A trypsin-sensitive, heat-labile, *N*-ethylmaleimide-sensitive factor in adipocyte post-microsomal supernatant which affects the assay of adipocyte glycerol phosphate acyltransferase activities

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1. Addition of adipocyte 100 000 *g* post-microsomal supernatant to assays of glycerol phosphate acyltransferase in isolated mitochondria or microsomal fractions decreased activity at lower concentrations of palmitoyl-CoA. At higher concentrations of palmitoyl-CoA, activation was observed on addition of post-microsomal supernatant. 2. The effect of post-microsomal supernatant to decrease activity at lower [palmitoyl-CoA] was abolished by heating or by trypsin treatment, and was also abolished by addition of *N*-ethylmaleimide to assays or by pretreatment of post-microsomal supernatant with *N*-ethylmaleimide. The stimulatory effect seen at higher [palmitoyl-CoA] was not sensitive to heat or trypsin treatment. 3. The effect of post-microsomal supernatant at lower [palmitoyl-CoA] cannot be attributed to palmitoyl-CoA hydrolase activity. 4. It was found that brief treatment of adipocyte mitochondria with low concentrations of trypsin was an effective way to remove contaminating microsomal glycerol phosphate acyltransferase activity. 5. Adipocyte post-microsomal supernatant was more effective than an equivalent quantity of liver post-microsomal supernatant protein in decreasing adipocyte microsomal glycerol phosphate acyltransferase activity. The effects of the supernatants from both tissues were decreased by flavaspodic acid. 6. Semi-purified Z-protein fraction from rat liver did not mimic the effect of adipocyte post-microsomal supernatant to decrease glycerol phosphate acyltransferase at lower [palmitoyl-CoA]. 7. Post-microsomal supernatants obtained from noradrenaline-treated adipocytes were less effective than those from control cells in decreasing glycerol phosphate acyltransferase activity in microsomal fractions at lower [palmitoyl-CoA]. 8. It is suggested that adipocyte cytosol may contain an acyl-CoA-binding protein or proteins differing from Z-protein in some respects. The physiological significance of the findings is briefly discussed.

Rider & Saggerson (1983) have investigated the dependence of NEM-sensitive (microsomal) GPAT on palmitoyl-CoA concentration. These measurements were made in crude homogenates prepared after incubation of adipocytes (see Fig. 2 of Rider & Saggerson, 1983), and it was found that the activity increased with increasing [palmitoyl-CoA] in a markedly sigmoidal manner. By contrast, when GPAT is assayed in microsomes (microsomal fraction) isolated from adipocytes, this sigmoidal

relationship is not apparent (Saggerson *et al.*, 1979; Rider & Saggerson, 1983). These findings suggested that factor(s) present in the whole homogenate, but absent from isolated microsomal fractions, might affect the response of GPAT to the acyl-CoA substrate. Accordingly, the effect of the 100 000 *g* post-microsomal supernatant on the GPAT assay was investigated.

Materials and methods

Animals and chemicals

These were supplied and treated as described by Rider & Saggerson (1983). In addition, trypsin (from bovine pancreas) and trypsin inhibitor (from soya bean) were from Boehringer (London) Ltd.,

Abbreviations used: GPAT, glycerol phosphate acyltransferase (EC 2.3.1.15); NEM, *N*-ethylmaleimide.

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Lewes, East Sussex, U.K. Heparin was supplied by Weddel Pharmaceuticals Ltd., London EC1A 9 HY, U.K., and Sephadex G-75 (superfine grade) was obtained from Pharmacia, Uppsala, Sweden. β -Flavaspidic acid *N*-methylglucamine, originally from Huhtamäki-Yhtymä Oy, Turku, Finland, was generously given by Dr. D. N. Brindley, Department of Biochemistry, University of Nottingham. [9,10(n)- ^3H]Palmitic acid was obtained from Amersham International, Amersham, Bucks., U.K., and subjected, before use, to a procedure to remove impurities. This procedure decreased background radioactivity during the identification of liver cytosolic fatty acid-binding proteins. The benzene solvent, in which the [^3H]palmitic acid was supplied, was evaporated under N_2 and the residue dissolved in 50 mM- NaHCO_3 in ethanol/water (1:1, v/v). The ethanolic bicarbonate solution was washed with 2×10 ml of hexane and the pH adjusted to 3 with 2.5 M-HCl. The palmitic acid was extracted with 10 ml of hexane and stored under N_2 in sealed glass ampoules at -20°C .

Laboratory preparations

Adipocytes were isolated from rat epididymal adipose tissues and incubated for 1 h as described by Rider & Saggerson (1983). Subcellular fractions (mitochondria, microsomes and 100 000 *g* post-microsomal supernatants) were prepared from incubated adipocytes as described by Rider & Saggerson (1983), by using 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA ('sucrose medium') as the extraction buffer.

Adipocyte mitochondrial preparations were in some cases treated with trypsin to inactivate contaminating microsomal GPAT. Portions (0.25 ml) of mitochondrial suspension in sucrose medium (approx. 1 mg of protein/ml) were incubated with 2.4 μg of trypsin at 30°C . After 2 min, 12 μg of soya-bean trypsin inhibitor was added to inactivate the proteinase and the mixture stored in ice.

Adipocyte post-microsomal supernatants were treated with trypsin by incubating 1.0 ml of the supernatant (approx. 5 mg of protein/ml) with 125 μg of trypsin at 30°C . After 30 min, 400 μg of soya-bean trypsin inhibitor was added and the mixture stored in ice. Undigested supernatants were prepared as controls in which the inhibitor protein was added before the proteinase. In some experiments, post-microsomal supernatants from adipocytes were heat-treated by incubation in a water bath at 95°C for 1 h.

NEM-treated adipocyte post-microsomal supernatant was prepared by incubating the supernatant with 10 mM-NEM at 30°C for 5 min. The supernatant was then dialysed overnight at 4°C against 200 vol. of sucrose medium. As controls, super-

natants were incubated without NEM and dialysed overnight.

To obtain post-microsomal supernatants from rat liver, homogenates were prepared in sucrose medium and then subjected to the centrifugation steps described by Bates & Saggerson (1979). When liver supernatants were to be used for the partial purification of Z-protein, rats were injected intraperitoneally with 0.15 ml of heparin solution (5000 i.u./ml) 10 min before being killed and the livers were flushed to remove blood by infusing 80 ml of ice-cold sucrose medium into the hepatic portal vein.

The method of Rüstow *et al.* (1978) was used to label liver post-microsomal supernatant with [^3H]palmitate for identifying fatty acid-binding proteins after gel filtration. [^3H]Palmitate (50–100 μCi) (treated to remove impurities as described above) was dispensed into a 50 ml flask and the hexane removed under N_2 with rotation of the flask in order to coat the glass wall with a thin film. Then 5–10 ml of rat liver post-microsomal supernatant was added and the flask shaken vigorously in a metabolic shaker for 30 min at 25°C (130 cycles/min). The labelled supernatant was cooled in ice and then loaded on a Sephadex G-75 column (2.5 cm $\times$ 100 cm) equilibrated with 0.15 M-potassium phosphate buffer, pH 7.4 at 4°C . For preparative purposes, larger volumes (20–30 ml) of unlabelled supernatant were subjected to gel filtration on the same column. Protein was eluted from the column with the same phosphate buffer at a flow rate of 25 ml/h; 5 ml fractions were collected and protein was measured by the A_{280} . Portions (50 μl) of each fraction were removed for liquid-scintillation counting in 2 ml of methanol + 10 ml of toluene containing 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen. Fractions containing Z-protein were identified from the elution profile of the labelled cytosol, which was similar to that found by Rüstow *et al.* (1978). Three fatty acid-binding fractions were identified (Fig. 1). Peak I was presumed to be albumin and peak III was assessed as containing Z-protein since β -flavaspidic acid *N*-methylglucamine decreased the binding of [^3H]palmitate to pooled peak-III fractions. This was established as follows: 1 ml of combined peak-III fractions (0.5 mg/ml of protein) was incubated at 0°C for 5 min with or without 31 μM -flavaspidic acid *N*-methylglucamine, followed by incubation with 50 μCi of [^3H]palmitate. Unbound palmitate was then removed by dialysis against 100 vol. of 50 mM-potassium phosphate, pH 7.4, for 24 h at 4°C . After liquid-scintillation counting and correction for the blank, it was found that the flavaspidic acid derivative had decreased [^3H]palmitate binding from 4.95×10^5 to 1.52×10^5 c.p.m. per 0.5 mg of peak-III protein. Fractions containing Z-protein were concentrated by dialysis against 200 vol. of 70% (w/v) sucrose in 50 mM-

Fig. 1. Partial purification of Z-protein from rat liver post-microsomal supernatant by gel filtration on Sephadex G-75. Post-microsomal supernatant (5 ml) was prepared and labelled with 75 μ Ci of [9,10(n - 3 H)]palmitic acid followed by gel filtration as described in the text. ● (—), A_{280} (protein); ■ (---), 3 H radioactivity (d.p.m.).

potassium phosphate, pH 7.4, for 12 h at 4°C. Finally the preparation was dialysed against 1 litre of 50 mM-potassium phosphate, pH 7.4, for 12 h and then stored at -20°C.

Analytical methods

GPAT activity was assayed in 1.0 ml volumes as described by Rider & Saggerson (1983). Glycerol phosphate was used at 1.0 mM in all cases, and albumin at 1.75 mg/ml. Concentrations of palmitoyl-CoA and other additions are indicated in individual Figures and Tables.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical methods

Statistical significance was determined by using Student's *t* test for paired samples.

Results and discussion

Adipocyte microsomes were assayed for GPAT activity in the presence and absence of approx. 0.5 mg of post-microsomal-supernatant protein/ml (Fig. 2). In the absence of supernatant, the GPAT activity followed a near-hyperbolic-type curve with increasing [palmitoyl-CoA], confirming other observations (Saggerson *et al.*, 1979; Rider & Saggerson, 1983). Substrate inhibition was apparent at higher [palmitoyl-CoA]. Addition of supernatant resulted in the curve for activity versus [palmitoyl-CoA] being shifted to the right and assuming a sigmoidal-type profile such that GPAT was inhibited between approx. 10 μ M- and 80 μ M-palmitoyl-CoA. In addition, the supernatant caused a small increase in GPAT activity at higher [palmitoyl-CoA] (100

Fig. 2. Effect of adipocyte post-microsomal supernatant on the response of adipocyte microsomal GPAT to palmitoyl-CoA concentration

Microsomes (10–20 μ g of protein) were assayed for GPAT in the absence (O) and presence (●) of 0.1 ml of post-microsomal supernatant (0.54 ± 0.06 mg of protein). The results are means for four separate experiments; the bars indicate S.E.M. Significance of difference between activity in the absence of supernatant and that at 10 μ M-, 40 μ M-, 70 μ M-, 100 μ M- and 130 μ M-palmitoyl-CoA: $P < 0.05$, <0.001 , <0.05 , <0.05 , <0.02 respectively.

and 130 μM). Although this latter effect could in part be attributed to relief of substrate inhibition, it should be noted that the resultant activity was higher than the highest activity obtained in the absence of supernatant. This stimulatory effect of supernatant, seen at high [palmitoyl-CoA], was somewhat variable between preparations, but was seen on several occasions (see Table 2 and Fig. 3*b*). The possibility that the stimulation could be due to the presence of GPAT in the post-microsomal supernatant was excluded, since this was found to be extremely low (0.16 nmol/min per mg of protein when measured with 1 mM-glycerol phosphate and 100 μM -palmitoyl-CoA).

When assayed at 40 μM -palmitoyl-CoA, under which condition microsomal GPAT was inhibited by the supernatant (Fig. 2), this action of the supernatant was abolished after heating or trypsin treatment (Table 1). It is therefore concluded that the inhibitory effect is likely to be due to a protein or proteins present in the cytosol. By contrast, the stimulatory effect of supernatant seen at high [palmitoyl-CoA] was not abolished either by heating or by trypsin digestion of the supernatant. This could be taken to imply that this effect was due to non-protein factor(s) present in the cytosol, or that this effect could be attributed to heat-stable trypsin-insensitive protein(s) in the supernatant fraction.

It was considered that the heat- and trypsin-

sensitive effect of the supernatant, namely to displace the curve for microsomal GPAT activity versus [palmitoyl-CoA], could be due either to long-chain fatty acyl-CoA-binding protein(s) or palmitoyl-CoA hydrolase activity. This second possibility was discounted, since, when 0.5 mg of post-microsomal supernatant protein was assayed for palmitoyl-CoA hydrolase (Norum, 1964) at 232 nm and 30°C in 1.0 ml of 0.1 M-Tris/HCl, pH 7.4, containing 0.7 mM-dithiothreitol, fatty acid-poor albumin (1.75 mg/ml) and 40 μM -palmitoyl-CoA, no activity was detected.

Intuitively, if a fatty acid-CoA-binding protein or proteins were responsible for the effect of the post-microsomal supernatant, then the supernatant should have a similar action on the response of mitochondrial GPAT activity to [palmitoyl-CoA]. Surprisingly, when mitochondrial GPAT was assayed (Fig. 3*a*), the inclusion of supernatant protein had no effect on the response of the activity to [palmitoyl-CoA]. These assays were performed in the presence of 10 mM-NEM to inhibit contaminating microsomal activity (Saggerson *et al.*, 1979, 1980), raising the possibility that the thiol-group reagent was inhibiting the effect of the supernatant. Nimmo (1979) has demonstrated that GPAT in intact rat liver mitochondria suspended in iso-osmotic medium is unaffected by trypsin. On the other hand, liver mitochondrial GPAT becomes trypsin-sensitive when the mitochondria are exposed to hypo-osmotic medium. This, together with other findings, was taken as evidence that, in liver, mitochondrial GPAT is associated with the inner aspect of the mitochondrial outer membrane. GPAT in liver microsomal preparations, on the other hand, is directly accessible to inactivation by trypsin (Bell *et al.*, 1979). Table 2 shows that when adipocyte mitochondrial preparations were briefly exposed to low concentrations of trypsin, the component of the GPAT activity that is NEM-insensitive was barely affected. On the other hand, the NEM-sensitive component of the activity, which is likely to represent contamination by the microsomal GPAT (Saggerson *et al.*, 1979), was inactivated by 92%. Fig. 3(*b*) shows that when trypsin treatment, rather than addition of NEM, was used to abolish microsomal contamination, effects of post-microsomal supernatant were evident in the GPAT assay. The curve describing the mitochondrial GPAT activity as a function of [palmitoyl-CoA] was shifted to the right such that the enzyme was inhibited between approx. 10 μM and 50 μM -palmitoyl-CoA (Fig. 3*b*). In addition, the activity of the mitochondrial enzyme was stimulated considerably by the supernatant at higher [palmitoyl-CoA]. This stimulation could be attributed neither to GPAT activity in the supernatant nor to relief of substrate inhibition, since the highest activity observed with supernatant was

Table 1. *Effects of heat-treatment and trypsin digestion on the action of adipocyte post-microsomal supernatant on adipocyte microsomal GPAT*

Before inclusion in GPAT assays, post-microsomal supernatants were heat-treated or trypsin-digested as described in the Materials and methods section. Microsomes (10–20 μg of protein) were assayed for GPAT without or with the addition of supernatants (0.45 $\pm$ 0.06 mg of protein/ml). The results are means $\pm$ S.E.M. for four separate experiments. *, **, *** indicate $P < 0.05$, < 0.02 , < 0.001 for effects of supernatants versus the control without supernatant.

Additions to microsomal GPAT assay	Microsomal GPAT activity (nmol/min per mg of protein)	
	At 40 μM -palmitoyl-CoA	At 130 μM -palmitoyl-CoA
None	31.2 $\pm$ 1.6	49.6 $\pm$ 7.7
0.1 ml of untreated supernatant	1.3 $\pm$ 0.5***	75.1 $\pm$ 3.6**
0.1 ml of heat-treated supernatant	35.3 $\pm$ 4.7	69.0 $\pm$ 5.2*
0.1 ml of undigested supernatant (trypsin inhibitor added before trypsin)	1.7 $\pm$ 0.8***	80.3 $\pm$ 8.8*
0.1 ml of trypsin-digested supernatant	30.1 $\pm$ 2.6	75.2 $\pm$ 5.5*

Fig. 3. Effect of adipocyte post-microsomal supernatant on the response of adipocyte mitochondrial GPAT to palmitoyl-CoA concentration

(a) Mitochondria (10–20 µg of protein) were assayed for GPAT with 10 mM-NEM in the absence (○) and presence (●) of 0.1 ml of post-microsomal supernatant (0.54 ± 0.06 mg of protein). The results are means for four separate experiments; the bars indicate S.E.M. (b) Trypsin-treated mitochondria (10 µg of protein) were assayed for GPAT in the absence (○) and presence (■) of 0.1 ml of post-microsomal supernatant (0.52 ± 0.03 mg of protein). The results are means for four separate experiments; the bars indicate S.E.M. Significance of differences between activity in the absence of supernatant and that at 10 µM-, 40 µM-, 70 µM-, 100 µM- and 130 µM-palmitoyl-CoA: $P > 0.2$, $P < 0.01$, $P < 0.1$, $P < 0.01$, $P < 0.05$ respectively.

Table 2. Susceptibility of NEM-sensitive and NEM-insensitive GPAT activities in adipocyte mitochondrial preparations to inactivation by trypsin

Adipocyte mitochondrial preparations were treated with trypsin as described in the Materials and methods section. Untreated and trypsin-treated mitochondria (10 µg of protein) were assayed for GPAT activity in the presence and absence of 10 mM-NEM, with 1.0 mM-glycerol phosphate and 100 µM-palmitoyl-CoA. NEM-sensitive activity was deduced by subtracting NEM-insensitive activity from total GPAT. The results are the means $\pm$ S.E.M. for four separate experiments.

Mitochondria	GPAT activity (nmol/min per mg of protein)		
	Total	NEM-insensitive	NEM-sensitive
Untreated	31.8 ± 4.4	9.3 ± 1.3	22.5 ± 4.3
Trypsin-treated	9.8 ± 1.8	8.1 ± 0.7	1.7 ± 0.4

substantially greater than that in the absence of the supernatant (Fig. 3b). It is also noteworthy that this stimulation at higher [palmitoyl-CoA] was not apparent in the presence of NEM (Fig. 3a).

These findings are relevant to the study by Rider & Saggerson (1983), in which GPAT activity was measured in crude homogenates in the presence and absence of NEM. These assays were performed in every case with 0.1 ml of crude homogenate, which was found to contain approx. 0.1 unit of lactate dehydrogenase activity. The post-microsomal supernatants used here contained approx. 4.5 mg of protein per unit of lactate dehydrogenase. Therefore the assays of GPAT in crude homogenates (Rider & Saggerson, 1983) contained equivalent concentrations of supernatant protein and equivalent palmitoyl-CoA/supernatant-protein ratios to those added here to microsomes (Fig. 2) or mitochondria (Fig. 3b). Although measurement of total GPAT in crude homogenates (Rider & Saggerson, 1983) is performed in the presence of approx. 0.5 mg of supernatant protein/ml, the effect of this supernatant protein is essentially abolished when NEM is added to crude extracts to facilitate measurement of the mitochondrial GPAT. Hence, at low [palmitoyl-CoA], the measurement of mitochondrial GPAT in crude extracts is an overestimate, and also the calculation, by difference from the total GPAT, of the microsomal activity is an underestimate.

To confirm that the putative fatty acyl-CoA binding protein(s) was sensitive to thiol-group reagents, post-microsomal supernatant was treated with NEM and then dialysed before its effect was tested on the microsomal enzyme (Fig. 4). The treatment with NEM abolished inhibition of microsomal GPAT at low [palmitoyl-CoA]. The curve in the presence of 10 mM-NEM-treated supernatant

Fig. 4. Effect of NEM-treated adipocyte post-microsomal supernatant on the response of adipocyte microsomal GPAT to palmitoyl-CoA concentration

Microsomes (10–20 μg of protein) were assayed for GPAT in the presence of 0.1 ml of untreated/dialysed (O) or 0.1 ml of NEM-treated/dialysed (●) post-microsomal supernatant. Treatment of supernatants is described in the Materials and methods section. The protein concentration of supernatants in GPAT assays was 0.42 ± 0.05 mg/ml. The results are means for three separate experiments. The bars indicate S.E.M. $P < 0.001$ and < 0.01 at $10 \mu\text{M}$ - and $40 \mu\text{M}$ -palmitoyl-CoA respectively for comparisons of the two treatments.

was essentially the same as that seen in the absence of any supernatant (Fig. 2). Treatment in the same way of supernatant with 1 mM-NEM was, however, ineffective in this respect (results not shown). Thus quite substantial concentrations of NEM appear to be required.

The Z-protein fraction of rat liver (Mishkin *et al.*, 1972) contains one or more proteins (Trulzsch & Arias, 1981) which bind long-chain fatty acids and acyl-CoA thioesters with high affinity (Mishkin & Turcotte, 1974a; Mishkin *et al.*, 1975). The Z-protein fraction described by Mishkin *et al.* (1972) is probably identical with the so-called 'fatty acid binding protein' of Ockner *et al.* (1972). Indeed, Z-protein or fatty acid-binding protein is probably

similar to 'A-protein' described by Ketterer *et al.* (1976), 'band C' described by Billheimer & Gaylor (1980) and the 'squalene or sterol carrier protein' (SCP) described by Dempsey *et al.* (1981), on the basis of similarities between the physical properties and amino acid compositions of the proteins (Ockner *et al.*, 1982). The earlier workers have attributed to the protein a role in the metabolism and/or intracellular transport of carcinogens, haem and cholesterol respectively. In addition, Z-protein has been implicated in mediating the uptake of bilirubin, sulphobromophthalein and other organic anions by the liver (Levi *et al.*, 1969). Flavaspodic acid, a phloroglucinol derivative extracted from the rhizomes of the male fern, has been shown to interfere with the uptake of bilirubin and sulphobromophthalein by the liver (Nosslin & Morgan, 1965) and competitively inhibits the binding of these substances (Levi *et al.*, 1969) and long-chain fatty acids (Mishkin *et al.*, 1972) to Z-protein.

Z-protein (M_r 12000) has been reported to account for as much as 4–5% (approximate concn. 0.4 mM) of the total liver cytosolic protein, and binds 60% of the cytosolic long chain fatty acids (Ockner *et al.*, 1982). It has been proposed that Z-protein facilitates and regulates the utilization of long-chain fatty acids (Mishkin & Turcotte, 1974a; Mishkin *et al.*, 1975; Ockner & Manning, 1976; Wu-Rideout *et al.*, 1976), from the finding that the protein stimulates microsomal acyl-CoA synthetase (Wu-Rideout *et al.*, 1976), microsomal GPAT (Mishkin & Turcotte, 1974b; Burnett *et al.*, 1979; Jamdar, 1979), microsomal diacylglycerol acyltransferase (O'Doherty & Kuksis, 1975), mitochondrial β -oxidation of fatty acids (Fournier *et al.*, 1978) and acetyl-CoA carboxylase (Lunzer *et al.*, 1977). The stimulation of microsomal GPAT by Z-protein has been attributed to the relief of palmitoyl-CoA-induced inhibition of the enzyme (Jamdar, 1979).

Z-Protein has been shown to be present in the cytosol fraction of many tissues, including liver, myocardium, skeletal muscle, intestinal mucosa, kidney and adipose tissue (Mishkin *et al.*, 1972). It was therefore decided to purify Z-protein from rat liver partially to see whether the protein would mimic the effect of adipocyte post-microsomal supernatant fractions on the response of microsomal GPAT to palmitoyl-CoA concentration.

Z-protein was partially purified from a rat liver post-microsomal supernatant by gel filtration on Sephadex G-75 (Rüstow *et al.*, 1978) as described in the Materials and methods section. Flavaspodic acid *N*-methylglucamine (31 μM) inhibited the binding of [^3H]palmitate to the preparation, confirming the identity of the fraction. Moreover, the preparation deinhibited liver microsomal GPAT activity when assayed in the absence of albumin (Fig. 5), as demonstrated by Jamdar (1979). In both that and

the present study $87\mu\text{M}$ -palmitoyl-CoA was used, which, in the absence of albumin, was inhibitory towards microsomal GPAT (Jamdar, 1979). Also, flavaspidic acid *N*-methylglucamate abolished this effect of the Z-protein preparation (Fig. 5).

Fig. 6 shows that the inclusion of 0.46 mg of partially purified liver Z-protein in assays of adipocyte microsomal GPAT (which contained the usual 1.75 mg of defatted albumin) did not have the same effect as the adipocyte high-speed supernatant on the kinetic response of the enzyme to low [palmitoyl-CoA]. The addition of Z-protein to GPAT assays did, however, relieve palmitoyl-CoA-induced inhibition of the enzyme at $100\mu\text{M}$ - and $130\mu\text{M}$ -palmitoyl-CoA (Fig. 6), consistent with its role as a

fatty acyl-CoA binding protein. In addition, Z-protein caused a slight stimulation of GPAT activity at $10\mu\text{M}$ -palmitoyl-CoA, which might have been due to the presentation of endogenously bound long-chain acyl-CoA species to the enzyme.

Since liver Z-protein fraction added in amounts equivalent to the total additions of adipocyte supernatant could not mimic the action of adipocyte supernatant, it is tentatively concluded that binding protein(s) different from liver Z-protein were responsible for the effects of adipocyte supernatant on GPAT assays at low [palmitoyl-CoA]. In support of this is the finding that NEM abolishes the effect of the adipocyte supernatant factor. Z-protein contains a single cysteine residue (Ockner *et al.*, 1982; Takahashi *et al.*, 1982), as does the similar or identical sterol- or squalene-carrier protein (Dempsey *et al.*, 1981). However, carboxymethylation of this cysteine residue does not affect the function of this sterol carrier protein (Dempsey *et al.*, 1981). It is also perhaps noteworthy that the

Fig. 5. Effect of partially purified rat liver Z-protein on the activity of liver microsomal GPAT, assayed in the absence of albumin

Rat liver microsomes were isolated as described by Bates & Saggerson (1979) and resuspended at a protein concentration of 5 mg/ml. Microsomal suspension (0.1 ml) was assayed for GPAT in a final volume of 1 ml containing 120 mM-KCl, 50 mM-Tris/HCl, pH 7.4, 0.7 mM-dithiothreitol, 1 mM-[U- ^{14}C]glycerol phosphate, $87\mu\text{M}$ -palmitoyl-CoA and the indicated concentrations of partially purified Z-protein. Assays were performed in the absence (○) and presence (●) of $31\mu\text{M}$ -flavaspidic acid *N*-methylglucamate.

Fig. 6. Effect of partially purified rat liver Z-protein on the response of adipocyte microsomal GPAT to palmitoyl-CoA concentration

Microsomes ($10\text{--}20\mu\text{g}$ of protein) were assayed for GPAT in the absence (○) and presence (●) of partially purified Z-protein (0.46 mg/ml); 1.75 mg of albumin/ml was present in all assays. The results are means of two separate experiments. The bars indicate the range of values.

binding of [3 H]oleic acid per mg of Z-protein fraction has been reported to be 10-fold greater to that isolated from the liver than to that obtained from adipose tissue (Mishkin *et al.*, 1972).

Whatever the nature of the protein factor in the adipocyte supernatant, it appeared to have a common property with Z-protein in that a relatively low concentration of the flavaspidic acid derivative (16 μ M) inhibited the effect of the supernatant in the microsomal GPAT assay (Fig. 7). An equivalent concentration of liver post-microsomal supernatant protein also inhibited adipocyte microsomal GPAT, although less effectively than the adipocyte supernatant. The flavaspidic acid derivative (16 μ M) also decreased this effect of the liver supernatant (Fig. 7). Concentrations of 80–100 μ M of the flavaspidic acid derivative were required to abolish totally the inhibitory effects of the liver or adipocyte supernatants (results not shown). The effects of these higher concentrations of the flavaspidic acid *N*-methylglucamine, however, were complex in that they also increased microsomal GPAT activities in the absence and presence of supernatants.

Fig. 7. Effect of flavaspidic acid *N*-methylglucamine on the actions of adipocyte or liver post-microsomal supernatants in the adipocyte microsomal GPAT assay

Adipocyte microsomes (10–20 μ g of protein) were assayed for GPAT with (closed symbols) or without (open symbols) 16 μ M-flavaspidic acid. Other additions were: Δ , $\blacktriangle$, adipocyte post-microsomal supernatant (0.5 mg of protein/ml); $\square$, $\blacksquare$, liver post-microsomal supernatant (0.5 mg of protein/ml); $\circ$, $\bullet$, no additions of supernatants. The results are the means of two separate experiments.

Post-microsomal supernatants were also prepared from adipocytes that had been incubated with 1 μ M-noradrenaline for 1 h. Fig. 8 shows that microsomal GPAT, assayed with 40 μ M-palmitoyl-CoA, was less sensitive to inhibition by supernatant from the hormone-treated cells than by that from control cells. The decreased capacity of supernatants from noradrenaline-treated cells to inhibit GPAT might be attributable to occupancy of binding sites on the putative acyl-CoA binding protein(s) with non-esterified fatty acids liberated as a result of hormone-stimulated lipolysis. Alternatively, hormone treatment of the cells might have resulted in some other form of modification of the supernatant protein(s). It is noteworthy that in rat adipose tissues the whole-tissue concentrations of long-chain fatty acyl-CoA thioesters change very

Fig. 8. Sensitivity of GPAT in microsomes prepared from control-incubated adipocytes to inhibition by post-microsomal supernatants obtained from control and noradrenaline-treated adipocytes

Adipocytes were incubated with or without 1 μ M-noradrenaline as described by Rider & Saggerson (1983). Microsomes were prepared from control cells, and post-microsomal supernatants were obtained from both sets of cells. Microsomes from control-incubated cells (10–20 μ g of protein) were assayed for GPAT by using 40 μ M-palmitoyl-CoA in the presence of the indicated concentrations of supernatant from control ($\circ$) and noradrenaline-treated ($\bullet$) cells. Each point is the mean for three separate experiments. The bars indicate S.E.M. $P < 0.05$ for comparison of the effects at 250 and 300 μ g of supernatant protein/ml.

little between various incubation conditions and physiological states, whereas alterations in the rates of fatty acid and triacylglycerol synthesis are often large (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970a,b). Halestrap & Denton (1974) have postulated the existence of a specific binding protein for fatty acyl-CoA thioesters in adipose tissue which is subject to regulation, thus allowing the free concentration of these metabolites to vary independently of their whole-tissue concentration. Such an effect may be apparent here.

It is concluded from the present study that adipocytes contain a cytosolic protein or proteins which bring about a sigmoidal relationship between GPAT activity in mitochondria or microsomes and the concentration of fatty acyl-CoA. The most obvious explanation is that this is due to sequestration of the acyl-CoA substrate, although it should be pointed out that binding of this sort has not been demonstrated directly. Although the effect is diminished by the flavaspidic acid derivative, there is some evidence to suggest that it is attributable to a factor or factors different from Z-protein preparations. The physiological significance of a factor that induces such a sigmoidal response making the GPAT reaction highly sensitive to changes in acyl-CoA concentration in the 'mid-range' is obvious. Furthermore, the effectiveness of this cytosolic factor may be modified by hormone treatment. The nature and properties of factors stimulating GPAT at higher [acyl-CoA] are unclear. Further work is necessary to establish whether this latter effect has any similarity to the effects of heat-stable cytosolic proteins isolated by Roncari & Mack (1981) which stimulate the conversion of phosphatidate into triacylglycerol.

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