Studies of rat adipose-tissue microsomal glycerol phosphate acyltransferase

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1. Incubation of rat adipose-tissue microsomal fractions with iodoacetate caused an inactivation of glycerol phosphate acyltransferase that could be prevented by the presence of palmitoyl-CoA. 2. A microsomal protein of subunit M_r 54000 was found to react with radioactively labelled iodoacetate in the absence, but not in the presence, of palmitoyl-CoA. It is suggested that this protein is a component of glycerol phosphate acyltransferase. 3. Incubation of rat adipose-tissue microsomal fractions with the catalytic subunit of cyclic AMP-dependent protein kinase, ATP and Mg^{2+} caused an inactivation of glycerol phosphate acyltransferase whose magnitude depended on the conditions used for assay of the acyltransferase. 4. Rat adipose tissue microsomal proteins were phosphorylated by using protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. One of the phosphorylated proteins was very similar, but not identical, in mobility to the M_r -54000 protein labelled by iodoacetate. 5. In contrast with a previous report [Sooranna & Saggerson (1976) *FEBS Lett.* **64**, 36–39], no changes could be detected in the activity of glycerol phosphate acyltransferase in adipocytes treated with adrenaline. 6. Adipocytes were labelled with $[\text{}^{32}\text{P}]\text{P}_i$ and treated with adrenaline, but no ^{32}P was incorporated into the M_r -54000 protein labelled by iodoacetate. 7. The results suggest that the activity of adipose-tissue microsomal glycerol phosphate acyltransferase is not directly controlled by phosphorylation.

White adipose tissue is able to store fatty acids as triacylglycerols and to release them again when required. These processes are under hormonal control. GPAT catalyses one of the key steps in the esterification pathway. Saggerson's group have shown that the addition of adrenaline, nor-adrenaline and other hormones to rat adipocytes causes a marked decrease in GPAT activity (Rider & Saggerson, 1983; Sooranna & Saggerson, 1976, 1978). Four other enzymes in the pathway of triacylglycerol synthesis are also regulated by catecholamines (see, e.g., Saggerson *et al.*, 1979). The mechanisms underlying these effects are not understood.

GPAT activity in mammalian tissues is found in both mitochondrial and microsomal fractions. The two forms of the enzyme differ in kinetic properties, in acyl-group specificity and in sensitivity to inhibition by thiol-group reagents [see, e.g., Nimmo (1979), Saggerson *et al.* (1980) and references therein]. It has been inferred that the

two forms are distinct isoenzymes of GPAT (Nimmo, 1979). Treatment of adipocytes with nor-adrenaline causes decreases in the activity of both forms of GPAT (Rider & Saggerson, 1983).

We found that treatment of rat adipose-tissue microsomes (microsomal fractions) with partially purified cyclic AMP-dependent protein kinase caused a decrease in GPAT activity that could be reversed by treatment with alkaline phosphatase or protein phosphatase-1 (Nimmo, 1980; Nimmo & Houston, 1978). In contrast, Rider & Saggerson (1983) treated rat adipocyte microsomes with the catalytic subunit of cyclic AMP-dependent protein kinase, but could detect no changes in GPAT activity. They found that the effects of noradrenaline on GPAT activity in adipocytes could be abolished by homogenization of the cells in the presence of bovine serum albumin. They concluded that the effects of noradrenaline on microsomal GPAT are not attributable to phosphorylation of the enzyme.

In the present paper we report that the effects of treating microsomal fractions with protein kinase on GPAT activity are critically dependent on the method used to assay GPAT. We show that a

Abbreviations used: GPAT, glycerol phosphate acyltransferase (EC 2.3.1.15); SDS, sodium dodecyl sulphate.

protein of M_r 54000 in rat adipose-tissue microsomes can be labelled by iodoacetate in the absence, but not in the presence, of palmitoyl-CoA, and we suggest that this protein bears the active site of GPAT. We were unable to detect phosphorylation of this protein either by purified protein kinase *in vitro* or in intact adipocytes treated with adrenaline.

Experimental

Materials

All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Adrenaline, bovine insulin, iodoacetic acid and palmitoyl-CoA were from Sigma (London) Chemical Co., Poole, Dorset, U.K. The catalytic subunit of cyclic AMP dependent protein kinase was prepared from ox liver by a minor modification (Meek & Nimmo, 1984) of the procedure of Sugden *et al.* (1976). Other materials were prepared or obtained as described previously (Borthwick *et al.*, 1984; Nimmo, 1979; Nimmo & Houston, 1978).

Preparation of microsomes

Microsomes were prepared from Wistar rats as described previously (Nimmo & Houston, 1978), washed once by centrifugation at 100000g for 60 min and resuspended in 100 mM-Tris/HCl (pH 7.5)/1 mM-EDTA to give a protein concentration of 1 mg/ml. For phosphorylation experiments, dithiothreitol was added to give a concentration of 1 mM. For reactions with iodoacetate, dithiothreitol was not added and the microsomes were used within 60 min of their preparation. Unless stated otherwise, 24 h-starved rats were used.

Reaction of microsomes with iodoacetate

Each incubation contained, in a final volume of 0.9 ml, 0.25 mg of microsomal protein/ml, 80 mM-Tris/HCl, pH 7.4, and 5 mM-iodoacetate. Palmitoyl-CoA was present as indicated in the text. The incubations were at 0°C and the reactions were started by the addition of iodoacetate. Samples (5 µl) were removed and immediately assayed for GPAT.

For measurement of the incorporation of carboxymethyl groups into protein, samples (0.1 ml) were removed and the reaction was quenched by the addition of 1 ml of ice-cold 5% (w/v) trichloroacetic acid. Bovine serum albumin (1 mg) was added as a carrier. Mixtures were left at 0°C for 10 min and the precipitated proteins were collected by centrifugation in an Eppendorf 5412 micro-centrifuge for 2 min. The pellets were washed with 5 × 1 ml of 5% trichloroacetic acid, dissolved in 0.1 ml of formic acid and counted for radioactivity in scintillant [4 g of diphenyl-oxazole/litre of toluene/Triton X-100 (2:1, v/v)].

For analysis by SDS/polyacrylamide-gel electrophoresis, samples (0.15 ml) were removed and the reaction was quenched by the addition of 1 ml of ice-cold 5% trichloroacetic acid and 5 µl of 2-mercaptoethanol. After 10 min, precipitated proteins were collected by centrifugation, washed once with 0.5 ml of acetone and then allowed to dissolve overnight at room temperature in 0.05 ml of 8 M-urea containing 2 µl of 2-mercaptoethanol. Finally 0.05 ml of SDS sample buffer [50 mM-Tris/HCl (pH 6.8), 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue and 1% (v/v) 2-mercaptoethanol] was added and the mixture was heated to 100°C for 2 min.

In the experiments shown in Figs. 1–3, iodo[2-³H]acetate (125 c.p.m./pmol) was used. For the samples used as markers in Figs. 5 and 6, iodo[2-¹⁴C]acetate (50 c.p.m./pmol) was used.

Incubation of microsomes with protein kinase

Each incubation contained, in a total volume of 0.6 ml, 0.25 mg of microsomal protein/ml, 50 mM-Tris/HCl, pH 7.4, 20 mM-NaF, 10 mM-MgCl₂, 1 mM-ATP and either 20 or 2 units of the catalytic subunit of cyclic AMP-dependent protein kinase/ml. Incubations were at 30°C and were started by the addition of ATP. Samples (5 µl) were assayed for GPAT activity. For the analysis of samples by gel electrophoresis (Fig. 5), [γ -³²P]ATP (100 c.p.m./pmol) was used. Samples were processed exactly as described for experiments with iodoacetate.

Polyacrylamide-gel electrophoresis

This was performed by the method of Laemmli (1970), with 10%-acrylamide separating gels. For experiments with iodo[2-³H]acetate (Figs. 2 and 3), tube gels (0.5 cm × 8 cm, with a 1 cm 3%-acrylamide stacking gel) were run, stained for protein with Coomassie Blue, destained and scanned densitometrically at 600 nm. The gels were then frozen and cut into 2 mm slices. Each slice was dissolved in 0.3 ml of 30% (w/v) H₂O₂ and counted for radioactivity in the scintillant described above. For the experiments with iodo[2-¹⁴C]acetate (Figs. 5 and 6), slab gels (1.5 mm thick) were run, dried and autoradiographed by using Kodak X-OMAT R film. Values of M_r were estimated by using the marker proteins listed by Borthwick *et al.* (1984).

Analysis of protein phosphorylation in adipocytes

Adipocytes from 120–140 g rats were prepared and washed as described by Nilsson & Belfrage (1981). Cells were incubated (50 µl packed cell volume/ml) in the low-phosphate medium (50 µM-P_i) described by Belfrage *et al.* (1980) at 37°C with 1 Ci of [³²P]P_i/mmol for 40 min before the addition of hormone. To terminate the incubations, cells

were separated from the medium by centrifugation (Eppendorf 5412) for 5 s through dinonyl phthalate. The cells were broken by vigorous vortex-mixing for 30 s in 1 ml of 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/25 mM-NaF/4 mM-EDTA/0.05% 2-mercaptoethanol. Mitochondria were removed by centrifugation of the extract at 12000g for 4 min in an Eppendorf 5412 micro-centrifuge. The resulting supernatant was centrifuged at 100000g for 60 min. The microsomal pellet was resuspended in 0.05 ml of SDS sample buffer (see above), heated to 100°C for 2 min and subjected to SDS/polyacrylamide-gel electrophoresis.

Effects of adrenaline on glycerol phosphate acyltransferase activity in adipocytes

Adipocytes were prepared and incubated as described by Sooranna & Saggerson (1976), except that the albumin concentration in the incubation medium was 30 mg/ml. Incubations were terminated by isolating the cells in 1 ml samples as described by Sooranna & Saggerson (1976). Cells were homogenized in 1 ml of ice-cold 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/25 mM-NaF/1 mM-EDTA/0.05% 2-mercaptoethanol with a Dounce homogenizer and then centrifuged at 1000g for 75 s. The resulting fat-free homogenate was assayed for GPAT and either lactate dehydrogenase or NADPH-cytochrome *c* reductase.

Hormonal responsiveness of adipocytes

The effects of insulin on the conversion of [^3H]glucose into lipids were measured as described by Nilsson & Belfrage (1981). The effects of adrenaline on lipolysis were monitored by measuring the release of non-esterified fatty acids into the incubation medium.

Assay of glycerol phosphate acyltransferase

Unless stated otherwise, GPAT was assayed by using exogenous palmitoyl-CoA. Routinely, incubations (0.1 ml) contained 75 mM-Tris/HCl, pH 7.4, 8 mM-NaF, 4 mM-MgCl₂, 1 mM-dithiothreitol, 2 mg of bovine serum albumin (fatty acid-free)/ml, 40 μM -palmitoyl-CoA, 0.5 mM-*sn*-[^3H]glycerol 3-phosphate (16000–20000 c.p.m./nmol) and enzyme. Incubations were at 30°C for 3–10 min. Reactions were stopped and the radioactive products were extracted and determined as described previously (Nimmo, 1979). In some experiments with adrenaline-treated adipocytes, the concentrations of substrates were altered as specified in the text. The conditions were chosen so that assays were linear with time and rates were proportional to enzyme concentration.

In some experiments, GPAT was assayed by using carnitine palmitoyltransferase as described by Nimmo & Houston (1978).

Other methods

Lactate dehydrogenase, NADPH-cytochrome *c* reductase, cyclic AMP-dependent protein kinase, non-esterified fatty acids and protein were determined by standard methods (Kornberg, 1955; Masters *et al.*, 1967; Witt & Roskoski, 1975; Itaya & Ui, 1965; Bradford, 1976). One unit of protein kinase activity is defined as the amount required to catalyse the incorporation of 1 nmol of phosphate into histone/min.

Results and discussion

Inactivation of microsomal glycerol phosphate acyltransferase by iodoacetate

The mitochondrial and microsomal forms of GPAT in rat liver and adipose tissue can be distinguished by the great sensitivity of the microsomal enzyme to thiol-group reagents such as *N*-ethylmaleimide and iodoacetate (Bates & Saggerson, 1979; Nimmo, 1979; Saggerson *et al.*, 1980). Nimmo (1979) showed that the microsomal GPAT of rat liver can be protected against inactivation by the presence of palmitoyl-CoA. We investigated whether a similar effect occurred with adipose-tissue microsomes and could be used to identify the microsomal acyltransferase by labelling microsomal proteins with radioactive reagent in the presence and absence of palmitoyl-CoA.

In agreement with the results of Saggerson *et al.*, (1980), we found that *N*-ethylmaleimide (1–10 mM) gave over 95% inhibition of the GPAT activity of rat adipose-tissue microsomes. However, the inactivation caused by *N*-ethylmaleimide was extremely rapid, reaching completion in less than 30 s, and as a consequence it was not possible to study protection by palmitoyl-CoA. On the other hand, the rate of inactivation of microsomal GPAT by iodoacetate was readily measurable. The results shown in Fig. 1 reveal that 100 μM -palmitoyl-CoA almost completely protects GPAT against inactivation and also slightly decreases the incorporation of ^3H from iodof[^3H]acetate into microsomal proteins. The degrees of inactivation of GPAT observed after incubation of microsomes with 5 mM-iodoacetate for 8 min were $51 \pm 9\%$ and $7 \pm 4\%$ (means $\pm$ S.D., $n = 4$) in the absence and presence of 100 μM -palmitoyl-CoA respectively.

GPAT activity could be protected against inactivation by iodoacetate for short periods by using concentrations of palmitoyl-CoA lower than 100 μM . For example, when 25 μM -palmitoyl-CoA was used, GPAT activity remained approximately constant for 2–4 min after the addition of iodoacetate, but thereafter it declined at a rate similar to that observed in the absence of palmitoyl-CoA (results not shown). This presumably reflects destruction of palmitoyl-CoA by endogenous hy-

drolases. Like the microsomal GPAT from rat liver (Nimmo, 1979), the adipose-tissue enzyme could not be protected against iodoacetate by methyl palmitate or CoA (results not shown). The results suggest that adipose tissue microsomal GPAT may contain a thiol group close to or at its active site.

Fig. 1. Effects of palmitoyl-CoA on the reaction of rat adipose-tissue microsomes with iodoacetate

Microsomes were incubated with iodo[2- ${}^3\text{H}$]acetate in the absence (■, ●) or presence (□, ○) of 100 μM -palmitoyl-CoA. The activity of GPAT (■, □) and the incorporation of ${}^3\text{H}$ into protein (●, ○) were measured as described in the Experimental section. The initial specific activity of GPAT was 51 nmol/min per mg of protein.

Identification of microsomal proteins that react with radioactively labelled iodoacetate

The rate of inactivation of adipose-tissue microsomal GPAT by iodoacetate is fast relative to the rate of reaction of free cysteine under similar conditions. This suggested that it might be possible to use iodoacetate to label microsomal GPAT with some degree of specificity. Experiments similar to those shown in Fig. 1 were carried out with either ${}^3\text{H}$ - or ${}^{14}\text{C}$ -labelled iodoacetate and the microsomal proteins were resolved by SDS/polyacrylamide-gel electrophoresis. Tritiated bands were identified after solubilization of gel slices (e.g. Fig. 2) and ${}^{14}\text{C}$ -labelled bands were identified by autoradiography (Figs. 5 and 6).

The labelling of microsomal proteins with iodo[2- ${}^3\text{H}$]acetate was investigated in four experiments; in each case similar results were obtained. Representative gels from one experiment are shown in Fig. 2. Samples incubated in the presence or absence of palmitoyl-CoA gave some minor peaks over a fairly uniform background incorporation. This background value increased with the time of incubation of the sample with iodoacetate (e.g. Figs. 2a and 2b), and it probably represents the incorporation of small amounts of [${}^3\text{H}$]carboxymethyl groups into a large number of proteins of different sizes. Samples incubated in the absence of palmitoyl-CoA gave, in addition, a heavily labelled band whose mobility corresponded to a subunit M_r of 54000 (Figs. 2a and 2b). Labelling of

Fig. 2. Labelling of microsomal proteins with iodo[2- ${}^3\text{H}$]acetate

Conditions were as described in the Experimental section. Each gel was loaded with 37.5 μg of protein. (a) No palmitoyl-CoA, 4 min incubation. (b) No palmitoyl-CoA, 8 min incubation. (c) Plus 100 μM -palmitoyl-CoA, 8 min incubation. The upper traces show densitometric scans of each gel and the bar represents 0.1 A_{600} unit. The GPAT activities corresponding to samples (a) and (b) are shown in Fig. 3. The arrows indicate the M_r values ($\times 10^{-3}$) of marker proteins.

this band was prevented by the presence of 100 μM -palmitoyl-CoA (Fig. 2c). Experiments with iodo[2- ^{14}C]acetate gave similar results. As is shown in Figs. 5 and 6, incubation of microsomes in the presence of 100 μM -palmitoyl-CoA gave a faint background with barely detectable minor bands. Incubation of microsomes in the absence of palmitoyl-CoA gave, in addition, a prominent band of M_r 54000.

In the experiment shown in Fig. 2, the amount of ^3H incorporated into the M_r -54000 band in the absence of palmitoyl-CoA was measured at several different times. GPAT activity was also measured in a parallel experiment in which unlabelled iodoacetate was used. The results (Fig. 3) show that the amount of ^3H incorporated into this band seems to be inversely related to the GPAT activity. This suggests that the M_r -54000 protein may contain the active site of GPAT.

Attempts to phosphorylate glycerol phosphate acyltransferase in vitro

We reported previously that the activity of rat adipose-tissue microsomal GPAT could be decreased by treatment with cyclic AMP-dependent protein kinase (Nimmo & Houston, 1978). In those experiments we used a partially purified preparation of the protein kinase, and we assayed GPAT using a method in which palmitoyl-CoA was

generated from palmitoylcarnitine by an excess of carnitine palmitoyltransferase. We have now repeated the experiments, using an essentially homogeneous preparation of protein kinase and assaying GPAT with exogenous palmitoyl-CoA as described in the Experimental section.

Incubation of rat adipose-tissue microsomes with the catalytic subunit of cyclic AMP-dependent protein kinase, ATP and Mg^{2+} as described in the Experimental section caused a time-dependent decrease in the activity of GPAT. The maximal extent of the inactivation was, however, much smaller than we reported previously (Nimmo & Houston, 1978); in the present experiments we observed only 25–40% inactivation ($31 \pm 5\%$, mean $\pm$ S.D. for five determinations). The difference was directly attributable to the altered assay conditions for GPAT. In the experiment shown in Fig. 4, microsomes were incubated with protein kinase, and GPAT activity was assayed either with exogenous palmitoyl-CoA (see the Experimental section) or with carnitine palmitoyltransferase (Nimmo & Houston, 1978). The former assay reproducibly gives 2–3-fold higher activities; this is probably caused by a difference between the two assay systems in the palmitoyl-CoA concentration, to which GPAT is very sensitive (see, e.g., Rider & Saggerson, 1983). Fig. 4 shows that incubation with protein kinase decreased the activity of GPAT as assessed by either assay system. The absolute magnitude of the inactivation was much lower when the former assay system was used, but the relative rates of decrease of GPAT activity were similar for the two assays. It may be that some component of the latter assay system, perhaps the palmitoylcarnitine, affects the microsomal membranes in such a way as to enhance the effect of protein phosphorylation on the activity of GPAT.

Our results are in contrast with those of Rider & Saggerson (1983), who were unable to detect any inactivation of GPAT in adipocyte microsomes from fed rats. This discrepancy was not caused by the nutritional state of the animals; we have observed inactivation of GPAT in microsomes from starved animals (e.g. Fig. 4) and from fed animals (results not shown). Both Rider & Saggerson (1983) and ourselves used purified catalytic subunits of cyclic AMP-dependent protein kinase, albeit from different sources, and so it seems unlikely that the nature of the protein kinase should be the cause of the discrepancy.

A more likely explanation of the discrepancy concerns the amounts of protein kinase activity used in the two sets of experiments. We routinely used 20 units of protein kinase/ml, a concentration that is within the physiological range (see, e.g., Soderling, 1975). The maximum incorporation of ^{32}P from [γ - ^{32}P]ATP into microsomal protein was

Fig. 3. Incorporation of ^3H into the M_r -54000 protein and inactivation of glycerol phosphate acyltransferase

Microsomes were incubated with iodo[2- ^3H]acetate and samples were analysed by gel electrophoresis as shown in Fig. 2. The amount of ^3H incorporated into the M_r -54000 band at different times (○) was obtained by subtraction of the average background radioactivity in gel slices 3–12. GPAT activity (■) was measured in an identical experiment with unlabelled iodoacetate. The initial specific activity of the GPAT was 43 nmol/min per mg of protein.

Fig. 4. Inactivation of glycerol phosphate acyltransferase by protein kinase

A preparation of microsomes was incubated with 20 units of protein kinase/ml. GPAT was assayed as described by Nimmo & Houston (1978) (●) or as given in the Experimental section (△). The initial specific activities of GPAT were 15.1 and 37 nmol/min per mg of microsomal protein respectively. In a separate experiment (□) the protein kinase activity was 2 units/ml and GPAT (initial specific activity 41 nmol/min per mg) was assayed as described in the Experimental section. In each case the activity of GPAT is expressed as a percentage of the value at zero time.

some 25 nmol/mg in these conditions (Nimmo, 1980). When we used only 2 units of protein kinase/ml, the maximal inactivation of GPAT that we observed was very small ($8 \pm 5\%$, $n = 3$) and was not statistically significant; the results of a representative experiment are shown in Fig. 4. The maximum incorporation of ^{32}P into microsomal protein was in the range 8–12 nmol/mg in these conditions. Rider & Saggerson (1983) used 4 units of protein kinase/ml, and the maximum incorporation of ^{32}P that they observed was 13 nmol/mg of microsomal protein. These values for ^{32}P incorporation are not directly comparable, because we isolated microsomes from whole adipose tissue, whereas Rider & Saggerson (1983) used adipocyte microsomes, and because different methods for determination of protein concentration were used. However, the data suggest strongly that inactivation of GPAT is observed only if a high concentration of protein kinase is used and consequently a high degree of microsomal protein phosphorylation is obtained.

We labelled adipose-tissue microsomal proteins by using protein kinase (20 units/ml) and [γ - ^{32}P]ATP as described in the Experimental section, and analysed the [^{32}P]phosphorylated proteins by SDS/polyacrylamide-gel electrophoresis. In preliminary experiments (Nimmo, 1981) in which we

ran tube gels and identified phosphorylated bands by liquid-scintillation counting of radioactivity of gel slices, we found that a protein of subunit M_r 54000 could be phosphorylated. This seemed to co-migrate with the protein labelled by iodoacetate, and we concluded that it was probably GPAT.

We have now increased the resolution of our analysis; we used ^{14}C - instead of ^3H -labelled iodoacetate to produce the marker protein, we deliberately over-ran slab gels to increase the resolution in the 50000–60000 range of M_r values, and we detected labelled proteins by autoradiography rather than by liquid-scintillation counting of gel slices. The results in Fig. 5 show that no rat adipose-tissue microsomal proteins were significantly phosphorylated in the absence of cyclic AMP-dependent protein kinase. However, this kinase was able to phosphorylate a microsomal protein that is slightly larger than the M_r -54000 protein that can be labelled by iodoacetate in the absence, but not in the presence, of palmitoyl-CoA. We estimate that the two proteins differ in M_r by 500–1000. We have considered the possibility that the slight difference between the mobilities of the [^{14}C]carboxymethylated and [^{32}P]phosphorylated bands might be caused by two different covalent modifications of a single protein. However, control experiments with *Escherichia coli* isocitrate dehydrogenase and rabbit muscle glyceraldehyde phosphate dehydrogenase (Borthwick *et al.*, 1984; H. G. Nimmo, unpublished work) rule out this possibility; the two labelled bands probably correspond to different proteins.

There is thus no evidence that the protein believed to bear the active site of GPAT can be phosphorylated *in vitro*. It is, of course, possible that the effects of the protein kinase on the activity of GPAT are mediated by phosphorylation of a regulatory subunit of the enzyme. However, a simpler explanation might be that the activity of GPAT is affected by the charge distribution on the surface of the microsomal membranes and that this is affected by phosphorylation. It is known that the activities of several mitochondrial and microsomal enzymes can be altered by phosphorylation in this way (Famulski *et al.*, 1979, 1983). Another possibility is that the incubation of microsomes with protein kinase results in either the production of an inhibitor or the destruction of an activator of GPAT. This seems unlikely, since the activity of GPAT is stable to dialysis both before and after treatment with protein kinase (Nimmo & Houston, 1978; Nimmo, 1980).

Activity and phosphorylation state of glycerol phosphate acyltransferase in adipocytes

Sooranna & Saggerson (1976, 1978) showed that

Fig. 5. *Phosphorylation of microsomal proteins in vitro* Microsomes were incubated with [γ - 32 P]ATP either with (tracks 1 and 3) or without (track 4) protein kinase for 20 min as described in the Experimental section. Incubation with protein kinase caused a 31% decrease in the activity of GPAT. Microsomes were incubated with iodo[2- 14 C]acetate either with (track 5) or without (track 2) 100 μ M-palmitoyl-CoA for 6 min. Incubation without palmitoyl-CoA caused a 49% decrease in the activity of GPAT. The gel was loaded with 30 μ g of protein (tracks 1, 3, 4) or 50 μ g of protein (tracks 2, 5). To increase the resolution in the M_r -50000–60000 region, electrophoresis was continued for 75 min after the dye front had reached the bottom of the gel. The numbers on the left indicate the M_r values ($\times 10^{-3}$) of marker proteins.

incubation of rat adipocytes with adrenaline caused a time- and dose-dependent decrease in GPAT activity. One of the objectives of the present study was to investigate whether phosphorylation of GPAT could be responsible for this. We therefore attempted to repeat the approach used by Sooranna & Saggerson (1976, 1978). We first tested the responsiveness of our adipocytes to adrenaline and insulin by measuring fatty acid release and triacylglycerol synthesis respectively. Adrenaline gave potent stimulation of lipolysis, with half-maximal effects at 0.3 μ M. Insulin gave an 8-fold stimulation of triacylglycerol synthesis, with half-maximal effects at 0.5 nM.

We carried out a series of experiments in which

Fig. 6. *Phosphorylation of microsomal proteins in adipocytes*

Microsomes were incubated with iodo[2- 14 C]-acetate either with (track 1) or without (track 2) 100 μ M-palmitoyl-CoA exactly as described in Fig. 5. Adipocytes were incubated with [32 P] P_i for 40 min (track 3). Adrenaline (5 μ M) was then added and further samples were taken immediately (track 4) and after 1, 3, 5 and 10 min (tracks 5–8 respectively). Approx. 40 μ g of protein was loaded in tracks 3–8. The numbers on the left indicate the M_r values ($\times 10^{-3}$) of marker proteins.

adipocytes were challenged with adrenaline (0.1–50 μ M) for up to 100 min in the presence or absence of 5 mM-glucose. GPAT was assayed under the routine conditions given in the Experimental section, with low concentrations of substrates (20 μ M-palmitoyl-CoA, 50 μ M-*sn*-glycerol 3-phosphate) or with high concentrations (125 μ M-palmitoyl-CoA, 0.5 mM-*sn*-glycerol 3-phosphate). The activity of GPAT was expressed relative to that of either lactate dehydrogenase (e.g. Rider & Saggerson, 1983) or NADPH-cytochrome c reductase, as a microsomal marker. No significant and reproducible inactivation of GPAT was observed (results not shown).

We also carried out experiments designed to show qualitatively whether the protein believed to bear the active site of GPAT could be phosphorylated in intact cells. In the experiment shown in Fig. 6, adipocytes were pre-labelled with [32 P] P_i and then challenged with 3 μ M-adrenaline for 10 min. Samples were removed during this incubation, and a 100000g pellet was isolated in the presence of NaF and EDTA to prevent dephos-

phorylation of proteins. This microsomal fraction was subjected to SDS/polyacrylamide-gel electrophoresis and the ^{32}P -containing bands were located by autoradiography. As expected, several ^{32}P -containing bands were detected in control cells. The degree of labelling of an M_r -65000 protein was markedly increased by adrenaline. This is in agreement with the results of several other groups (Avruch *et al.*, 1976; Benjamin & Clayton, 1978; Counis *et al.*, 1978). There was also a small increase in the degree of labelling of an M_r -42000 protein. It is known that pyruvate dehydrogenase can be inactivated in response to adrenaline in adipocytes (Sooranna & Saggerson, 1976). The microsomal fraction used in our experiments probably contains some mitochondria, and the M_r -42000 band may represent the α -subunit of pyruvate dehydrogenase.

A careful examination of Fig. 6 shows that none of the ^{32}P -labelled proteins corresponds exactly in mobility to the M_r -54000 protein that can be labelled by iodoacetate in the absence, but not in the presence, of palmitoyl-CoA. This was true even when adipocytes were incubated with adrenaline for 60 min (results not shown). The specific radioactivities of the iodo[2- ^{14}C]acetate and the [^{32}P]P_i used in Fig. 6 were such that the presence of 0.01–0.02 phosphate group per subunit of the 54000- M_r protein should have been detectable. We conclude that this protein is not phosphorylated in adipocytes in these conditions.

Concluding remarks

Our failure to observe inactivation of GPAT in adipocytes in response to adrenaline is in marked contrast to the results obtained by Saggerson's group. Sooranna & Saggerson (1976, 1978) observed a decrease of up to approx. 50% in the total GPAT activity of adipocytes incubated with adrenaline over periods of 60–75 min. Rider & Saggerson (1983) observed similarly slow decreases in the activities of both the mitochondrial and the microsomal forms of the enzyme in response to noradrenaline. These experiments were carried out with adipocytes incubated either with (Rider & Saggerson, 1983) or without (Sooranna & Saggerson, 1976, 1978) adenosine deaminase. Thus our failure to observe inactivation of GPAT in adipocytes cannot be attributed to the fact that we did not use adenosine deaminase. At present we cannot explain this discrepancy between our results and those of Saggerson's group. Rider & Saggerson (1983) showed that the effects of noradrenaline on the microsomal form of GPAT could be abolished by the inclusion of albumin in the homogenization buffer. They suggested that the effects of noradrenaline might be mediated by changes in the amount of an inhibitor of GPAT that can be removed by binding to albumin. Our

results support their conclusion that phosphorylation of microsomal GPAT is not responsible for its inactivation by catecholamines; there is no evidence that the M_r -54000 protein tentatively identified as a component of GPAT (Figs. 1–3) can be phosphorylated either *in vitro* by cyclic AMP-dependent protein kinase or in adipocytes challenged with adrenaline.

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References

- Avruch, J., Leone, G. R. & Martin, D. B. (1976) *J. Biol. Chem.* **251**, 1511–1515
- Bates, E. J. & Saggerson, E. D. (1979) *Biochem. J.* **182**, 751–762
- Belfrage, P., Fredrikson, G., Nilsson, N. O. & Stralfors, P. (1980) *FEBS Lett.* **111**, 120–124
- Benjamin, W. B. & Clayton, N. L. (1978) *J. Biol. Chem.* **253**, 1700–1709
- Borthwick, A. C., Holms, W. H. & Nimmo, H. G. (1984) *Eur. J. Biochem.* **141**, 393–400
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Counis, R., Mongongu, S., Pierre, M., Loeb, J. E. & Jutisz, M. (1978) *FEBS Lett.* **96**, 69–74
- Famulski, K. S., Nalecz, M. J. & Wojtczak, L. (1979) *FEBS Lett.* **103**, 260–264
- Famulski, K. S., Nalecz, M. J. & Wojtczak, L. (1983) *FEBS Lett.* **175**, 124–128
- Itaya, K. & Ui, M. (1965) *J. Lipid Res.* **6**, 16–20
- Kornberg, A. (1955) *Methods Enzymol.* **1**, 441–443
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Masters, B. S. S., Williams, C. H. & Kamin, H. (1967) *Methods Enzymol.* **10**, 565–573
- Meek, D. W. & Nimmo, H. G. (1984) *Biochem. J.* **222**, 125–130
- Nilsson, N. O. & Belfrage, P. (1981) *Methods Enzymol.* **72**, 319–325
- Nimmo, H. G. (1979) *Biochem. J.* **177**, 283–288
- Nimmo, H. G. (1980) *Mol. Aspects Cell. Regul.* **1**, 135–152
- Nimmo, H. G. (1981) *Biochem. Soc. Trans.* **9**, 238P
- Nimmo, H. G. & Houston, B. (1978) *Biochem. J.* **176**, 607–610
- Rider, M. H. & Saggerson, E. D. (1983) *Biochem. J.* **214**, 235–246
- Saggerson, E. D., Sooranna, S. R. & Cheng, C. H. K. (1979) *INSERM Colloq.* **87**, 223–238
- Saggerson, E. D., Carpenter, C. A., Cheng, C. H. K. & Sooranna, S. R. (1980) *Biochem. J.* **190**, 183–189
- Soderling, T. R. (1975) *J. Biol. Chem.* **250**, 5407–5412
- Sooranna, S. R. & Saggerson, E. D. (1976) *FEBS Lett.* **64**, 36–39
- Sooranna, S. R. & Saggerson, E. D. (1978) *FEBS Lett.* **90**, 141–144
- Sugden, P. H., Holladay, L. A., Reimann, E. M. & Corbin, J. D. (1976) *Biochem. J.* **159**, 409–422
- Witt, J. J. & Roskoski, R. (1975) *Anal. Biochem.* **66**, 253–258