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Both insulin and epidermal growth factor stimulate lipogenesis and acetyl-CoA carboxylase activity in isolated adipocytes

Importance of homogenization procedure in avoiding artefacts in acetyl-CoA carboxylase assay

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1. Epidermal growth factor (EGF) stimulates lipogenesis by 3–4-fold in isolated adipocytes, with a half-maximal effect at 10 nM-EGF. In the same batches of cells insulin stimulated lipogenesis by 15-fold. 2. Freezing and prolonged homogenization of adipocytes results in release of large quantities of pyruvate carboxylase from broken mitochondria, and sufficient pyruvate can be carried through into assays for this enzyme to cause significant interference with assays of acetyl-CoA carboxylase in crude adipocyte extracts. This may account for the high amount of citrate-independent acetyl-CoA carboxylase activity reported to be present in adipocyte extracts in some previous publications. This problem may be eliminated by homogenizing very briefly without freezing. 3. By using the modified homogenization procedure, EGF treatment of adipocytes was shown to produce an effect on acetyl-CoA carboxylase activity almost identical with that of insulin. Both messengers increase $V_{max.}$ without significant effect on the K_a for the allosteric activator, citrate.

INTRODUCTION

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the first step committed to fatty acid synthesis, and is generally believed to be rate-limiting in the synthesis of fatty acids from cytosolic acetyl-CoA. The activity of acetyl-CoA carboxylase is controlled both by allosteric regulation (e.g. activation by citrate and inhibition by long-chain acyl-CoA) and by reversible phosphorylation (Hardie, 1980; Hardie *et al.*, 1984). 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 and inactivated by cyclic AMP-dependent protein kinase, and there is good evidence that this also occurs during inhibition of fatty acid synthesis by hormones which increase cyclic AMP in hepatocytes and adipocytes, i.e. glucagon and adrenaline (Brownsey & Hardie, 1980; Holland *et al.*, 1984, 1985).

Insulin stimulates fatty acid synthesis in both adipocytes and hepatocytes (Hardie, 1980), i.e. it has the opposite effect to agents which increase cyclic AMP. It might therefore be expected that insulin would bring about a dephosphorylation of acetyl-CoA carboxylase. However, several groups have found that insulin stimulates the phosphorylation of acetyl-CoA carboxylase at a site distinct from that phosphorylated by cyclic AMP-dependent protein kinase (Brownsey & Denton, 1982; Witters *et al.*, 1983; Holland & Hardie, 1985). Brownsey *et al.* (1984) have also found increased acetyl-CoA carboxylase kinase activity in supernatant fractions after insulin treatment of isolated adipocytes, and have suggested that insulin treatment releases a protein (serine) kinase from the plasma membrane which activates acetyl-CoA carboxylase by phosphorylation. However, it is not clear that the increased phosphorylation is the cause of enzyme activation, because the effect of insulin on enzyme activity does not survive enzyme purification on avidin–Sepharose, unlike the effect of insulin on phosphorylation of the enzyme (Witters *et al.*, 1983; Holland & Hardie, 1985).

The plasma-membrane receptor for insulin is now known to share many similarities with receptors for polypeptide growth factors such as EGF, including amino acid sequence homologies, and the fact that the cytoplasmic region of the receptor contains a protein (tyrosine) kinase that is activated by binding of hormone or growth factor (Ullrich *et al.*, 1985; Petruzzelli *et al.*, 1984; Buhrow *et al.*, 1982). In addition, insulin and EGF cause increased phosphorylation of the same set of intracellular proteins on serine residues, e.g. ribosomal protein S6 (Martin-Perez *et al.*, 1984) and unidentified polypeptides of M_r 22000 (Blackshear *et al.*, 1982) and 46000 (Holland & Hardie, 1985).

We have previously shown that insulin and EGF stimulate both fatty acid synthesis and the phosphorylation of ATP citrate lyase and acetyl-CoA carboxylase in isolated hepatocytes (Holland & Hardie, 1985). In order to determine whether EGF stimulates acetyl-CoA carboxylase activity, we have now turned our attention to isolated adipocytes, where insulin treatment of cells has been reported to give an increase in enzyme activity that can be measured in cell extracts (Halestrap & Denton, 1973). During the course of this work, we found that methods used previously to examine effects of hormone treatment of cells on acetyl-CoA carboxylase activity were subject to interference by pyruvate carboxylase. We show that this interference can be avoided if an appropriate homogenization procedure is used. Using this procedure, we also found that insulin and EGF have very similar effects on acetyl-CoA carboxylase activity in isolated adipocytes.

Abbreviation used: EGF, epidermal growth factor.

EXPERIMENTAL

Animals

Male Wistar rats (body wt. 120–160 g) were used, and were allowed unrestricted access to standard pelleted diet and water.

Preparation of isolated adipocytes

Isolated adipocytes were prepared by a modification of the method of Rodbell (1964). The whole epididymal and perirenal fat-pads from six rats were digested without mincing in 10 ml of Buffer A [collagenase (0.5 mg/ml), bovine serum albumin (3.5%, w/v), 590 mM-NaCl, 0.2 mM-NaH_2PO_4, 2.6 mM-$CaCl_2$, 4.8 mM-KCl, 1.2 mM-$MgSO_4$, 24 mM-Hepes, 0.5 mM-glucose, adjusted to pH 7.4 with NaOH]. Digestion was carried out in 50 ml polyethylene flasks for 90 min at 37 °C in a reciprocating shaker (100 cycles/min).

Cells were harvested by centrifugation in a 15 ml polypropylene centrifuge tube (300 *g* for 30 s), with removal by aspiration of the infranatant medium. They were washed five times by resuspension in 10 vol. of buffer A without collagenase and with serum albumin at 1% (Buffer B), with harvesting each time by centrifugation. The cells were counted in a Neuberg chamber and diluted to 10^6 cells/ml in buffer B. They were then incubated in 20 ml polyethylene scintillation vials in a reciprocating shaker (30 cycles/min) at 37 °C. Cells were preincubated for 30 min before hormone treatment, which was for 15 min unless stated otherwise.

Measurement of lipogenesis and fatty acid synthesis

Incorporation of radioactivity from [^{3}H]glucose into total lipid was determined by incubating cells in [^{3}H]-glucose (0.5 mM; 0.18 mCi/mol) and terminating the incubation by adding 1 ml of cell suspension to 10 ml of toluene-based scintillation fluid (Optiscint 'T'). After thorough mixing, the mixture was left for 60 min at room temperature to allow the aqueous and toluene phases to separate. The radioactivity in the upper phase was then determined by scintillation counting: β-particles derived from the aqueous phase do not cross the phase boundary to any significant extent, and it is not necessary to remove the aqueous phase. Saponification, extraction and counting of fatty acids were as described previously (Holland *et al.*, 1984).

Preparation of extracts and enzyme assays

Cells were harvested as described above and the infranatant medium was removed by aspiration. Two methods for the preparation of homogenates were used. (1) The packed cells were frozen by immersing the tube in liquid N_2 and were stored at −70 °C. The cell pellets were thawed and 0.5 ml of ice-cold homogenization buffer was added (0.25 M-mannitol, 50 mM-NaF, 2 mM-EDTA, 1 mM-EGTA, 50 mM-Tris/HCl, pH 7.4 at 0 °C). Homogenization was for 30–40 s with a Polytron homogenizer at setting 3. (2) As (1), except that cells were homogenized for only 5 s and without freezing and storage at −70 °C. In both cases the homogenate was centrifuged (14000 *g* for 30 s) and the infranatant extract removed from the pellet and floating fat-cake by aspiration. Protein content was determined by the method of Bradford (1976). For the assay of acetyl-CoA carboxylase, the extract was diluted with an equal volume of 20% bovine serum albumin and assayed as described previously (Holland *et al.*, 1984).

Ion-exchange and reversed-phase h.p.l.c. analysis of acetyl-CoA carboxylase assay reaction products

The normal assay was scaled up 5-fold, was stopped with 0.2 vol. of 6M-HCl and the mixture dried down in a Savant Speedvac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.). The samples were redissolved in 1 ml of 10 mM-potassium phosphate (pH 4.0) and analysed on a Whatman Partisil-10 SAX analytical column, eluted with a gradient of 10 mM–1 M-potassium phosphate, pH 4.0 (30 min linear gradient at 1 ml/min); 1 ml fractions were collected and samples were counted for radioactivity in Optiphase 'MP' scintillation fluid. Standards ([^{14}C]aspartate, [^{14}C]malonate, [^{14}C]malonyl-CoA) were dried down and analysed in the same way as samples.

The putative aspartate peak in the experimental samples (see Fig. 3*a*) was also analysed by using the Waters Pico-Tag amino acid analysis system after pre-column derivative formation with phenyl isothiocyanate (Millipore/Waters Chromatography Division, Harrow, Middx., U.K.).

Materials

Adrenaline and bovine serum albumin (essentially fatty-acid-free) were from Sigma Chemical Co., Poole, Dorset, U.K.; collagenase, ATP and acetyl-CoA were from Boehringer Mannheim, Mannheim, Germany; insulin (human Actrapid) was from Novo Industri, Copenhagen, Denmark; Hepes (ultra-pure) was from Schwarz–Mann, Cambridge, MA, U.S.A.; $NaH^{14}CO_3$, [^{3}H]glucose, [^{14}C]malonyl-CoA and [^{14}C]aspartate were from Amersham International, Amersham, Bucks., U.K. [^{14}C]Malonate was made by hydrolysis of [^{14}C]malonyl-CoA for 1 h in 1 M-NaOH at room temperature. Optiscint 'T' and Optiphase 'MP' scintillation fluids were from Fisons, Loughborough, Leics., U.K. EGF purified from mouse submaxillary gland either was from Sigma Chemical Co. or was a gift from Dr. Peter Parker, Imperial Cancer Research Fund, London.

Expression of results and statistical significance

Unless stated otherwise, results are expressed as means ± S.E.M., with the numbers of experiments shown in parentheses, and the significance of differences from control values was determined by the paired *t* test.

RESULTS

Effects of insulin and EGF on lipogenesis in isolated adipocytes

Lipogenesis was routinely monitored as incorporation of radioactivity from [^{3}H]glucose into total toluene-extractable lipid. Very similar results were obtained if fatty acids were extracted into light petroleum after saponification and neutralization (results not shown). In 11 experiments the basal rate of lipogenesis was 0.73 ± 0.08 nmol of glucose units incorporated/min per 10^6 cells, and EGF (17 nM) stimulated this rate by 3.4 ± 0.35-fold ($P < 0.001$); in the same batches of cells insulin (0.8 nM) stimulated lipogenesis by 14.6 ± 1.8-fold ($P < 0.001$). EGF had a half-maximal effect at 10 nM (Fig. 1), and the half-maximal effect of insulin occurred

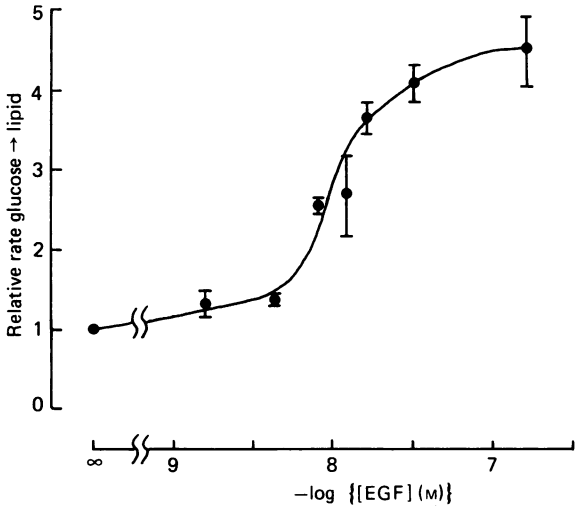

Fig. 1. Effect of EGF on incorporation of radioactivity from [^{3}H]glucose into lipid

Results are expressed as incorporation relative to controls in the absence of EGF.

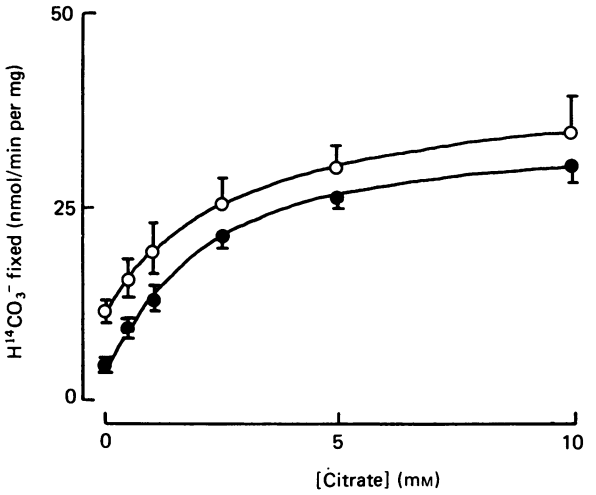

Fig. 2. Citrate-dependence of apparent acetyl-CoA carboxylase activity in adipocyte extracts measured by the $H^{14}CO_3^-$-fixation assay

○, Cells frozen and then homogenized for 30–40 s; ●, cells homogenized for 3–5 s without freezing.

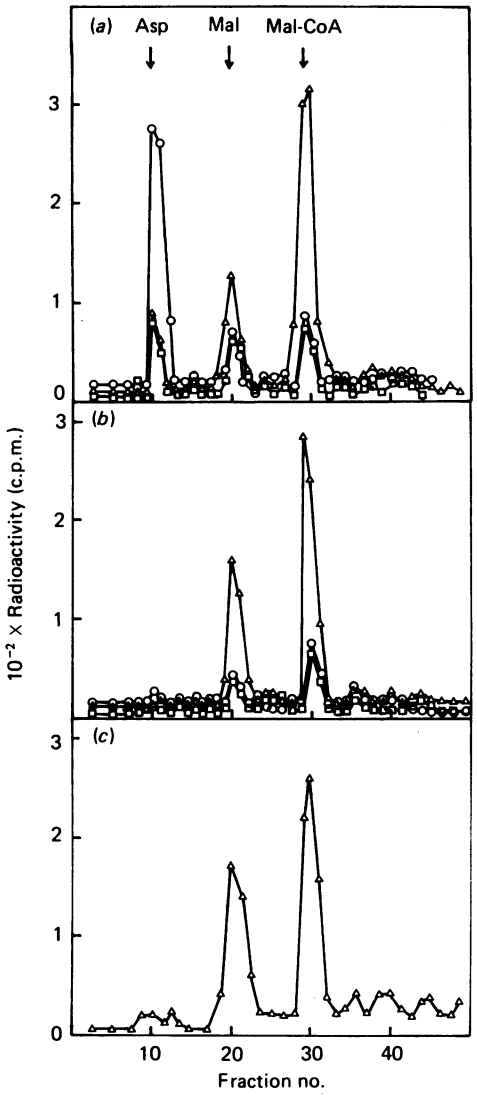

Fig. 3. Analysis of the products of the ^{14}C-fixation assay by ion-exchange h.p.l.c.

Arrows show the retention time of markers: aspartic acid (Asp), malonic acid (Mal) and malonyl-CoA (Mal-CoA). (*a*) Assays in crude extracts of adipocytes which had been frozen and then homogenized for 30–40 s; (*b*) assays in crude extracts of adipocytes which had been homogenized for 3–5 s without freezing; (*c*) assays of acetyl-CoA carboxylase purified to homogeneity from rat mammary gland (Munday & Hardie, 1984). ○, Assays in the presence of 5 mM-pyruvate; △, assays in the presence of 5 mM-citrate; □, assays in the absence of exogenous citrate or pyruvate.

at 0.5 nM (results not shown). If the effect of the preparation of EGF was due to contaminating insulin, the contamination would therefore have to be around 5% (by wt.). Radioimmunoassay of the EGF preparation used, which should have detected much lower contamination, did not indicate the presence of insulin in the preparation. Similar results were also obtained with two different preparations of EGF.

Validation of acetyl-CoA carboxylase assay in crude adipocyte extracts

If adipocytes were frozen in liquid N_2, homogenized in a Polytron for 30–40 s, and acetyl-CoA carboxylase was assayed in infranatant fractions prepared from these homogenates by the $H^{14}CO_3^-$-fixation assay, the allosteric activator citrate was found to stimulate the rate by only 2–3-fold (Fig. 2; ○). This is in marked contrast with results obtained with acetyl-CoA carboxylase purified to homogeneity from mammary gland, which is stimulated at least 20-fold by citrate (Hardie & Guy, 1980). We therefore examined the specificity of the $H^{14}CO_3^-$-fixation assay by analysing the products of the reaction by using ion-exchange h.p.l.c. When purified acetyl-CoA carboxylase was assayed, the acid-fixed non-volatile products were recovered as two peaks which exactly co-migrated

with malonate and malonyl-CoA markers (Fig. 3c). The ratio of malonate to malonyl-CoA recovered varied somewhat from experiment to experiment, and appeared to increase if the samples were dried down at an elevated temperature. The malonate is therefore assumed to be derived from breakdown of malonyl-CoA during the drying-down procedure. With the purified enzyme, the fixation of radioactivity into non-volatile products was negligible in the absence of added citrate (results not shown).

When the products of the assays of the adipocyte extracts were analysed in the same manner, the malonate and malonyl-CoA peaks were again observed, and the total radioactivity recovered in these two peaks was increased 6-fold by inclusion of 5 mM-citrate in the assay (Fig. 3a). However, a substantial amount of radioactivity was recovered as an additional peak eluted before malonate. In the absence of added citrate, this peak accounted for 36% of the total radioactivity recovered. This radioactive product has been tentatively identified as aspartate, produced apparently by the successive actions of pyruvate carboxylase and aspartate aminotransferase on endogenous pyruvate. The evidence for this may be summarized as follows: (1) the product co-migrated with authentic aspartic acid not only in this ion-exchange system but also in an amino acid-analysis system using reversed-phase h.p.l.c. of phenyl isothiocyanate derivatives (see the Experimental section); (2) addition of 5 mM-pyruvate to the assay increased the radioactivity recovered in this peak by 6-fold (Fig. 3a). By contrast, 5 mM-citrate had no significant effect on this peak, showing that it does not result from acetyl-CoA carboxylase action.

These results show that the freezing and/or homogenization procedure used released a considerable amount of pyruvate carboxylase activity into the infranatant, and that sufficient pyruvate was carried through into the assay to cause serious interference with the assay of acetyl-CoA carboxylase. To circumvent this problem, we homogenized very briefly (3–5 s) without prior freezing of cells, a technique which should result in much less mitochondrial breakage (R. M. Denton, personal communication). Under these conditions the amount of radioactivity recovered in the putative aspartate peak was negligible, and addition of pyruvate to the assay had no significant effect (Fig. 3b). When extracts were prepared in this manner, the total radioactivity recovered was stimulated 6-fold by 10 mM-citrate, with a citrate-dependence curve much more typical of those obtained with purified acetyl-CoA carboxylase (Fig. 2; ●).

Effects of insulin, EGF and adrenaline on acetyl-CoA carboxylase activity in isolated adipocytes

Using the gentle homogenization procedure which obviated interference from pyruvate carboxylase, we examined the effects of treatment of isolated cells with insulin, EGF or adrenaline on the activity of acetyl-CoA carboxylase measured in a subsequently prepared extract. Cells were homogenized in the presence of EDTA and F^- to inhibit protein kinases and phosphatases, which might modify the phosphorylation state of the enzyme subsequent to homogenization. Insulin and EGF had very similar effects on acetyl-CoA carboxylase activity, i.e. a stimulation which was observed at all citrate concentrations used in the assay, but which was most significant at high citrate concentration. Adrenaline caused an inhibition of acetyl-CoA carboxylase at all citrate concentrations (Fig. 4).

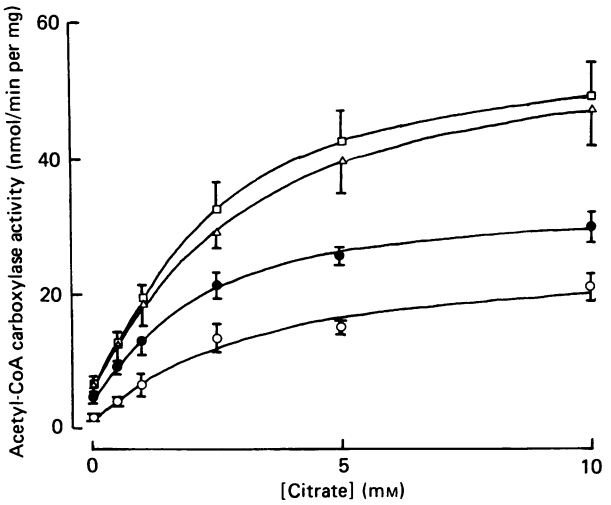

Fig. 4. Effects of treatment of isolated adipocytes with insulin, EGF or adrenaline on the activity of acetyl-CoA carboxylase measured in a crude supernatant fraction prepared from the cells

●, Control; □, insulin (0.9 nM); △, EGF (16 nM); ○, adrenaline (1 μM).

Table 1. Kinetic parameters of acetyl-CoA carboxylase measured in a cell supernatant fraction after treatment of isolated adipocytes with insulin, EGF or adrenaline

Values are derived from the data in Fig. 4 by computer fitting of the values from seven separate cell preparations of velocity (v) at different citrate concentrations ([C]). Data were fitted (Holland *et al.*, 1984) to the equation

$$v = V_0 + (V_{max.} - V_0)\,[C]^h/(K_a + [C]^h)$$

where V_0 is the velocity in the absence of added citrate. $V_{max.}$ values are expressed per mg of total supernatant protein. Values significantly different from control values are indicated: $*P < 0.02$; $**P < 0.01$.

	$V_{max.}$ (nmol/min per mg)	K_a (mM)	h
Control	29.9±1.6	2.40±0.54	1.63±0.16
EGF	**68.3±13.1	4.63±1.16	1.16±0.13
Insulin	*58.3±6.8	3.46±0.75	1.34±0.09
Adrenaline	23.2±4.7	5.12±1.97	2.39±0.43

Kinetic analysis showed that both insulin and EGF significantly increased $V_{max.}$, with no significant effect on the K_a for citrate (Table 1).

DISCUSSION

As reported previously for hepatocytes (Holland & Hardie, 1985), both insulin and EGF cause a marked stimulation of lipogenesis in isolated adipocytes, although in adipocytes the effects of EGF are much smaller than

those of insulin (3–4-fold, as against 15-fold). Stimulation of lipogenesis by EGF was not due to contamination of the EGF preparation with insulin, and there is no evidence that EGF can activate insulin receptors directly (see, e.g., Avruch *et al.*, 1982). Receptors for EGF have been previously detected on rat adipocytes, albeit in low abundance (Pessin *et al.*, 1983).

EGF has been reported to stimulate deoxyglucose uptake in 3T3 cells and human fibroblasts (Barnes & Colowick, 1976), although no effect of EGF was found on 3-*O*-methylglucose uptake in human fibroblasts (Hollenberg & Cuatrecasas, 1975). It is therefore possible that stimulation of glucose transport by EGF could account for some of the stimulation of lipogenesis in adipocytes. However, since EGF also stimulates fatty acid synthesis in hepatocytes (Holland & Hardie, 1985), a process which does not involve glucose uptake (Holland *et al.*, 1984), it seemed likely that EGF also affected a more distal step such as that catalysed by acetyl-CoA carboxylase.

During our initial experiments to answer this question, we used a widely used protocol for examining effects of hormone treatment on adipocyte acetyl-CoA carboxylase, in which cells are frozen in liquid N_2 and/or homogenized for 30–40 s in a Polytron-type homogenizer (Halestrap & Denton, 1973; Brownsey *et al.*, 1979; Zammit & Corstorphine, 1982; Witters *et al.*, 1983). We were perturbed by the high citrate-independent activity both in our own data (Fig. 1; ○) and in many of these previous reports (e.g. Halestrap & Denton, 1973; Witters *et al.*, 1983), since purified acetyl-CoA carboxylase is almost completely dependent on citrate under our assay conditions (Hardie & Guy, 1980). In addition, Davies *et al.* (1982) and Buechler *et al.* (1984) have reported that only part of the radioactivity recovered when the ^{14}C-fixation assay is used in crude liver extracts is malonyl-CoA. We therefore examined the specificity of the assay by analysing the reaction products by h.p.l.c. The results shown in Fig. 3 clearly indicate that pyruvate carboxylase can interfere with the assay of acetyl-CoA carboxylase in crude adipocyte extracts. The presence of pyruvate carboxylase was not unexpected, since breakage of mitochondria was likely to occur during freezing and/or prolonged homogenization of the cells. More surprising was the observation that sufficient pyruvate could be carried through into the assay to lead to fixation of $H^{14}CO_3$ by pyruvate carboxylase, since homogenization of adipocytes results in very considerable dilution of the cytoplasm. However, we also found (T. A. J. Haystead, unpublished work) that the citrate-independent activity was not decreased by 'centrifuge desalting', i.e. the rapid centrifugation of the extract through a plug of Sephadex G-25, which is able to separate proteins from small molecules within seconds (McCarthy & Hardie, 1982). These results suggest that pyruvate remains because it binds tightly to protein(s) (perhaps pyruvate carboxylase) and that the rate constant for its dissociation is sufficiently low that it is not removed by rapid dilution or gel filtration. However, the interference in the assay is obviated by avoiding the freezing of cells and by homogenizing very briefly (3–5 s), which results in negligible release of pyruvate carboxylase into the infranatant, with no apparent effect on recovery of acetyl-CoA carboxylase (Fig. 3). This method was used for all subsequent studies.

Although some previous reports in the literature may also have been subject to interference by pyruvate carboxylase (e.g. Halestrap & Denton, 1973; Witters *et al.*, 1983), the results in Fig. 4 show that insulin treatment of adipocytes does indeed lead to activation of acetyl-CoA carboxylase which is measurable in a crude extract. They also show that insulin and EGF have very similar effects on acetyl-CoA carboxylase activity, i.e. an increase in $V_{max.}$ without a significant effect on the K_a for citrate. We have previously shown that insulin and EGF have almost identical effects on the phosphorylation of acetyl-CoA carboxylase (and ATP citrate lyase) in isolated hepatocytes (Holland & Hardie, 1985). However, a causal relationship between the increased phosphorylation and the activation of acetyl-CoA carboxylase has not been established, because any effects of insulin and EGF on acetyl-CoA carboxyase activity do not survive purification on avidin–Sepharose, unlike the effects on phosphorylation (Witters *et al.*, 1983; Holland & Hardie, 1985). By contrast, the effects of glucagon or adrenaline on acetyl-CoA carboxylase activity, for which there is good evidence that a change in phosphorylation is responsible, are stable to purification on avidin–Sepharose (Holland *et al.*, 1984, 1985).

Since the effects of insulin and EGF on fatty acid synthesis are so different in magnitude, it is notable that the effects of these two agents on acetyl-CoA carboxylase activity are similar. A likely explanation for this apparent anomaly is that insulin and EGF may differ in their effects on earlier steps in the pathway, such as glucose transport or the conversion of pyruvate into acetyl-CoA, one or both of which may be partially rate-limiting in adipocytes.

The physiological roles of EGF are still not clear: the plasma concentration in the mouse is normally around 0.2 nM, although it can rise to around 20 nM after intravenous treatment with phenylephrine (Byyny *et al.*, 1974). Alternatively, EGF may have a much more local, paracrine, effect on proliferating cells. It is interesting that fatty acid synthesis is stimulated 2-fold in regenerating rat liver, apparently by unidentified extrahepatic factors (Gove & Hems, 1978). Whatever the significance of EGF effects on adipocytes, stimulation of lipogenesis by EGF may be one facet of the general stimulation of biosynthetic pathways by growth factors. Our results also contribute to the growing awareness that the effects of insulin and polypeptide growth factors on cells are very similar and may operate through common pathways.

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