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Hormonal Regulation of Adipose-Tissue Acetyl-Coenzyme A Carboxylase by Changes in the Polymeric State of the Enzyme

THE ROLE OF LONG-CHAIN FATTY ACYL-COENZYME A THIOESTERS AND CITRATE

By ANDREW P. HALESTRAP and RICHARD M. DENTON
Department of Biochemistry, Medical School, University Walk, Bristol BS8 1TD, U.K.

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1. Acetyl-CoA carboxylase activity was measured in extracts of rat epididymal fat-pads either on preparation of the extracts (initial activity) or after incubation of the extracts with citrate (total activity). In the presence of glucose or fructose, brief exposure of pads to insulin increased the initial activity of acetyl-CoA carboxylase; no increase occurred in the absence of substrate. Adrenaline in the presence of glucose and insulin decreased the initial activity. None of these treatments led to a substantial change in the total activity of acetyl-CoA carboxylase. A large decrease in the initial activity of acetyl-CoA carboxylase also occurred with fat-pads obtained from rats that had been starved for 36h although the total activity was little changed by this treatment. 2. Conditions of high-speed centrifugation were found which appear to permit the separation of the polymeric and protomeric forms of the enzyme in fat-pad extracts. After the exposure of the fat-pads to insulin (in the presence of glucose), the proportion of the enzyme in the polymeric form was increased, whereas exposure to adrenaline (in the presence of glucose and insulin) led to a decrease in enzyme activity. 3. These changes are consistent with a role of citrate (as activator) or fatty acyl-CoA thioesters (as inhibitors) in the regulation of the enzyme by insulin and adrenaline; no evidence that the effects of these hormones involve phosphorylation or dephosphorylation of the enzyme could be found. 4. Changes in the whole tissue concentration of citrate and fatty acyl-CoA thioesters were compared with changes in the initial activity of acetyl-CoA carboxylase under a variety of conditions of incubation. No correlation between the citrate concentration and the initial enzyme activity was evident under any condition studied. Except in fat-pads which were exposed to insulin there was little inverse correlation between the concentration in the tissue of fatty acyl-CoA thioesters and the initial activity of acetyl-CoA carboxylase. 5. It is suggested that changes in the concentration of free fatty acyl-CoA thioesters (which may not be reflected in whole tissue concentrations of these metabolites) may be important in the regulation of the activity of acetyl-CoA carboxylase. The possibility is discussed that the concentration of free fatty acyl-CoA thioesters may be controlled by binding to a specific protein with properties similar to albumin.

Recent studies have shown that insulin causes an activation of two key lipogenic enzymes in rat epididymal fat-pads, pyruvate dehydrogenase (EC 1.2.4.1) (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971) and acetyl-CoA carboxylase (EC 6.4.1.2) (Halestrap & Denton, 1973). The increased activity of pyruvate dehydrogenase is the result of an increase in the proportion of the enzyme in the active non-phosphorylated form and may involve activation of pyruvate dehydrogenase phosphate phosphatase or inhibition of pyruvate dehydrogenase kinase (Denton *et al.*, 1972; Martin *et al.*, 1972; Taylor *et al.*, 1973; Severson *et al.*, 1974). It has been established for some time that purified preparations of acetyl-CoA carboxylase from avian and mammalian tissues can exist either as an inactive protomer or an active polymer. The interconversion of these forms has been studied by using density-gradient centrifugation and electron microscopy. The formation of the active polymeric form of the enzyme is enhanced by citrate, but is inhibited by fatty acyl-CoA thioesters or by carboxylation of the enzyme-bound biotin (Numa *et al.*, 1965, 1967; Numa & Ringelmann, 1965; Gregolin *et al.*, 1968; Lane *et al.*, 1970; Moss *et al.*, 1972). These observations have led to the hypothesis that the enzyme may be regulated by changes in the concentrations of long-chain fatty acyl-CoA thioesters and citrate within the cell (see Vagelos, 1971; Moss & Lane, 1971; Volpe & Vagelos, 1973; Goodridge, 1973*a*,*b*). In addition, Carlson & Kim

(1973) have proposed that the rat liver enzyme may be regulated by a phosphorylation–dephosphorylation mechanism.

In a previous paper (Halestrap & Denton, 1973), we suggested that the activation of acetyl-CoA carboxylase in rat fat-pads which had been treated with insulin may be the result of the decrease in the concentration of long-chain fatty acyl-CoA thioesters in the tissue which occurs under these conditions (Denton & Halperin, 1968). It was shown that the inhibition by long-chain fatty acyl-CoA thioesters has the necessary properties of reversibility and concentration dependence to be a physiological effector of the enzyme, which is in agreement with the results of Goodridge (1972) on the chicken liver enzyme. In the present paper we report that exposure of rat fat-pads to adrenaline in the presence of insulin greatly diminishes the activity of acetyl-CoA carboxylase in extracts which were subsequently prepared from the fat-pads. Evidence from centrifugation studies is presented which indicates that the effects of insulin and adrenaline on the activity of acetyl-CoA carboxylase may be accompanied by changes in the proportion of the enzyme in the polymeric form. This appears to be the first direct evidence for polymer–protomer transitions being involved in the physiological regulation of the activity of acetyl-CoA carboxylase and is consistent with a role for long-chain fatty acyl-CoA thioesters or citrate in this regulation. No evidence for a control mechanism involving phosphorylation and dephosphorylation was found. Changes in the whole tissue concentrations of fatty acyl-CoA thioesters and citrate have been compared with the activity of acetyl-CoA carboxylase extracted from rat fat-pads incubated under a variety of conditions. The role of fatty acyl-CoA thioesters in the regulation of this enzyme is discussed in the light of these results and suggestions are made as to the cellular disposition of these metabolites.

Experimental

Materials

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

Chemicals. Except where stated, enzymes, substrates, coenzymes and triethanolamine hydrochloride were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Adrenaline and all other chemicals were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

L-Malic acid, palmitoyl-CoA and bovine serum albumin (fraction V, essentially fatty acid- and citrate-free) were obtained from Sigma 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 3mM-HCl to yield a stock solution of 10 units/ml. Sodium itaconate was a gift of Pfizer Ltd., Sandwich, Kent. Acetyl-CoA was prepared by the method of Simon & Shemin (1953); excess of acetic anhydride and acetic acid were removed by two extractions with diethyl ether after acidification with HCl to pH4.5.

2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was purified from pig heart by the method of Sanadi *et al.* (1952). Long-chain fatty acyl-CoA hydrolase (EC 3.1.2.2) was partially purified from rat brain as follows. Fresh brain (1g wet wt.) was homogenized in 3ml of buffer [100mM-potassium phosphate buffer containing EDTA (2mM), adjusted to pH7.4 with 1M-KOH] in an all-glass tissue grinder. After centrifugation at 100000*g* for 1h the supernatant was adjusted to 50% saturation with $(NH_4)_2SO_4$. The protein fraction precipitating between 50 and 65%-satd. $(NH_4)_2SO_4$ was collected by centrifugation and made up in 1ml of the phosphate buffer described above. This preparation contained fatty acyl-CoA hydrolase at a concentration of 18 units/ml and had a specific activity of approx. 2.5 units/mg of protein (for definition of units see below).

Media. Fat-pads were incubated in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5). Additions were made as indicated in the text and tables.

Methods

Extractions of fat-pads for enzyme analysis. After incubation, fat-pads were lightly blotted and rapidly frozen and ground in liquid N_2. Samples of the frozen powders were extracted at 0°C in phosphate buffer (50mM-potassium phosphate–2mM-EDTA–4mM-GSH, adjusted to pH7.3 with 1M-KOH) in a Polytron PT10 OD homogenizer for 30s at half-maximum speed, by using approx. 300mg of powder/ml of phosphate buffer. This extraction procedure gave results identical with those described previously when an all-glass tissue grinder was used (Halestrap & Denton, 1973). After extraction the homogenate was centrifuged at 15000$g_{av.}$ for 2min in an Eppendorf 3200 centrifuge and the infranatant used for enzyme assays and further experiments.

In some experiments high-speed centrifugation of the extracts was performed at 4°C in an MSE 65 refrigerated centrifuge with a 10×10ml titanium rotor and polycarbonate screw-cap tubes at 52000rev./min 260000$g_{max.}$ for 1h. The resulting supernatant was removed by syringe and the pellet made up in the original volume of phosphate buffer

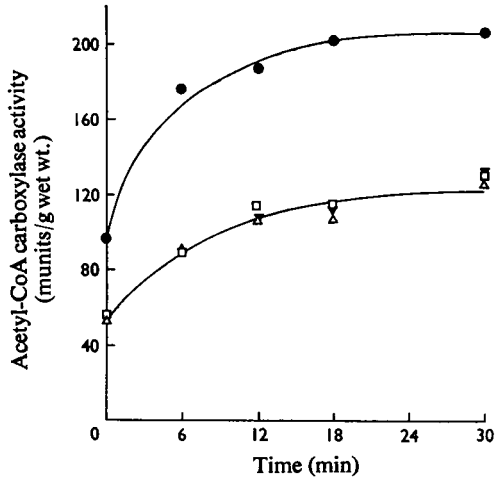

Fig. 1. *Effects of citrate, albumin and palmitoyl-*DL*-carnitine on the activation of acetyl-CoA carboxylase by preincubation*

Extracts of 12 frozen fat-pads which had not been incubated were made with the phosphate extraction buffer described in the text. The following additions were made to samples of the extract before they were incubated at 30°C for the times indicated: none (▼), albumin (10mg/ml) (△); 0.1mM-palmitoyl-DL-carnitine (□); 20mM-potassium citrate (●). In all cases acetyl-CoA carboxylase activity was assayed in the presence of albumin (10mg/ml) and citrate (2mM).

containing additions of citrate and albumin as indicated in the Table legends.

Enzyme assays. $NADP^+$–malate dehydrogenase (EC 1.1.1.40) was assayed as described by Martin & Denton (1970). Long-chain fatty acyl-CoA hydrolase was assayed in Tris–HCl buffer (100mM), pH7.4, containing 0.1mM-5,5′-dithiobis-(2-nitrobenzoate) and 5μM-palmitoyl-CoA. The increase in adsorption at 412nm due to the reaction of 5,5′-dithiobis-(2-nitrobenzoate) with the free CoA released was measured.

Acetyl-CoA carboxylase was assayed by measuring the incorporation of [^{14}C]bicarbonate into acid-stable material in 3min as described previously (Halestrap & Denton, 1973). Extracts were only preincubated before assay where indicated; additions to the assay medium are given in the legends to the Tables and Figures.

Enzyme activities are given as units (1μmol of substrate converted/min) measured at 30°C. Since $NADP^+$–malate dehydrogenase is a purely cytoplasmic enzyme (Martin & Denton, 1970) in this tissue the activity of this enzyme has been used in some experiments as an index of recovery of acetyl-CoA carboxylase activity.

The total activity of acetyl-CoA carboxylase is taken to be the activity present in extracts after incubation at 30°C for 30min with 20mM-potassium citrate. Activation by citrate was complete in approx. 20min (Fig. 1). As found previously, some activation of acetyl-CoA carboxylase was observed when extracts were incubated in the absence of citrate (Halestrap & Denton, 1973). The initial activities of acetyl-CoA carboxylase were measured within 15min of extraction unless otherwise stated.

Determination of the concentrations of citrate and long-chain fatty acyl-CoA thioesters in whole tissue. The spectrophotometric assays described below were performed with a sensitive split-beam spectrophotometer designed and built in this Department. The sensitivity of this instrument allows changes in the concentration of NADH in the cuvette of 0.5–2.0μM to be measured to better than 5% accuracy.

Samples (1g) of frozen, ground fat-pad were extracted with ice-cold 5% (w/v) $HClO_4$ (2ml) and diethyl ether (10ml) as described by Denton & Halperin (1968). After homogenization and centrifugation the diethyl ether phase was removed by aspiration. The extraction procedure was then repeated twice with fresh 10ml portions of diethyl ether. The remaining $HClO_4$ extract and precipitate were freed from diethyl ether by passing a stream of N_2 through the solution, which was then centrifuged to sediment the insoluble protein and acid-insoluble CoA. The supernatant was neutralized with 2M-KOH containing 0.1M-triethanolamine hydrochloride and assayed for citrate spectrophotometrically as described by Moellering & Gruber (1966). Long-chain fatty acyl-CoA thioesters were assayed as acid-insoluble CoA (Denton & Halperin, 1968). The $HClO_4$-insoluble precipitate was suspended in 0.75ml of 50mM-potassium phosphate buffer, pH7.0, containing 25mM-2-mercaptoethanol, and adjusted to pH13 by the addition of 10M-KOH. After incubation at this pH for 8min at 55°C (conditions which were found to be optimum for the recovery of CoA) the solution was acidified with 70% (w/v) $HClO_4$, cooled on ice for 10min, centrifuged and the supernatant neutralized with 2M-KOH containing 0.1M-triethanolamine hydrochloride. The released CoA was assayed by using 2-oxoglutarate dehydrogenase as described by Garland *et al.* (1965).

Results

Effects of incubation of rat epididymal fat-pads with insulin, adrenaline, glucose and fructose on the activity of acetyl-CoA carboxylase

Fat-pads were incubated under a number of conditions with greatly differing rates of fatty acid synthesis. The activity of acetyl-CoA carboxylase was measured either immediately on extraction (control

activity) or after incubation of the extract with citrate (total activity). The results are summarized in Table 1. As previously reported (Halestrap & Denton, 1973) the initial activity of acetyl-CoA carboxylase is markedly increased after exposure of fat-pads to insulin in the presence of glucose; in the present series of experiments the initial activity (measured in the presence of albumin but not citrate) increased from about 14 to 27% of the total activity. In the presence of fructose, the effect of insulin appeared to be slightly less than in the presence of glucose, and in the absence of substrate insulin had little or no effect. The initial activity in the absence of substrate may be somewhat greater than that with glucose or fructose.

The addition of adrenaline in the presence of insulin decreases the rate of fatty acid synthesis in fat-pads (Saggerson & Greenbaum, 1970*a*; Denton & Halperin, 1968) and also the activity of pyruvate dehydrogenase (Coore *et al.*, 1971; Taylor *et al.*, 1973). Marked inhibitory effects can also be demonstrated on the initial activity of acetyl-CoA carboxylase. In the experiments reported in Table 1 adrenaline decreased the initial activity by more than half. A small (but not significant) diminution in the total activity of acetyl-CoA carboxylase was seen with adrenaline; no changes in total activity were apparent with insulin, glucose or fructose.

Changes in the sedimentation of acetyl-CoA carboxylase in fat-pad extracts during high-speed centrifugation

The results shown in Table 2 indicate that exposure of extracts from fat-pads to citrate, ATP or palmitoyl-CoA leads not only to changes in the initial activity of acetyl-CoA carboxylase but also to parallel changes in the proportion of the total acetyl-CoA carboxylase activity sedimented during subsequent high-speed centrifugation. The ratio of the acetyl-CoA carboxylase activity in the pellet to that in the supernatant was increased nearly fivefold after exposure to citrate. By contrast, exposure to palmitoyl-CoA or ATP decreased the ratio by approx. 50% and 25% respectively. In all cases the recovery of total acetyl-CoA carboxylase activity in the pellet plus supernatant fractions was nearly 100%.

It is well established that only the polymeric form of acetyl-CoA carboxylase is active (Moss *et al.*, 1972). Studies involving electron microscopy and centrifugation of the enzyme through sucrose density gradients have indicated that the changes in acetyl-CoA carboxylase activity induced by treatment with ATP, citrate or palmitoyl-CoA are accompanied by changes in the polymeric state of the enzyme (Numa *et al.*, 1965, 1967; Numa & Ringelmann, 1965; Gregolin *et al.*, 1968; Lane *et al.*, 1970). Thus the findings of the experiments reported in Table 2 appear

Table 1. *Effects of incubation with insulin, adrenaline, glucose and fructose on acetyl-CoA carboxylase activity in epididymal fat-pads*

In each experiment fat-pads from 14 animals were evenly distributed between seven incubation flasks (four fat-pads per flask) containing 10ml of bicarbonate-buffered medium. After preincubation with shaking for 30min the fat-pads were transferred to fresh incubation medium containing, as appropriate, glucose (10mM) or fructose (10mM), insulin (10munits/ml) and adrenaline (1μg/ml) and incubated with shaking for a further 30min. The fat-pads were removed, blotted and frozen in liquid N_2. Samples of frozen and ground fat-pads were extracted and assayed for initial and total acetyl-CoA carboxylase activity as described under 'Methods'. Acetyl-CoA carboxylase activity was assayed in the presence of albumin (10mg/ml) but in the absence of citrate. $NADP^+$–malate dehydrogenase activity was also assayed; this enzyme acted as a cytoplasmic marker and the activity ratio of acetyl-CoA carboxylase to $NADP^+$–malate dehydrogenase was used to compensate for differences in the efficiency of extraction. Results are expressed as the mean ± S.E.M. of observations from four separate experiments. The statistical significance of each result was determined by Student's *t* test and the values are as follows: the effects of insulin versus the appropriate substrate control values, * $P<0.05$, ** $P<0.02$, *** $P<0.01$; the effects of adrenaline versus the control incubation containing glucose plus insulin, † $P<0.001$.

Additions to the incubation medium	Acetyl-CoA carboxylase activity (munits/g wet wt. of tissue)		$10^3\times$Activity ratio (acetyl-CoA carboxylase/$NADP^+$–malate dehydrogenase)		
	Initial	Total	Initial	Total	100×Initial / Total
None	11.8±0.35	68.3±3.98	6.7±0.43	38.2±1.12	17.5±1.17
Insulin	14.8±1.58	79.7±5.64	8.5±1.09	45.3±3.54	18.5±1.04
Glucose	9.8±1.0	71.7±5.85	5.7±0.43	42.0±0.41	13.6±0.96
Glucose plus insulin	18.1±2.30**	67.6±3.86	11.2±1.68**	41.6±2.28	26.9±3.10***
Glucose plus insulin plus adrenaline	6.3±1.30†	57.3±11.14	3.7±0.54†	29.6±3.78	12.4±0.90†
Fructose	10.4±1.52	72.9±10.74	5.5±0.66	38.1±2.82	14.4±1.37
Fructose plus insulin	15.2±4.10	71.1±12.99	8.7±0.99*	42.5±1.56	20.3±1.93*

Table 2. *Effects of incubation with citrate, palmitoyl-CoA and ATP on the sedimentation of acetyl-CoA carboxylase activity in extracts of fat-pads during high-speed centrifugation*

Extracts of powdered frozen fat-pads which had not been incubated were prepared in phosphate buffer (see under 'Methods') containing albumin (10mg/ml), and additions of citrate, palmitoyl-CoA and ATP were made as indicated. Samples were stored at 0°C for 90min or were subjected to high-speed centrifugation as described under 'Methods'. After centrifugation the pellets were resuspended to the original volume in phosphate buffer containing albumin (10mg/ml). Acetyl-CoA carboxylase activity was measured in all cases in the presence of albumin (10mg/ml) and citrate (2mM). The initial activity was the activity present after storage of the extract for 90min at 0°C except where citrate was present, when the extract was incubated for 30min with citrate at 30°C before being stored on ice. The total activity, pellet activity and supernatant activity were measured after incubation of the original extract, resuspended pellet and supernatant respectively with citrate as described under 'Methods'. All results are the mean of results from two separate experiments (each using the fat-pads from 12 animals) which agreed to within 5%.

Additions to the extract	Acetyl-CoA carboxylase activity: In the extract (as munits/g wet wt. of fat-pad)						
	Initial	Total	$\frac{\text{Initial activity} \times 100}{\text{Total activity}}$	$\frac{\text{Pellet activity} \times 100}{\text{Total activity}}$	$\frac{\text{Supernatant activity} \times 100}{\text{Total activity}}$	$\frac{\text{Pellet activity}}{\text{Supernatant activity}}$	$\frac{\text{Supernatant plus pellet activities} \times 100}{\text{Total activity}}$
None	110	204	54	85	28	3.0	113
Citrate	203	233	87	100	7	14.3	107
Palmitoyl-CoA (20μM)	42	143	30	64	40	1.6	104
ATP (5mM) plus $MgCl_2$ (5mM)	67	180	37	70	31	2.3	101

to indicate that similar changes occur in crude extracts of fat-pads and that simple high-speed centrifugation gives some discrimination between the polymeric and protomeric forms of acetyl-CoA carboxylase. The separation did not appear to be complete, since the total activity found in the pellet after centrifugation is rather greater than the initial activity in the extract. By contrast the total activity in the supernatant was correspondingly less than the difference between total and initial activities in the extract (i.e. the amount of enzyme initially present in the protomeric form). Thus under these conditions of centrifugation some of the protomeric form of acetyl-CoA carboxylase is probably sedimented. However, any decrease in the *g*-min used during centrifugation appeared to lead to a markedly lower recovery of the polymeric form in the pellet fraction. The change in the polymeric state of acetyl-CoA carboxylase after treatment of extracts with palmitoyl-CoA, citrate or ATP is reflected in the activity of the enzyme as determined in a 3 min assay. For example, treatment of an extract with palmitoyl-CoA leads to inhibition of the enzyme even when citrate is present in the assay medium at concentrations up to 5 mM (Fig. 2*a*). However, if citrate is present in the extract when palmitoyl-CoA is added little or no inhibition is observed (Fig. 2*b*). This implies that citrate is effective in protecting the enzyme from depolymerization by either palmitoyl-CoA in the extract or by carboxylation during the assay. However, in the short time-period of the assays used in these studies (3 min) citrate does not appear to promote appreciable polymerization.

By using the same centrifugation conditions as those described in Table 2 it was found that the changes in the initial acetyl-CoA carboxylase activity in extracts of fat-pads that had been previously exposed to insulin or to insulin plus adrenaline were associated with parallel changes in the proportion of the enzyme sedimented during centrifugation (Table 3). Exposure to insulin, in the presence of glucose, doubled the ratio of the total activity of acetyl-CoA carboxylase in the pellet fraction to that in the supernatant fraction. By contrast exposure to adrenaline in the presence of insulin and glucose halved this ratio. Thus the changes in the initial activity produced by the hormones would appear to involve changes in the proportion of the enzyme in an active polymeric form. It should be noted that the recovery of total activity in the pellet plus supernatant fractions was close to 100% and that hormonal treatment had only marginal effects on the total enzyme activity in tissue extracts. In these experiments the changes in enzyme activity have to be maintained in the extract during storage or centrifugation at 4°C for 1–2 h. Separate experiments (not shown) have indicated that in extracts stored at 4°C the activity of acetyl-CoA carboxylase declines slowly (about 5–10%/h) but that the effect of insulin is largely maintained. The decline would appear to be caused by slow depolymerization of the active form, since the total activity does not decrease appreciably over this time-period.

Studies on the possibility that changes in acetyl-CoA carboxylase activity caused by insulin and adrenaline might involve phosphorylation and dephosphorylation of the enzyme

The results of Carlson & Kim (1973) suggest the possibility that acetyl-CoA carboxylase from rat liver may be inactivated by phosphorylation which is

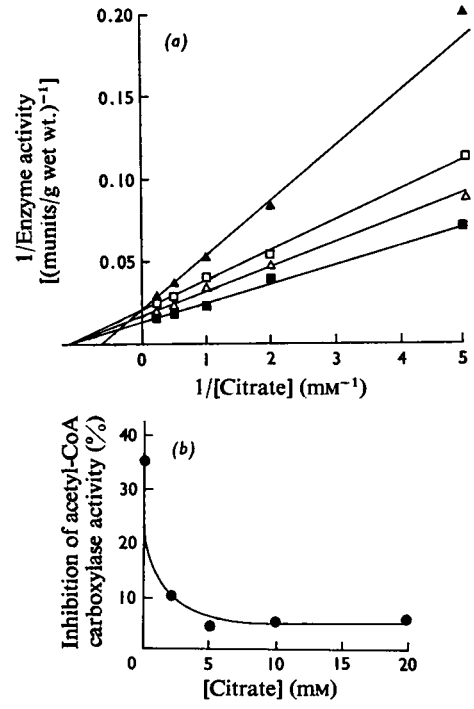

Fig. 2. *Effects of citrate on the inhibition of acetyl-CoA carboxylase by palmitoyl-CoA*

Extracts of 12 fat-pads which had not been incubated were prepared in phosphate buffer containing EDTA (2 mM) and albumin (10 mg/ml). In (*a*), palmitoyl-CoA was added to samples of an extract at 0°C at the following concentrations; (■), 0 μM; (△), 20 μM; (□), 50 μM; (▲), 100 μM. After 5 min acetyl-CoA carboxylase activity was assayed in the samples in the presence of albumin (10 mg/ml) and various concentrations of citrate. In (*b*) palmitoyl-CoA (50 μM) was added to an extract at 30°C together with various concentrations of citrate. After 5 min, acetyl-CoA carboxylase activity was assayed in the presence of albumin (10 g/ml) and citrate (2 mM) and the results are expressed as a percentage of the activity found in the absence of added palmitoyl-CoA.

Table 3. *Effects of incubation of fat-pads with insulin and adrenaline on the sedimentation of acetyl-CoA carboxylase in extracts during high-speed centrifugation*

Paired groups of four fat-pads were preincubated with shaking for 30min in bicarbonate-buffered medium (10ml) at 37°C and then transferred to fresh medium containing glucose (10mM) and other additions as indicated. Conditions for the preparation of extracts, high-speed centrifugation and assay of initial total, pellet and supernatant activity are given in Table 2. Results are given as the mean±S.E.M. of four separate observations. * $P<0.05$; ** $P<0.01$. Initial activity was measured after storage of extracts at 0°C for 90min.

Additions to the incubation medium	Acetyl-CoA carboxylase activity: In the extract (as munits/g wet wt. of fat-pad)		Initial activity×100	Pellet activity×100	Supernatant plus pellet activities×100
	Initial	Total	Total activity	Supernatant activity	Total activity
None	73.3±3.4	135.5±7.2	54.0±2.7	1.56±0.26	97.1±4.7
Insulin	107.6±13.5*	146.1±12.9	73.1±3.5**	2.99±0.49**	99.0±4.3
Insulin	142.2±6.3	212.7±7.9	66.9±1.5	4.63±0.44	112.8±3.8
Insulin plus adrenaline	68.1±3.8**	182.0±7.2*	37.3±0.6**	2.46±0.19**	108.9±2.3

catalysed by an ATP-requiring kinase. Reactivation appeared to involve the action of a phosphatase which required Mg^{2+} ions and was inhibited by F^- ions. Further, the presence of phosphate in purified liver acetyl-CoA carboxylase has been reported (Inoue & Lowenstein, 1972).

The effects of Mg^{2+} chelators and of F^- ions on the activation of acetyl-CoA carboxylase during preincubation of fat-pad extracts in the absence of citrate are shown in Fig. 3. EDTA, EGTA [ethanedioxybis(ethylamine)tetra-acetate] and F^- ions all increase the rate and extent of activation, whereas Mg^{2+} ions considerably inhibit the activation. These results are the exact converse of those expected if the activation observed on incubation of fat-pad extracts involved dephosphorylation along the lines proposed by Carlson & Kim (1973). Lee *et al.* (1973) have also reported that the presence of Mg^{2+} ions does not enhance activation of adipose-tissue acetyl-CoA carboxylase during incubation.

Incubation of fat-pad extracts with ATP and Mg^{2+} ions led to inactivation of the enzyme (Fig. 4), as has been described previously for this enzyme in liver extracts (Greenspan & Lowenstein, 1967, 1968; Carlson & Kim, 1973). In fat-pad extracts, inhibition by ATP was pronounced when the ATP was added after activation of the enzyme by preincubation (Fig. 4*a*), but was much less apparent when 20mM-citrate was present (Fig. 4*b*). This suggests that the effect of ATP is to cause depolymerization of the enzyme, as indicated by the centrifugation data in Table 1. This inhibition may involve ATP-dependent carboxylation of the biotin of acetyl-CoA carboxylase by endogenous HCO_3^-. Carboxylation is known to lead to dissociation and inhibition of the enzyme in the absence but not in the presence of citrate (Numa *et al.*, 1967; Moss & Lane, 1972). Since the inhibition demonstrated by these workers was with the pure avian enzyme, it would seem unlikely to involve phosphorylation.

Relationship between the concentrations of citrate and fatty acyl-CoA thioesters and the activity of acetyl-CoA carboxylase in whole tissue

In Table 4 we report the concentrations of citrate and acid-insoluble CoA in whole tissue found under the same incubation conditions as used in Table 1. The effects of pyruvate and of starvation on the concentration of these metabolites and on the activity of acetyl-CoA carboxylase are shown in Tables 5 and 6. The tissue concentrations of citrate and acid-insoluble CoA together with the initial activity of acetyl-CoA carboxylase are expressed as percentages of the relevant control in Table 7, which serves to summarize the data of Tables 1 and 4–6.

In Table 4 the only significant change in the whole-tissue concentration of citrate was seen when pads

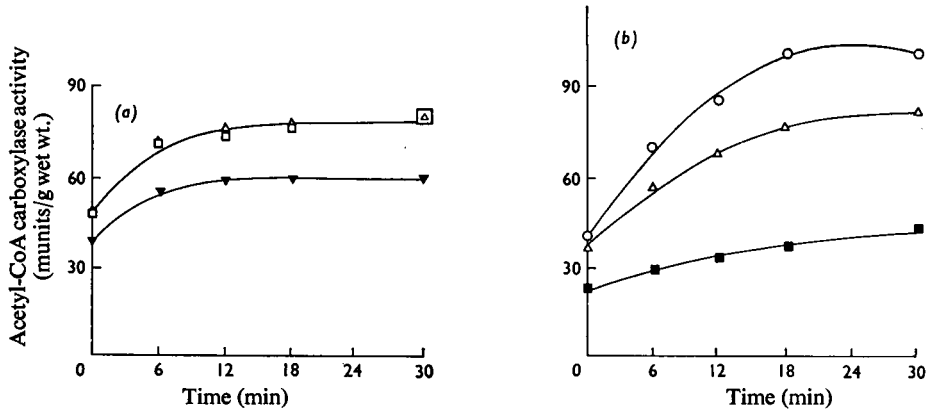

Fig. 3. *Effects of incubation in the presence of albumin, Mg^{2+}, F^- and bivalent-metal-chelating agents on the activity of acetyl CoA carboxylase in extracts of rat epididymal fat-pads*

Extracts of 12 fat-pads which had not been incubated were prepared in phosphate buffer containing 5mM-GSH but no EDTA. The following additions were made to samples of the extract before incubation at 30°C for the times indicated: none (▼), 2mM-EDTA (△), 2mM-EGTA (□), 20mM-NaF (○), 20mM-$MgCl_2$ (■). The conditions used for the enzyme assay are as described in Fig. 1.

which had been incubated with glucose and insulin were exposed to adrenaline. The citrate concentration increased nearly threefold, which agrees with the results of Denton & Halperin (1968) and Saggerson & Greenbaum (1970*a*), but the acetyl-CoA carboxylase activity fell by more than 50%. There may have been a slight decrease in the concentration of citrate when fat-pads which had been incubated with fructose were exposed to insulin; acetyl-CoA carboxylase was activated under these conditions. Pads incubated with glucose and 20mM-pyruvate showed an eightfold increase in whole-tissue concentration of citrate, whereas the activity of acetyl-CoA carboxylase only increased very slightly (Table 5). In this experiment the assay of acetyl-CoA carboxylase proved difficult because the presence of pyruvate in the extract led to some pyruvate carboxylase activity also being measured during the assay of acetyl-CoA carboxylase. This was largely overcome by exposing the extracts to NADH and lactate dehydrogenase before assay and by adding itaconate, which is an inhibitor of pyruvate carboxylase (A. P. Halestrap, unpublished work), to the assay. Citrate was also added to the assay system, since this should increase the activity of acetyl-CoA carboxylase relative to that of pyruvate carboxylase, without overcoming any effect on the activity of acetyl-CoA carboxylase resulting from incubating the fat-pads with pyruvate. It is still possible, even with the precautions taken, that the small apparent increase in the activity of acetyl-CoA carboxylase in extracts of tissues which had been previously incubated with pyruvate was due to pyruvate carboxylase activity.

Table 6 shows that fat-pads taken from starved animals had a slightly raised tissue concentration of citrate compared with fed animals, which agrees with the results of Saggerson & Greenbaum (1970*b*), whereas the initial activity of acetyl-CoA carboxylase was greatly decreased. Thus under the various conditions reported here there is no evidence of any correlation between the activity of acetyl-CoA carboxylase and the concentration of citrate in the whole tissue. Indeed increases in the tissue concentration of citrate are often accompanied by decreases in acetyl-CoA carboxylase activity, and vice versa. The possibility of compartmentation of citrate and its bearing on these results will be considered below.

Concentrations of acid-insoluble CoA in whole tissue have been taken to be a measure of the concentration in whole tissue of long-chain fatty acyl-CoA thioesters. Under several conditions an inverse relationship appears to exist between the concentrations of acid-insoluble CoA and the initial activity of acetyl-CoA carboxylase. In the presence of either fructose or glucose, insulin caused a decrease in the concentration of acid-insoluble CoA and an increase in acetyl-CoA carboxylase activity (Table 1), whereas in the absence of substrate neither parameter changed appreciably with insulin. When fat-pads incubated with glucose and insulin were exposed to adrenaline very little increase in the concentration of acid-insoluble CoA in whole tissue was apparent. However, in similar experiments Denton & Halperin (1968) were able to show a significant increase in the concentration of acid-insoluble CoA; the activity of acetyl-CoA carboxylase decreased by more than 50%

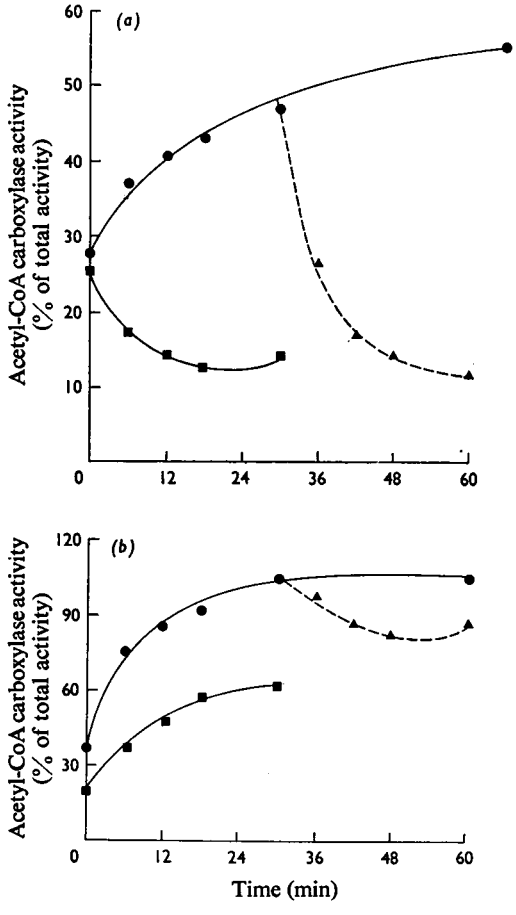

Fig. 4. *Effect of ATP–Mg on the activation of acetyl-CoA carboxylase in crude homogenates of fat-pads*

Extracts were prepared from fat-pads (which had not been incubated) as described in Table 2 and were preincubated at 30°C before assay for the times shown with (■) or without (●) 5mM-ATP–Mg and in the absence (*a*) or presence (*b*) of 20mM-citrate. After 30min incubation 5mM-ATP–Mg was added to part of the control extracts and the incubation continued (▲). The conditions used for the enzyme assay are as described in Fig. 1.

under these conditions (Table 1). The addition of either glucose or fructose to fat-pads incubated in the absence of substrate gave a slight but significant decrease in the concentration of acid-insoluble CoA (Table 4), although the acetyl-CoA carboxylase activity may also have decreased slightly under these conditions (Table 7). A much more clear-cut breakdown in the inverse correlation between concentrations of acid-insoluble CoA and acetyl-CoA carboxylase activity was apparent in pads taken from rats which had been starved for 36h (Table 6). Both the initial enzyme activity and the concentration of acid-insoluble CoA were decreased by 36 and 77% respectively. The total activity of acetyl-CoA carboxylase decreased very little after this short period of starvation; much greater decreases are observed in extracts of both liver and adipose tissue after more prolonged (i.e. 48–72h) starvation (Majerus & Kilburn, 1969; Nakanishi & Numa, 1970; Saggerson & Greenbaum, 1970*b*). The concentration of acid-insoluble CoA in liver has been reported to be increased after 48h starvation (Tubbs & Garland, 1964).

Table 4. *Effects of incubation with insulin, adrenaline, glucose and fructose on whole tissue concentrations of acid-insoluble CoA and citrate in epididymal fat-pads*

Acid-insoluble CoA and citrate were measured in neutralized $HClO_4$ extracts prepared from samples of frozen powdered pads which had been incubated as described in Table 1. The results are given as mean±S.E.M. for four observations, each observation involving four fat-pads from separate animals.

Additions to the incubation medium	Whole tissue concentration (nmol/g wet wt. of tissue)	
	Acid-insoluble CoA	Citrate
None	4.49±0.38	8.54±0.27
Insulin	4.49±0.36	9.85±1.37
Glucose	3.75±0.20	8.16±0.80
Glucose plus insulin	2.68±0.24	6.85±0.43
Glucose plus insulin plus adrenaline	2.89±0.44	20.43±1.26
Fructose	3.72±0.36	7.73±0.68
Fructose plus insulin	2.75±0.29	5.93±0.57

Thus there is some evidence of an inverse correlation between the concentration of whole-tissue long-chain fatty acyl-CoA thioesters and the initial activity of acetyl-CoA carboxylase. However, in fat-pads taken from animals starved for 36h and in fat-pads incubated with adrenaline in the presence of insulin, this correlation appears to break down; in both cases, lipolysis is increased.

Discussion

The stimulation by insulin of the rate of fatty acid synthesis from glucose in rat epididymal fat-pads and its antagonism by adrenaline are well established (Cahill *et al.*, 1960; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970*a*). In the present paper we have shown that insulin and adrenaline have opposing effects on acetyl-CoA carboxylase activity which parallel the effects of these hormones not only on the rate of fatty acid synthesis but also on the

Table 5. *Effect of pyruvate on the activity of acetyl-CoA carboxylase and on the whole-tissue citrate concentration of fat-pads incubated with glucose*

Paired groups of four fat-pads were preincubated with shaking in 10ml of bicarbonate-buffered medium at 37°C for 30min and then transferred to fresh medium containing glucose (10mM) and where indicated, pyruvate (20mM). Fat-pads were extracted for enzyme assay or citrate determinations as described under 'Methods'. Acetyl-CoA carboxylase was assayed in the presence of citrate (2mM), sodium itaconate (1mM) and albumin (10mg/ml). Before assay extracts were incubated with NADH (1mM) and lactate dehydrogenase (50 units/ml) for 15min at 0°C. Enzyme activities and whole tissue citrate concentrations were measured in different experiments. Results are given as the mean ± S.E.M. of four separate observations. Effect of pyruvate versus control: ** $P<0.001$; * $P<0.05$.

Additions to the incubation medium	Acetyl-CoA carboxylase activity (munits/g wet wt.) Initial activity	Total activity	Initial activity × 100 / Total activity	Whole-tissue citrate concentration (nmol/g wet wt.)
Glucose	28.6 ± 1.57	75.43 ± 5.45	39.0 ± 4.0	10.0 ± 1.5
Glucose plus pyruvate	33.56 ± 3.22	62.99 ± 6.91	54.0 ± 4.8*	80.0 ± 2.5**

Table 6. *Effect of starvation for 36h on the fat-pad whole tissue concentration of fatty acyl-CoA and citrate and on the activity of acetyl-CoA carboxylase*

Sixteen rats which were well matched for weight and age were divided into two groups of eight, one of which was allowed free access to food and water while the other group were deprived of food for 36h before death (11.0 a.m.). Rats were killed in pairs by decapitation and the fat-pads removed and rapidly frozen in liquid N_2. The extraction of the fat-pads and the assay of acetyl-CoA carboxylase activity and the concentrations of citrate and acid-insoluble CoA were performed as described in Tables 4 and 5. Results are given as the mean ± S.E.M. for four sets of pads. * $P<0.05$; ** $P<0.02$; *** $P<0.01$.

Dietary status	Acetyl-CoA carboxylase activity (munits/g wet wt.) Initial activity	Total activity	Initial activity × 100 / Total activity	Whole tissue concentrations (nmol/g wet wt.) Acid insoluble-CoA	Citrate
Normal fed animals	44.7 ± 7.3	207 ± 19	22.0 ± 3.6	2.83 ± 0.15	35.22 ± 2.27
Animals starved for 36h	13.3 ± 1.2***	167 ± 11	8.0 ± 0.6***	2.17 ± 0.14**	48.65 ± 4.54*

Table 7. *Relationships between the concentrations of acid-insoluble CoA and citrate in whole tissue and the initial activity of acetyl-CoA carboxylase in epididymal fat-pads*

The whole-tissue concentrations are taken from: (*a*) Table 4; (*b*), Denton & Halperin (1968); (*c*), Table 5; or (*d*) Table 6; the initial acetyl-CoA carboxylase activities are taken from the values expressed as the percentage of the total activity in Tables 1, 5 and 6. All the results are given as the concentration or activity in experimental tissues expressed as a percentage of that in control tissues. *** $P<0.01$, ** $P<0.02$, * $P<0.05$ when compared with control values (i.e. 100%).

Experimental	Control	Source	Experimental whole-tissue concentration (as % of control value) Acid-insoluble CoA	Citrate	Experimental acetyl-CoA carboxylase initial activity (as % of control value)
None	Insulin	a	100	115	105
None	Glucose	a	83**	95	78*
None	Fructose	a	83**	91	82
Glucose plus insulin	Glucose	a	71**	84	198***
		b	52***	95	—
Fructose plus insulin	Fructose	a	74*	77**	141*
Glucose plus insulin plus adrenaline	Glucose plus insulin	a	108	298	46***
		b	152***	263***	—
Glucose plus pyruvate	Glucose	c	—	800***	141*
Starved	Fed	d	77**	138*	36***

activity of pyruvate dehydrogenase (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971). Further, we report that using strictly controlled conditions of high-speed centrifugation we have been able to demonstrate that fatty acyl-CoA thioesters or ATP decrease the acetyl-CoA carboxylase activity in crude homogenates of fat-pads sedimented during centrifugation, whereas citrate increases the activity of acetyl-CoA carboxylase in this fraction (Table 2). Although this technique does not allow complete separation between polymeric and protomeric forms the separation would appear sufficient to demonstrate that extracts from insulin-treated fat-pads contained a greater proportion of the enzyme in its polymeric form. By contrast, prior exposure of the pads to adrenaline increased the proportion of the enzyme in the protomeric form. These results appear to provide the first direct evidence that regulation of the activity of acetyl-CoA carboxylase within cells may involve polymer/protomer transitions.

The question arises as to how insulin and adrenaline exert their effects on the polymeric state of acetyl-CoA carboxylase. It has been suggested that a phosphorylation–dephosphorylation cycle may be involved in the regulation of the activity of acetyl-CoA carboxylase (Carlson & Kim, 1973; Lee *et al.*, 1973), as is known to occur for pyruvate dehydrogenase (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971). However, we have been unable to find any evidence for this. The inhibitory effects of ATP observed with crude homogenates of adipose tissue (Fig. 4) and liver (Greenspan & Lowenstein, 1967, 1968; Carlson & Kim, 1973) appear to be the result of carboxylation of the enzyme-bound biotin by endogenous HCO_3^-.

It seems more likely that adrenaline and insulin regulate acetyl-CoA carboxylase activity through changes in the cellular concentrations of either citrate (which promotes polymerization) or fatty acyl-CoA thioesters (which inhibit polymerization). Attempts to study this possibility are limited by the possible compartmentation of these metabolites in fat-cells. The degree of correlation between the whole-tissue concentrations of long-chain fatty acyl-CoA thioesters and citrate and the activity of acetyl-CoA carboxylase is summarized in Table 7. Although we cannot totally rule out the possibility that citrate may regulate the activity of the enzyme because of the compartmentation of citrate between the mitochondria and cytoplasm, the complete lack of correlation between the whole-tissue concentration of citrate and enzyme activity strongly implies that regulation by citrate may not be of physiological importance under the conditions studied. Most strikingly, in pads incubated with glucose the addition of pyruvate resulted in an eightfold increase in the tissue concentration of citrate, without any significant rise in the initial acetyl-CoA carboxylase activity. In liver, too, there seems to be no consistent correlation between the rate of fatty acid synthesis and the whole-tissue concentration of citrate (Spencer & Lowenstein, 1967; Guynn *et al.*, 1972; Goodridge, 1973*b*).

On the other hand there was some evidence of an inverse correlation between whole-tissue concentration of fatty acyl-CoA thioesters and the initial activity of acetyl-CoA carboxylase. In the presence of either glucose or fructose, insulin decreased the concentration of fatty acyl-CoA thioesters in the fat-pad while causing activation of acetyl-CoA carboxylase. In our experiments, the most serious departures from an inverse correlation were seen when fat-pads which had been incubated with glucose and insulin were exposed to adrenaline, and when fat-pads from animals which had been starved for 36h were compared with those obtained from fed animals. In both these cases the rates of lipolysis are increased. Yeh & Leveille (1971) and Goodridge (1973*a*,*b*) have also reported some inverse correlation between the tissue concentration of fatty acyl-CoA thioesters and the rate of fatty acid synthesis by extracts from chicken liver and by isolated chicken liver cells. Similar results have been reported for extracts from rat liver (Guynn *et al.*, 1972).

There have been objections to postulating a physiological role for the regulation of enzymes by fatty acyl-CoA thioester, since this metabolite at high concentrations can inhibit many enzymes irreversibly, probably because of its detergent properties (Taketa & Pogell, 1966).

However, in previous work we have shown (Halestrap & Denton, 1973) that inhibition of acetyl-CoA carboxylase in extracts of fat-pads by fatty acyl-CoA thioesters is reversible, as has been reported for the chicken liver enzyme (Goodridge, 1972). Although insulin treatment of fat-pads activates acetyl-CoA carboxylase, whereas palmitoyl-CoA treatment of the enzyme inhibits it, there are striking similarities between these effects. Both effects are reversed by preincubation of the extracts, and this reversal is unaffected by the presence of albumin or citrate in the assay and is accompanied by changes in the polymeric state of the enzyme.

It is probable that long-chain fatty acyl-CoA thioesters are largely bound to proteins and/or membranes within the cell (Sümper & Trauble, 1973), since if it were assumed that no binding occurred, then the concentration of long-chain fatty acyl-CoA thioesters within the cell (50–150μM; see Halestrap & Denton, 1973) would be sufficient to inhibit many enzymes irreversibly. In the experiments reported here we have as a routine used albumin (normally at 10mg/ml) to simulate the binding of fatty acyl-CoA thioesters to cellular proteins. To estimate the extent to which palmitoyl-CoA is bound to albumin we have used a preparation of palmitoyl-CoA hydrolase from rat brain (see under 'Methods'). This preparation had a K_m for palmitoyl-CoA of $2.5 \pm 0.3 \mu$M and

was irreversibly inhibited by palmitoyl-CoA at concentrations above 5 μM. The rate of hydrolysis of palmitoyl-CoA in the presence of albumin was greatly decreased. For example, at an albumin concentration of 10 mg/ml and a total palmitoyl-CoA concentration of 100 μM, the rate of hydrolysis was compatible with the concentration of free palmitoyl-CoA being less than 0.1 μM. Under these conditions acetyl-CoA carboxylase is inhibited by about 60% (Halestrap & Denton, 1973), which would suggest that the K_i of the enzyme for palmitoyl CoA is less than 0.1 μM. Goodridge (1972) obtained a K_i value for the partially purified chicken liver enzyme of 0.2 μM, assuming that 98% of palmitoyl-CoA binds to albumin (3.75 mg/ml).

This extremely tight binding of fatty acyl-CoA thioesters to acetyl-CoA carboxylase might suggest that the activation of the enzyme on preincubation of tissue extracts is due to removal of the inhibitor. However, the activation was not increased by the presence of albumin or palmitoyl-DL-carnitine (Fig. 1), both of which are thought to remove fatty acyl-CoA thioesters from the enzyme (Fritz & Hsu, 1967; Goodridge, 1972; Moss *et al.*, 1972). While studying the activity of long-chain fatty acyl-CoA hydrolase of fat-pads (approx. 0.7 unit/g wet wt. of fat-pad at 30°C) we found that the enzyme is competitively inhibited by $MgCl_2$, the K_m for palmitoyl-CoA increasing from a control value of 4.0 ± 0.5 μM in the absence of $MgCl_2$ to 53 ± 11 μM in the presence of 20 mM-$MgCl_2$. In Fig. 3(*b*) we reported that 20 mM-$MgCl_2$ also inhibits the activation, by incubation at 30°C, of acetyl-CoA carboxylase in crude homogenates of adipose tissue. It therefore seems possible that removal of fatty acyl-CoA thioester from acetyl-CoA carboxylase requires deacylase activity. However, we have been unable to enhance the rate of activation of acetyl-CoA carboxylase in extracts of fat-pads by the addition of fatty acyl-CoA hydrolase from rat brain.

The apparent K_i of acetyl-CoA carboxylase for palmitoyl-CoA (less than 0.1 μM) is three orders of magnitude less than the concentration of fatty acyl-CoA thioesters in fat-cells when calculated on the basis of the metabolite being evenly distributed throughout the cell. It appears that extensive binding of fatty acyl-CoA thioesters must occur within the cell. The apparent coexistence of fatty acyl-CoA thioesters with the appreciable activity of long-chain fatty acyl-CoA hydrolase supports this conclusion. Indeed, it seems possible that a specific binding protein for fatty acyl-CoA thioester may exist and that the binding may be subject to regulation. This would allow the concentration of free fatty acyl-CoA thioesters to vary independently of the whole-tissue concentration of these metabolites, and thus offer an explanation of why the whole-tissue concentration of fatty acyl-CoA thioesters may not always vary inversely with the acetyl-CoA carboxylase activity. Albumin seems to be a possible model for such a binding protein, since it binds long-chain fatty acyl-CoA thioester extremely tightly. Albumin also binds unesterified fatty acids and a similar, but distinct, binding protein for unesterified fatty acids has been recognized in a number of tissues (Ockner *et al.*, 1972; Mishkin *et al.*, 1972). By using fatty acyl-CoA hydrolase to estimate binding of palmitoyl-CoA to albumin as described above, it is possible to demonstrate the displacement of palmitoyl-CoA from albumin when palmitate is added (A. P. Halestrap, unpublished work). If such competition for binding sites also occurred with an intracellular binding protein, the intracellular concentrations of free fatty acyl-CoA thioesters might be increased under conditions of enhanced rates of lipolysis without changes in the whole-tissue content of fatty acyl-CoA thioesters. This is a possible explanation for the lack of inverse correlation seen between whole-tissue concentrations of fatty acyl-CoA thioesters and the initial activity of acetyl-CoA carboxylase seen after starvation and on incubation of pads with adrenaline.

The underlying interrelationship between the hormonal regulation of acetyl-CoA carboxylase to that of pyruvate dehydrogenase remains to be elucidated.

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