissues (see Moss & Lane, 1971) and this has been used as part of the standard assay procedure for the

enzyme. Usually citrate has been added to the pre-incubation medium, since it has been reported that the enzyme from rat liver and mammary gland, as well as adipose tissue, requires lengthy exposure to citrate to become fully active (see Moss & Lane, 1971). On the other hand, the enzyme from bovine adipose tissue and avian liver has been reported to be instantaneously activated by citrate (Moss *et al.*, 1969; Gregolin *et al.*, 1966). Under the conditions used in this study it appears that the activation by citrate is instantaneous also, and that the enhancement of activity observed during incubation of extracts is not accelerated by the presence of citrate. Thus the time-course of activation of acetyl-CoA carboxylase activity in an extract incubated with 20 mM-citrate (giving 2 mM-citrate in the assay) was very close to that in an extract incubated in the absence of citrate but assayed also in the presence of 2 mM-citrate (Fig. 4). Further, the K_m for citrate was not significantly changed by incubation (Fig. 5).

Activation of acetyl-CoA carboxylase by incubation in the absence of citrate has also been reported for the rat liver enzyme by Swanson *et al.* (1968). It should be noted that our studies have been carried out with a crude homogenate of adipose tissue, which has been centrifuged for 1500g-min only. Other studies where citrate appeared to be essential for activation on incubation have used either particle-free high-speed supernatants or purified preparations. It has been suggested that acetyl-CoA carboxylase may be associated with microsomal material in lactating rat mammary gland and rat liver (Smith *et al.*,

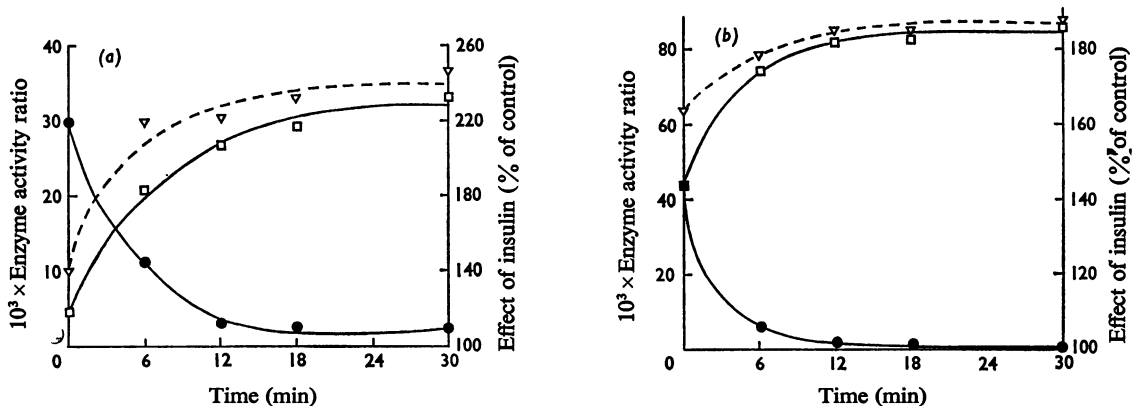


Fig. 3. Effects of incubation on the activity of acetyl-CoA carboxylase in extracts prepared from control (\square) or insulin-treated (∇) fat-pads

The procedure for incubation of fat-pads and preparation of extracts was as given in Table 2. Extracts were incubated for the time indicated at 30°C and then samples were taken for the assay of acetyl-CoA carboxylase activity in the presence of citrate (2 mM) (a) without or (b) with albumin (10 mg/ml). Effect of insulin on acetyl-CoA carboxylase activity as a percentage of the control is shown (\bullet). The enzyme activity ratio is given by acetyl-CoA carboxylase/NADP-malate dehydrogenase.

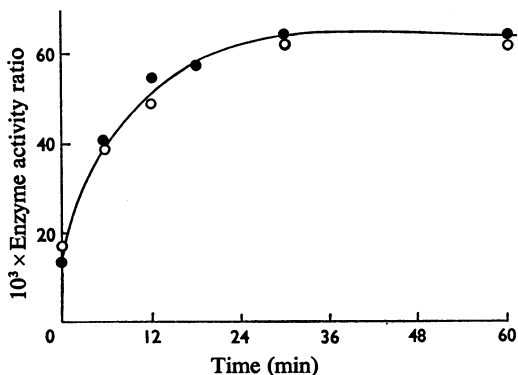


Fig. 4. Effect of citrate on the time-course of activation of adipose-tissue acetyl-CoA carboxylase during incubation of fat-pad extract at 30°C

The procedure for incubation of fat-pads and preparation of extract was as given in Table 2. Samples of extract were incubated at 30°C for the time indicated in the presence (○) or the absence (●) of citrate (20mM). In both cases acetyl-CoA carboxylase was then immediately assayed in the presence of 2mM-citrate. The enzyme activity ratio is given by acetyl-CoA carboxylase/NADP-malate dehydrogenase.

1966; Margolis & Baum, 1966; Easter & Dils, 1968) and it appears that for the rat liver enzyme sensitivity to citrate is much greater in high-speed supernatants than in extracts containing mitochondria and microsomal fractions (Ilfie & Myant, 1970).

Inhibition of acetyl-CoA carboxylase by palmitoyl-CoA

The physiological role of the well-established inhibition of acetyl-CoA carboxylase by long-chain fatty acyl-CoA (see Marquis *et al.*, 1968; Moss & Lane, 1971; Vagelos, 1971) has been questioned because of the apparently non-specific nature of the inhibition and the high concentrations of other proteins likely to bind fatty acyl-CoA in cells (Taketa & Pogell, 1966; Fang & Lowenstein, 1967). However, liver acetyl-CoA carboxylase is still very sensitive to inhibition even in the presence of high concentrations of albumin (Marquis *et al.*, 1968; Goodridge, 1972). Moreover, it has been suggested that the inhibition of fatty acid synthesis in homogenates of liver from rats treated with anti-insulin serum may be the result of inhibition of acetyl-CoA carboxylase by long-chain fatty acyl-CoA (Kalkhoff & Kipnis, 1966). The sensitivity of adipose-tissue enzyme to palmitoyl-CoA under the conditions of this study is shown in Fig. 6.

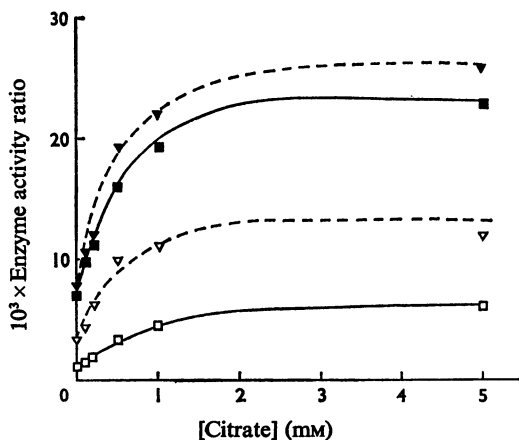


Fig. 5. Comparison of the activation by citrate of acetyl-CoA carboxylase from non-incubated (□, ▽) and incubated (■, ▼) extracts of control (□, ■) and insulin-treated (▽, ▼) fat-pads

The procedure for incubation of fat-pads and preparation of extracts was as described in Table 2. Acetyl-CoA carboxylase was assayed in the presence of various citrate concentrations in samples of extracts before and after incubation of the extracts for 30min at 30°C. The enzyme activity ratio is given by acetyl-CoA carboxylase/NADP-malate dehydrogenase.

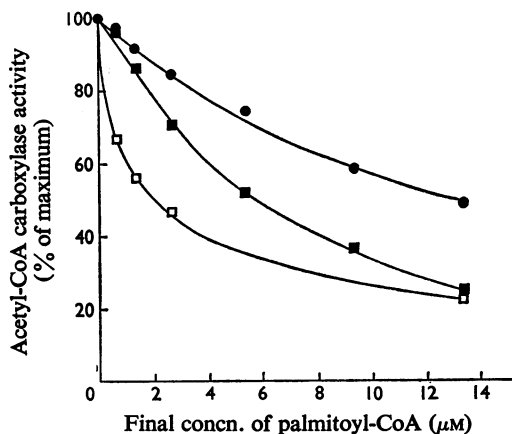


Fig. 6. Effects of palmitoyl-CoA on the activity of acetyl-CoA carboxylase in extracts of fat-pads

Extracts were prepared from non-incubated fat-pads as described in Table 2. Palmitoyl-CoA was added either directly to the assay of acetyl-CoA carboxylase (●), which contained citrate (2mM) and albumin (10mg/ml), or directly to the extract at 0°C in the presence (■) or the absence (□) of albumin (10mg/ml) at a concentration tenfold that to be present in the final assay, 1 min before the extract was diluted tenfold into the assay.

If palmitoyl-CoA was added directly to the assay in the presence of 10mg of albumin/ml the activity of acetyl-CoA carboxylase was approximately halved on addition of $14\mu\text{M}$ -palmitoyl-CoA. More surprisingly, the inhibition was markedly greater if palmitoyl-CoA was added to the fat-pad extract before it was diluted tenfold into the assay. For example, addition of $2.67\mu\text{M}$ -palmitoyl-CoA directly to the assay resulted in a 15% decrease in acetyl-CoA carboxylase activity; however, if $26.7\mu\text{M}$ -palmitoyl-CoA was added to the extract and then after 1 min the extract was diluted tenfold into the assay, the activity of the enzyme was inhibited by about 30 or 55%, depending on the presence or absence respectively of albumin in the extract (Fig. 6). It appears that some of the inhibition caused by the higher concentration of palmitoyl-CoA in the extract persists when the extract is diluted into the assay.

The inhibition by palmitoyl-CoA appeared to be reversible (Fig. 7). Addition of palmitoyl-CoA ($100\mu\text{M}$) to an extract incubated at 30°C in the presence of albumin (10mg/ml) led to a 34% inhibition of enzyme activity 6min after the addition. However, little or no inhibition was discernible 18 or more minutes after the addition, presumably because the palmitoyl-CoA had been hydrolysed by deacylase activity present in the crude extract.

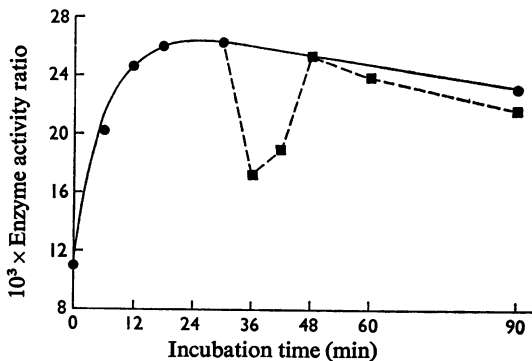


Fig. 7. Reversal of palmitoyl-CoA inhibition of acetyl-CoA carboxylase by incubation at 30°C

An extract containing albumin (10mg/ml) was prepared from non-incubated fat-pads as described in Table 2 and incubated at 30°C . Samples were removed and assayed for acetyl-CoA carboxylase activity (●) at various times. After 30 min palmitoyl-CoA ($100\mu\text{M}$) was added to half the extract and again samples were removed for assay of acetyl-CoA carboxylase activity (■). Assays for acetyl-CoA carboxylase were performed in the presence of albumin (10mg/ml) and citrate (2mM). The enzyme activity ratio is given by acetyl-CoA carboxylase/NADP-malate dehydrogenase.

General conclusions

In this study brief exposure of fat-pads to insulin in the presence of glucose consistently led to a doubling or more in the activity of acetyl-CoA carboxylase in extracts freshly prepared from the incubated tissue. This effect of insulin was evident whether or not extracts were prepared with albumin, and was not noticeably diminished by the presence of citrate or albumin or both in the assay. Incubation of extracts before assay led to activation of acetyl-CoA carboxylase and marked diminution in the insulin effect. The earlier failure to observe an activation of acetyl-CoA carboxylase activity in this laboratory (Coore *et al.*, 1971) was probably due to the much longer time allowed to elapse between extraction of the fat-pads and completion of the enzyme assay.

Whole-tissue concentrations of citrate in fat-pads incubated with glucose are not significantly altered by insulin (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). It is unlikely therefore that the activation of acetyl-CoA carboxylase by insulin involved citrate, especially since the effect in extracts was not abolished by the further addition of citrate. Rather it would appear that the activation of acetyl-CoA carboxylase is a possible explanation of the absence of increase in citrate concentration in fat-pads despite the activation of pyruvate dehydrogenase and therefore a greater rate of citrate formation.

Equally, it would seem unlikely that the effect of insulin observed in this study can be explained in terms of an alteration in the amount of enzyme protein. The 30min exposure to insulin was probably too brief for effects due to changes in rates of enzyme synthesis or degradation to be observed and, moreover, incubation of extracts often led to complete loss of the effect of insulin. Long-term changes in the activity of acetyl-CoA carboxylase in rat liver can, however, be explained in terms of changes in the amount of enzyme protein (Majerus & Kilburn, 1969; Nakanishi & Numa, 1970).

The most likely mechanism for the effect of insulin would appear to be one involving inhibition of the enzyme by long-chain fatty acyl-CoA. The adipose-tissue enzyme is very sensitive to reversible inhibition by palmitoyl-CoA even in the presence of substantial concentrations of albumin. Moreover, the tissue content of long-chain fatty acyl-CoA is decreased by insulin (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). Denton & Halperin (1968) found that, under the same conditions as those used in the present study, the tissue content of long-chain fatty acyl-CoA decreased from 3.25 to 1.68nmol/g wet wt. of tissue (or if the intracellular space of fat-pads is taken to be 2%, from approx. 160 to 85 μM). On the basis of these values, the extracts prepared in this study would contain only about 1.0 and 0.5 μM -fatty acyl-CoA respectively, and

only one-tenth of these concentrations would be present in the assay. Direct addition of such low amounts of palmitoyl-CoA to extracts or assay caused little inhibition (Fig. 6). However, it has also been observed that the inhibition of acetyl-CoA carboxylase by palmitoyl-CoA persists on dilution (Fig. 6). Thus it seems possible that the increase in enzyme activity in extracts reflects an increased enzyme activity in the intact fat-cell caused by a decrease in the concentration of long-chain fatty acyl-CoA. The observed activation of acetyl-CoA carboxylase and loss of the insulin effect on incubation of fat-pad extracts may then be explained by a slow dissociation of fatty acyl-CoA from acetyl-CoA carboxylase. Fritz & Hsu (1967) have previously concluded that acetyl-CoA carboxylase in homogenates of rat livers may be masked by fatty acyl-CoA (or some other hydrophobic inhibitor) and that this masking was lost on incubation with palmitoyl-D-carnitine.

Clearly, further studies are needed to substantiate the proposed role of fatty acyl-CoA in the observed activation of fat-cell acetyl-CoA carboxylase by insulin, particularly on the nature, specificity and reversibility of the inhibition of acetyl-CoA carboxylase by palmitoyl- and other fatty acyl-CoA derivatives. It should also be demonstrated under a variety of conditions *in vivo* and *in vitro* that changes in concentrations of fat-cell long-chain fatty acyl-CoA are associated with opposing changes in fat-cell acetyl-CoA carboxylase activity.

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