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A Study of the Glycerol Phosphate Acyltransferase and Dihydroxyacetone Phosphate Acyltransferase Activities in Rat Liver Mitochondrial and Microsomal Fractions

RELATIVE DISTRIBUTION IN PARENCHYMAL AND NON-PARENCHYMAL CELLS, EFFECTS OF *N*-ETHYLMALEIMIDE, PALMITOYL-COENZYME A CONCENTRATION, STARVATION, ADRENALECTOMY AND ANTI-INSULIN SERUM TREATMENT

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1. GPAT (glycerol phosphate acyltransferase) and DHAPAT (dihydroxyacetone phosphate acyltransferase) activities were measured both in subcellular fractions prepared from fed rat liver and in whole homogenates prepared from freeze-stopped pieces of liver. 2. GPAT activity in mitochondria differed from the microsomal activity in that it was insensitive to *N*-ethylmaleimide, had a higher affinity towards the palmitoyl-CoA substrate and showed a different response to changes in hormonal and dietary status. 3. Starvation (48h) significantly decreased mitochondrial GPAT activity. The ratio of mitochondrial to microsomal activities was also significantly decreased. The microsomal activity was unaffected by starvation, except after adrenalectomy, when it was significantly decreased. Mitochondrial GPAT activity was decreased by adrenalectomy in both fed and starved animals. 4. Acute administration of anti-insulin serum significantly decreased mitochondrial GPAT activity after 60min without affecting the microsomal activity. 5. A new assay is described for DHAPAT. The subcellular distribution of this enzyme differed from that of GPAT. The highest specific activity of DHAPAT was found in a 23000$g_{av.}$ pellet obtained by centrifugation of a post-mitochondrial supernatant. This fraction also contained the highest specific activity of the peroxisomal marker uricase. DHAPAT activity in mitochondrial fractions or in the 23000$g_{av.}$ pellet was stimulated by *N*-ethylmaleimide, whereas that in microsomal fractions was slightly inhibited by this reagent. The GPAT and DHAPAT activities in mitochondrial fractions had a considerably higher affinity for the palmitoyl-CoA substrate. 6. Total liver DHAPAT activity was significantly decreased by starvation (48h), but was unaffected by administration of anti-insulin serum. 7. The specific activities of GPAT and DHAPAT were lower in non-parenchymal cells compared with parenchymal cells, but the GPAT/DHAPAT ratio was 5–6-fold higher in the parenchymal cells.

It is currently thought that the biosynthesis of glycerolipids can be initiated by the acylation of either glycerol phosphate or dihydroxyacetone phosphate. The relative contributions of these two metabolic routes in liver or any other tissue are not fully established (see review by Hajra, 1977). GPAT and DHAPAT activities are both found in cellular membranes. In rat liver, GPAT activity is generally found in both mitochondrial and microsomal fractions, although there is disagreement about the relative proportions of the mitochondrial and microsomal forms (see review by van den Bosch, 1974). When rat liver is simply fractionated into mitochondrial and microsomal fractions DHAPAT activity is found in both. However, in guinea-pig liver a peroxisomal location for this enzyme has been claimed (Jones & Hajra, 1977).

The properties of GPAT in mitochondrial and microsomal fractions differ in several respects (van den Bosch, 1974). Studies (Bates & Saggerson, 1977; Bates *et al.*, 1977) have suggested that the mitochondrial, but not the microsomal, activity is affected by insulin status and appears to be acutely regulated by the hormone. Here we have investigated, in the long term, the effects of starvation and adrenalectomy and, in the short term, the effects of anti-insulin serum administration on GPAT activity. Again, the mitochondrial and microsomal activities were found to differ in their responses to these treatments.

Abbreviations used: GPAT, glycerol phosphate acyltransferase (EC 2.3.1.15); DHAPAT, dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42).

Materials and Methods

Animals

Male rats of the Sprague–Dawley strain were used throughout. All unoperated rats were bred in the animal colony at University College London and had constant access to water and GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.). Fed rats weighed 170–190g at time of death. Starved rats (48h) had constant access to drinking water and weighed 135–150g at time of death.

Adrenalectomized rats and their sham-operated controls were purchased from Charles River (Margate, Kent, U.K.) 1 day after being operated on at 130–140g body wt. These animals were kept for a further 5–8 days before use. Adrenalectomized rats were given 0.15M-NaCl in tap water to drink.

Fed rats were treated with anti-insulin serum or control serum under sodium pentobarbitone anaesthesia (intraperitoneal, 70mg/kg body wt., in 0.15M-NaCl): 0.5ml lots of sera were injected into the femoral vein. Blood samples were taken from the tail.

Male guinea pigs of the Dunkin and Hartley strain were purchased from Bantin and Kingman (Hull, U.K.) and maintained on R.G.P. diet with ascorbic acid (E. Dixon and Sons).

Chemicals

All enzymes (except collagenase), cytochrome *c* (from horse heart), fructose 1,6-bisphosphate (trisodium salt), *sn*-glycerol 3-phosphate (dicyclohexylammonium salt), phosphoenolpyruvate (potassium salt) and 2-oxoglutarate (disodium salt) were from Boehringer Corp. (London) Ltd. (Lewes, Sussex, U.K.). Collagenase (type 1 from *Clostridium histolyticum*), ADP, NADH, $NADP^+$, NADPH and palmitoyl-CoA (potassium salt) were from International Enzymes Ltd. (Windsor, Berks., U.K.). *N*-Ethylmaleimide, tyramine hydrochloride, dihydroxyacetone phosphate (lithium salt), uric acid (sodium salt) and bovine plasma albumin (fraction V) were purchased from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Dithiothreitol was from Cyclochemical Travenol Laboratories (Los Angeles, CA, U.S.A.) and 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen from CIBA (A.R.L.) Ltd. (Duxford, Cambridge, U.K.). Radiochemicals were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Bovine insulin (6× recrystallized) was from Boots Pure Drug Co. (Nottingham, U.K.) and heparin (mucous) B.P. was supplied by Weddel Pharmaceuticals (London E.C.1, U.K.). Sodium pentobarbitone (Nembutal) powder was a gift from Abbott Laboratories (Queenborough, Kent, U.K.). *Haemophilus pertussis* vaccine (one human dose/ml) was a gift from Dr. J. Green of the Lister Institute of Preventive Medicine (Elstree, Herts., U.K.).

Bovine plasma albumin was subjected to a defatting procedure before use (Chen, 1967), with minor modifications (Saggerson, 1972). Antiserum to bovine insulin was raised in guinea pigs by the method of Wright *et al.* (1968) by using heat-killed *H. pertussis* vaccine as an adjuvant. The antiserum (1ml) was found to bind 3.3units of ^{125}I-labelled insulin by the method of Wright & Malaisse (1966). Control serum was obtained from guinea pigs treated with adjuvant alone.

Preparation of parenchymal and non-parenchymal cells from rat liver

Fed rats were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (70mg/kg body wt.) in 0.15M-NaCl 5min before an intraperitoneal injection of 0.2ml of heparin (38mg/ml). Parenchymal cells were prepared essentially by the method of Krebs *et al.* (1974), except that hyaluronidase was omitted from the preparation. The resulting cells were pelleted by centrifugation at $50g_{av.}$ for 2min and washed once with Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 1.27mM-Ca^{2+} and fatty acid-poor albumin (25mg/ml). The cells were re-centrifuged at $50g_{av.}$ as above and two similar washings were performed with Krebs–Ringer bicarbonate buffer alone. Finally, a portion of the parenchymal-cell preparation (approx. 100mg wet wt.) was placed in a silicone-treated Ultra-Turrax homogenizer tube, centrifuged at $1700g_{av.}$ for 90s, the washing medium decanted and the cell pellet frozen in liquid N_2.

Non-parenchymal cells were isolated by perfusion of the liver with collagenase followed by destruction of the parenchymal cells with Pronase (2.5mg/ml for 1h) (Mills & Zucker-Franklin, 1969). No pellet was obtained on centrifuging at $50g_{av.}$ for 2min (see above) after this treatment, indicating the absence of parenchymal cells. The remaining non-parenchymal cells were collected by centrifugation for 5min at $600g_{av.}$ (van Berkel & Kruijt, 1977). These were washed three times as for parenchymal cells, except that all centrifugations were at $600g_{av.}$ for 5min. Finally, the total yield of non-parenchymal cells from one liver (200–400mg wet wt.) was centrifuged at $1700g_{av.}$ and frozen in liquid N_2 as described above.

Preparation of subcellular fractions from rat liver

Rats were killed by cervical dislocation. The entire liver was removed, minced with scissors in ice-cold 0.25M-sucrose medium containing 10mM-Tris/HCl buffer, pH7.4, and 1mM-EDTA, followed by three washes with approx. 50ml of the same ice-cold medium. The liver was homogenized in a further

80ml of the same medium by using a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle (0.19mm radial clearance). The homogenate was centrifuged at 0–4°C at $620g_{av.}$ for 10min in a Sorvall Superspeed RC2-B centrifuge fitted with an SS-34 rotor ($r_{av.}$ 10.8cm). The resulting supernatant was centrifuged at $7250g_{av.}$ for 10min to yield a mitochondrial pellet. This was washed twice with 30ml of the homogenization buffer by resuspension and centrifugation at $9200g_{av.}$ for 10min. The washed mitochondrial fraction (M) was then resuspended in 0.25M-sucrose medium containing 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA and 1mM-dithiothreitol at 4–5mg of protein/ml. Unless otherwise stated, a microsomal fraction (E) was obtained by centrifuging the $7250g_{av.}$ post-mitochondrial supernatant at $105000g_{av.}$ for 1h in a Beckman Spinco model L ultracentrifuge. The microsomal pellet was resuspended in 0.25M-sucrose medium containing 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA and 1mM-dithiothreitol at a protein concentration of 6–8mg/ml. In some experiments a further fraction was obtained by centrifugation of the $7250g_{av.}$ post-mitochondrial supernatant at $23000g_{av.}$ for 10min. The resulting pellet was washed twice by resuspension in approx. 30ml of the homogenization buffer, followed by centrifugation at $25000g_{av.}$ for 10min. Finally, this fraction (P) was suspended in 0.25M-sucrose medium containing 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA and 1mM-dithiothreitol to give a final protein concentration of 2–3mg/ml.

Freeze-stopping and extraction of rat liver

Pieces (approx. 1g) of the left lateral lobe were removed either immediately after cervical dislocation of the animals or under sodium pentobarbitone anaesthesia. The liver samples were freeze-clamped between aluminium plates precooled in liquid N_2 (Wollenberger *et al.*, 1960) and then stored under liquid N_2. Tissue extracts were prepared by grinding 400–600mg of the frozen liver to a fine powder in a mortar under liquid N_2 followed by homogenization with an Ultra-Turrax tissue disintegrator (Janke und Künkel, Staufen, German Federal Republic). This was performed at 0–4°C for three 10s periods in 0.25M-sucrose medium containing 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA and 1mM-dithiothreitol (85mg of powdered liver/ml). Ultra-Turrax homogenates were similarly obtained from the frozen preparations of isolated cells (see above) by homogenization in 1ml of this medium.

Analytical methods

DHAPAT activity (EC 2.3.1.42) was measured at 30°C in a final volume of 1ml containing 120mM-KCl, 50mM-Tris/HCl buffer, pH7.4, 4mM-$MgCl_2$, 8mM-NaF, fatty acid-poor albumin (4mg/ml), 65μM-palmitoyl-CoA, 0.5mM-[U-^{14}C]fructose 1,6-bisphosphate (0.4μCi/ml), 50μg of aldolase (0.45unit) and 3μg of triose phosphate isomerase (15units). These conditions gave a concentration of 0.45mM-dihydroxyacetone phosphate in the assay (see Fig. 1 below). Before use, the coupling enzymes were dialysed at 4°C overnight against 750vol. of 240mM-KCl/100mM-Tris/HCl buffer, pH7.4, to remove $(NH_4)_2SO_4$. A 0.9ml portion of the reaction mixture was preincubated for 16min at 30°C before addition of *N*-ethylmaleimide (where indicated) or 0.1ml of tissue extracts (1–1.5mg of protein for Ultra-Turrax homogenates, 0.4–0.6mg of protein for mitochondria or 0.5–0.7mg of protein for microsomal fraction). Assays were normally performed for 6 or 8min in duplicate and were terminated by the addition of 3.5ml of chloroform/methanol (1:2, v/v) (Hajra, 1974). After centrifugation for 5min at $1500g_{av.}$ the resulting supernatant was decanted and 1.0ml of chloroform was added, followed by 1.0ml of 2M-KCl in 0.2M-H_3PO_4. After mixing and centrifugation for 5min at $1000g_{av.}$ the top layer was discarded and the lower chloroform layer washed by mixing with 4ml of water and 0.5ml of 2M-KCl in 0.2M-H_3PO_4. After centrifugation at $1000g_{av.}$ for 5min a 1.0ml portion of the chloroform layer was evaporated to dryness in a glass scintillation vial under a stream of O_2-free N_2. Liquid-scintillation counting of the samples was performed in toluene containing 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen (4g/litre). Palmitoyl-CoA was omitted from blank assays. Incorporation of ^{14}C into chloroform-soluble material was linear with time and quantity of tissue-extract protein.

Glutamate dehydrogenase (EC 1.4.1.2) and GPAT (EC 2.3.1.15) activities were measured as described by Bates & Saggerson (1977). Unless otherwise stated, GPAT assays in isolated mitochondria or Ultra-Turrax homogenates of whole liver or of cells were performed for 4min at 30°C in the presence of albumin (6mg/ml) and 65μM-palmitoyl-CoA, whereas assays in microsomal fractions were performed with albumin (1.75mg/ml) and 65μM-palmitoyl-CoA. Monoamine oxidase activity (EC 1.4.3.4) was measured at 30°C by the method of Aas (1971), with [*side-chain*-2-^{14}C]tyramine hydrochloride as substrate. $NADP^+$–cytochrome *c* reductase (EC 1.6.2.4) was assayed by the method of Phillips & Langdon (1962) and uricase (EC 1.7.3.3) by the method of Leighton *et al.* (1968). Pyruvate kinase activity (EC 1.2.4.1) was measured with 1mM-phosphoenolpyruvate in the presence and absence of 0.5mM-fructose 1,6-bisphosphate as described by van Berkel & Kruijt (1977).

For measurement of glycogen content, 100–200mg samples of powdered frozen liver were extracted as

described by Good *et al.* (1933). Glucose was measured enzymically in glycogen hydrolysates or in samples of blood plasma by the method of Slein (1963). Dihydroxyacetone phosphate was measured enzymically by the method of Michal & Beutler (1974) and glycerol phosphate by the method of Michal & Lang (1974). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chromatographic methods

Indentification of products of the DHAPAT assay was performed by t.l.c. on silica-gel G plates (Macherey, Nagel and Co., Düren, German Federal Republic) in chloroform/methanol/acetic acid/aq. 5% (w/v) Na_2SO_4 (25:10:3:1, by vol.). The migration of the radioactive peaks was compared with synthetic monopalmitoylglycerol phosphate and bispalmitoylphosphatidic acid.

Statistical methods

Analysis of data was performed by using Student's *t* test modified by Bessel's correction factor for small samples.

Results and Discussion

Assay of DHAPAT

Radioactively labelled dihydroxyacetone phosphate is not commercially available. Although this enzyme activity may be measured by introducing a radioactive label via the fatty acyl substrate, separation of labelled products from the precursor is rather complicated (Dodds *et al.*, 1976). Alternatively ^{32}P-labelled dihydroxyacetone phosphate has been synthesized and used in several studies (Hajra, 1968*a*; Schlossman & Bell, 1977). In the present study dihydroxy[^{14}C]acetone phosphate was generated *in situ* from commercially available [U-^{14}C]fructose 1,6-bisphosphate. This method appears to be both reproducible and simple.

Dihydroxy[^{14}C]acetone phosphate was generated during a 16min preincubation period and reached an equilibrium concentration by this time (results not shown). Dihydroxyacetone phosphate was found to vary linearly with (fructose 1,6-bisphosphate concentration)$^{\frac{1}{2}}$, except at low concentrations (Fig. 1). Fig. 1 was used as a calibration curve to calculate the dihydroxy[^{14}C]acetone phosphate concentration in all subsequent experiments. The formation of dihydroxyacetone phosphate was unaffected by NaF, albumin or dithiothreitol. In a typical assay, variation of the quantity of ^{14}C-labelled fructose 1,6-bisphosphate in the presence of a fixed amount of unlabelled fructose 1,6-bisphosphate had no effect

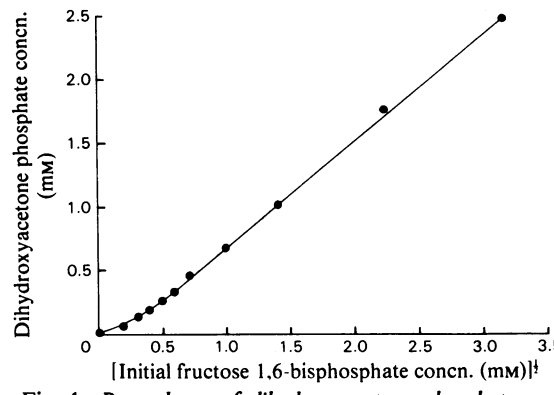

Fig. 1. *Dependence of dihydroxyacetone phosphate concentration in the DHAPAT assay on the initial concentration of fructose* 1,6-*bisphosphate*

Preincubations were performed as described in the Materials and Methods section for 16min with the indicated additions of fructose 1,6-bisphosphate after which the incubations were deproteinized and dihydroxyacetone phosphate was measured.

on the calculated incorporation of dihydroxyacetone phosphate carbon into chloroform-soluble products, suggesting that isotope-discrimination effects were negligible. The product of the assay was assumed to be monopalmitoyldihydroxyacetone phosphate, since it was retarded relative to monopalmitoylglycerol phosphate on t.l.c. in a solvent system containing $NaHSO_3$ (Hajra, 1968*b*), but migrated in the position of monopalmitoylglycerol phosphate in the absence of $NaHSO_3$. There was no formation of phosphatidic acid unless NADPH was included in the incubation medium. There was negligible formation of glycerol 3-phosphate during a typical assay performed with an Ultra-Turrax homogenate.

Effect of N-ethylmaleimide on GPAT and DHAPAT activities

Microsomal GPAT from various tissues has been reported to be sensitive to inhibition by thiol reagents (Lands & Hart, 1965; Monroy *et al.*, 1972; Haldar & Pullman, 1975), whereas mitochondrial activity is reported to be insensitive to, or possibly slightly stimulated by, these agents (Monroy *et al.*, 1972; Haldar & Pullman, 1975). Assay with and without a suitable concentration of *N*-ethylmaleimide should therefore give a quick and convenient measurement of the mitochondrial and microsomal forms of GPAT in unfractionated freeze-stopped liver, which could facilate study of acute regulation of this enzyme. Fig. 2 demonstrates the concentration-dependence of the effect of *N*-ethylmaleimide on mitochondrial and microsomal GPAT activities. The percentage effects

of *N*-ethylmaleimide were essentially the same with fresh unfrozen preparations or with preparations that had been frozen in liquid N_2 and subjected to Ultra-Turrax homogenization to mimic the procedure used in preparation of extracts from freeze-stopped liver. Fig. 2 confirms previous observations (Monroy *et al.*, 1972; Haldar & Pullman, 1975) that mitochondrial GPAT activity is essentially *N*-ethylmaleimide-insensitive. On the other hand, the activity measured in the microsomal fraction was 80–90% inhibited by *N*-ethylmaleimide (0.5–10mM). By using glutamate dehydrogenase activity as a marker to correct for mitochondrial contamination of the microsomal fraction, it was found that 10mM-*N*-ethylmaleimide decreased the microsomal activity by 92% and 91% in fresh and frozen Ultra-Turrax-homogenized preparations respectively. In other experiments (results not shown), the effect of *N*-ethylmaleimide (10mM) on microsomal GPAT was seen to be maximal within 1min and was constant for at least 10min. Mitochondrial GPAT activity showed a small progressive decrease with 10mM-*N*-ethylmaleimide. This was 13% after 4min, which was the chosen assay time. It was concluded that *N*-ethylmaleimide-insensitive activity is a reasonable estimate of the mitochondrial GPAT. It is noteworthy that freezing followed by Ultra-Turrax homogenization decreased mitochondrial or microsomal GPAT activity measured at 0.5mM-glycerol phosphate. This effect was more pronounced in microsomal preparations (see legend to Fig. 2 and Table 1). At 5mM-glycerol phosphate mitochondrial GPAT activity was unchanged by freezing and homogenization (Table 1). The reason for this is not understood at present. In subsequent experiments (results not shown) it was found the mitochondrial activity at 0.5mM-glycerol phosphate could also be decreased by Ultra-Turrax homogenization alone, but not by freezing alone or by sonication. DHAPAT activity in mitochondrial or microsomal fractions was not decreased by freezing and homogenization (Table 1).

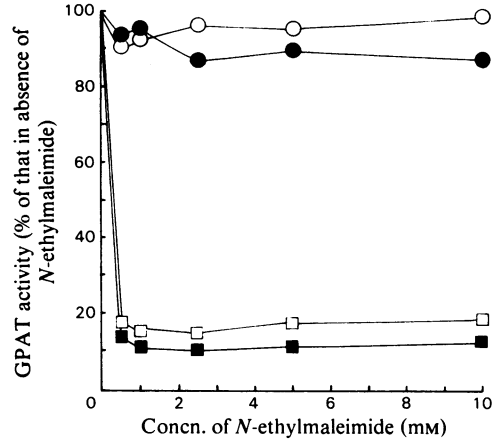

Fig. 2. *Effect of N-ethylmaleimide on mitochondrial and microsomal GPAT activities*

Mitochondria (fraction M) (○, ●) and microsomal fractions (fraction E) (□, ■) were either used directly (closed symbols) or were firstly subjected to freezing, thawing and Ultra-Turrax homogenization (open symbols). Absolute activities of GPAT in the absence of *N*-ethylmaleimide, expressed as nmol/min per mg of protein were: fresh mitochondria, 2.77; frozen homogenized mitochondria, 1.79; fresh microsomal fraction, 1.9; frozen, homogenized microsomal fraction, 0.73.

Schlossman & Bell (1977) have observed similar effects of *N*-ethylmaleimide on rat liver microsomal GPAT and DHAPAT, but the effect of thiol reagents on DHAPAT in mitochondrial fractions does not appear to have been investigated. Table 2 shows that DHAPAT activity in fractions M and P was stimulated by 10mM-*N*-ethylmaleimide. This effect was maximal within 1min and was constant for at least 10min (results not shown). On the other hand, the activity in a microsomal fraction obtained from the post-23000$g_{av.}$ supernatant was slightly inhibited by *N*-ethylmaleimide (Table 2). It is clear that the effects of *N*-ethylmaleimide on DHAPAT activity are not as clear-cut as on the two types of GPAT and that

Table 1. *Effect of Ultra-Turrax homogenization on GPAT and DHAPAT activities*

Preparation of liver M and E fractions, enzyme assays and Ultra-Turrax homogenization were as described in the Materials and Methods section. The activities are expressed as nmol of glycerol phosphate or dihydroxyacetone phosphate incorporated into products/min per mg of protein. The numbers of separate experiments are shown in parentheses.

	GPAT activity			DHAPAT activity	
	In mitochondria		In microsomal fraction		
Treatment	0.5mM-Glycerol phosphate	5mM-Glycerol phosphate	0.5mM-Glycerol phosphate	In mitochondria	In microsomal fraction
Fresh preparation	3.00±0.17 (4)	5.15 (2)	3.15±0.45 (4)	0.49±0.07 (3)	0.27±0.02 (3)
Frozen and homogenized	1.79±0.13 (4)	5.21 (2)	1.70±0.36 (4)	0.59±0.11 (3)	0.34±0.01 (3)

Table 2. *Subcellular distribution of GPAT and DHAPAT activities*

Enzyme activities are expressed as nmol/min per mg of protein and are shown as means ± S.E.M., except where only two measurements were made, in which case the range of values is given. The numbers of independent measurements are shown in brackets. The numbers in parentheses indicate the percentage changes caused by *N*-ethylmaleimide. Microsomal fractions were isolated by centrifugation of the post-23000$g_{av.}$ supernatant for 1 h at 105000$g_{av.}$ Latent glutamate dehydrogenase represents the difference between the activity in fresh extracts before and after the addition of Triton X-100 (1 mg/ml).

	Fraction M	Fraction P	Microsomal fraction
DHAPAT [3]	0.49 ± 0.02	0.92 ± 0.19	0.20 ± 0.01
+ *N*-Ethylmaleimide (10mM)	0.77 ± 0.07 (+57 ± 1%)	1.55 ± 0.25 (+72 ± 11%)	0.15 ± 0.00 (−24 ± 2%)
GPAT [3]	3.85 ± 0.29	2.41 ± 0.56	4.31 ± 0.24
+ *N*-Ethylmaleimide (10mM)	3.42 ± 0.31 (−11 ± 2%)	1.47 ± 0.20 (−36 ± 6%)	0.45 ± 0.02 (−90 ± 0%)
Monoamine oxidase [3]	7.37 ± 1.42	3.87 ± 1.07	1.87 ± 0.33
Latent glutamate dehydrogenase [3]	3501 ± 233	509 ± 60	42 ± 4
Uricase [2]	23 ± 3	87 ± 12	5 ± 1
NADP–cytochrome *c* reductase [2]	25 ± 6	100 ± 9	177 ± 9
Total protein content of fraction (mg) [3]	58.0 ± 7.3	16.8 ± 2.0	125.6 ± 10.7

differential sensitivity to the reagent could not be used conveniently as a tool to quantify different forms of the enzyme in unfractionated tissues. Table 2 shows that the distribution, in fractions produced by centrifugation, of DHAPAT (with or without *N*-ethymaleimide) was very different from that of GPAT. The highest specific activity of DHAPAT was found in fraction P, which also contained the highest specific activity of the peroxisomal marker uricase. The distribution of DHAPAT differed considerably from those of glutamate dehydrogenase (latent activity), monoamine oxidase or the microsomal marker $NADP^+$–cytochrome *c* reductase. Jones & Hajra (1977) have suggested that DHAPAT is a peroxisomal enzyme in guinea-pig liver. The present findings with the rat appear to be similar. Table 2 does, however, show a good correlation between the distributions of *N*-ethylmaleimide-insensitive GPAT, glutamate dehydrogenase and monoamine oxidase and also between the distributions of *N*-ethylmaleimide-sensitive GPAT and $NADP^+$–cytochrome *c* reductase. Schlossman & Bell (1977) found considerable (approx. 60%) inhibition of DHAPAT in rat liver microsomal fraction with low concentrations of *N*-ethylmaleimide (up to 0.5mM). The present findings might appear to be at variance with this. However, in our hands most of the DHAPAT activity appears to be non-microsomal and to be *N*-ethylmaleimide-stimulated. Contamination of microsomal fractions with this activity may mask an *N*-ethylmaleimide-inhibited 'true' microsomal activity. Fig. 3 shows that maximal stimulation of DHAPAT in fraction M needed relatively high concentrations of N-ethylmaleimide (2.5mM). It is likely that in the experiments of Schlossman & Bell (1977) *N*-ethylmaleimide activation of a contaminating non-microsomal activity would not have been observed. However, further studies are needed to explain these apparent inconsistencies.

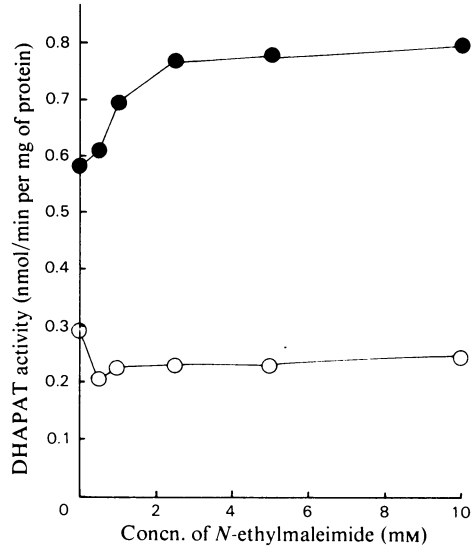

Fig. 3. *Effects of N-ethylmaleimide on DHAPAT activities in mitochondrial and microsomal fractions*

Fresh mitochondria (fraction M) (●) and microsomal fractions (fraction E) (○) were assayed for DHAPAT activity in the presence of the indicated concentrations of *N*-ethylmaleimide.

Effect of palmitoyl-CoA concentration on GPAT and DHAPAT activities in mitochondrial and microsomal fractions

In preliminary experiments in which albumin concentration was varied in the presence of a fixed concentration of palmitoyl-CoA (65 μM), it was observed that GPAT activity in mitochondrial (M) fractions was barely affected, whereas variation of the concentration of this acyl-binding protein considerably affected the rate of glycerol phosphate acylation by microsomal (E) preparations. This suggested that the dependence of GPAT activity on palmitoyl-CoA concentration in the presence of a fixed albumin concentration would differ between the two fractions. This was found to be so, as shown in Fig. 4(*a*). Since high concentrations of palmitoyl-CoA resulted in inhibition of GPAT, it was not really feasible to determine K_m values for this substrate. However, the decrease in the ratio of fraction-M/fraction-E GPAT specific activities seen with increasing palmitoyl-CoA concentration suggested that the mitochondrial form of the enzyme has a higher 'affinity' for palmitoyl-CoA ($P<0.01$ for this ratio at 65, 87, 108 and 130 μM compared with that at 22 μM). These findings may have physiological implications in that the relative rates of glycerol phosphate acylation by the mitochondrial and microsomal enzymes will be affected by changes in the intracellular fatty acyl-CoA content and by the binding of these metabolites to intracellular binding sites. Fig. 4(*b*) shows that DHAPAT activity in fraction M was saturated by palmitoyl-CoA at concentrations as low as 22 μM, whereas this was not the case for the activity observed in fraction E. Again the ratio of fraction-M/fraction-E specific activity decreased with increasing palmitoyl-CoA concentration ($P<0.01$ for 65, 87, 108 and 130 μM compared with 22 μM). It is suggested that the *N*-ethylmaleimide-stimulated DHAPAT activity (Table 2), which appears to be sedimented at lower centrifugal fields, has a high affinity for the palmitoyl-CoA substrate, whereas the *N*-ethylmaleimide-inhibited activity (Table 2), which may represent microsomal activity, and which is probably contaminated by the *N*-ethylmaleimide-stimulated enzyme (Table 2), has a much lower affinity for this acyl-CoA substrate.

All these experiments were performed with freshly prepared unfrozen fractions.

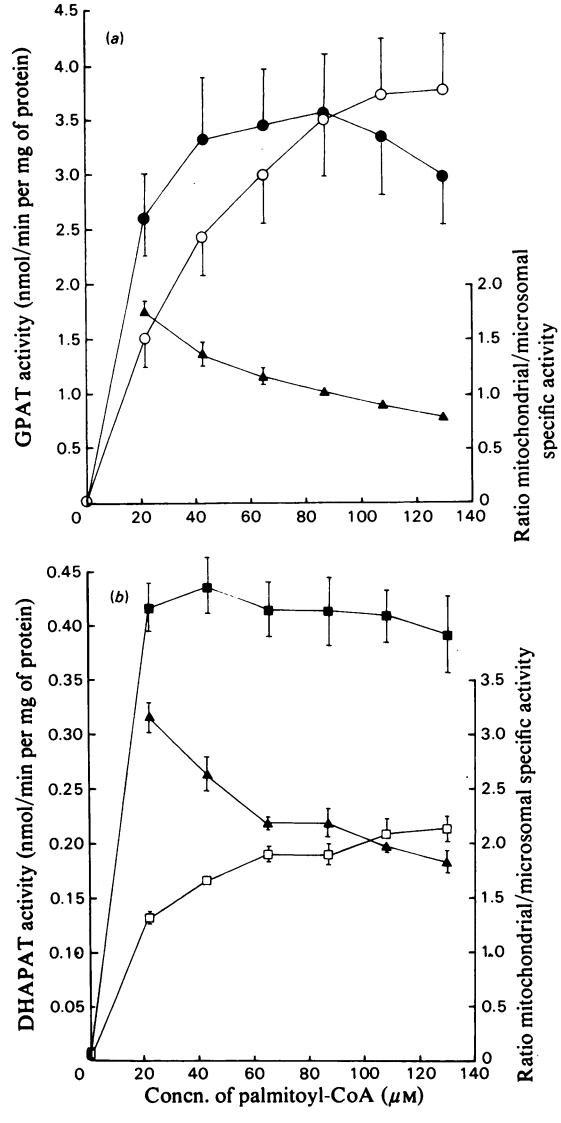

Fig. 4. *Dependence of GPAT and DHAPAT activities on palmitoyl-CoA concentration*

Fresh M and E fractions were assayed for both enzyme activities in the presence of 4 mg of albumin/ml and the indicated concentrations of palmitoyl-CoA. The values are means ± S.E.M. of four separate experiments. (*a*) GPAT activity in mitochondrial (●) and microsomal (○) fractions; ratio of fraction-M/fraction-E specific activities (▲). (*b*) DHAPAT activity in mitochondrial (■) and microsomal (□) fractions; ratio of fraction-M/fraction-E specific activities (▲).

Effect of dietary and hormonal status on GPAT and DHAPAT activities

N-Ethylmaleimide-insensitive (mitochondrial) GPAT activity is increased by 34% in perfused rat liver by treatment with insulin for 30 min (Bates *et al.*, 1977). No change in *N*-ethylmaleimide-sensitive (microsomal) activity was seen. These results suggested the possibility of a selective acute control of the mitochondrial enzyme by insulin. GPAT activity is also decreased in mitochondria, but not microsomal

fraction, isolated by conventional fractionation procedures from livers of streptozotocin-diabetic rats (Bates & Saggerson, 1977). This would imply that some or all of the decrease in the mitochondrial activity in response to this long-term change in insulin status is relatively stable. It is quite possible, though, that in the time taken for isolation of subcellular fractions some alteration in enzyme activity owing to acute control might be lost. In the present study therefore mitochondrial and microsomal GPAT activities have been differentiated on the basis of *N*-ethylmaleimide-sensitivity in extracts from freeze-clamped liver. Assays were performed immediately after homogenization, thus possibly permitting the detection of acute as well as long-term changes.

N-Ethylmaleimide-insensitive (mitochondrial) GPAT activity was significantly decreased by starvation (48h) to 60% of the value in fed animals (Table 3). The total content of liver protein was also decreased. On the other hand, the *N*-ethylmaleimide-sensitive (microsomal) activity was unchanged. As a result of this, the ratio of mitochondrial to microsomal GPAT activity was also significantly decreased. The assay conditions used (65μM-palmitoyl-CoA and albumin at 6mg/ml) were optimal for mitochondrial but not for microsomal GPAT (Fig. 4*a*). For this reason, the ratio of mitochondrial to microsomal activities is higher than expected from previous findings (Stoffel & Schiefer, 1968; Shephard & Hübscher, 1969; Daae, 1973; Van Tol, 1974; Bjerve *et al.*, 1976).

In four of the nine experiments comparing livers of fed and starved animals, M and E fractions were also isolated. GPAT activity in fresh M fractions was lowered by 42% after starvation, compared with a decrease of 35% in *N*-ethymaleimide-insensitive GPAT measured in freeze-stopped liver. Starvation had no effect on the GPAT activity measured in fresh E fractions. These findings are taken as further validation of the use of *N*-ethylmaleimide to differentiate between mitochondrial and microsomal GPAT. It is also noteworthy that *N*-ethylmaleimide-insensitive GPAT activity per total liver measured in these freeze-stopped preparations was approx. 60% of the activity per total liver measured in isolated mitochondria. This correlates with the 40% decrease in GPAT (measured with 0.5mM-glycerol phosphate) that is observed on freezing and homogenizing mitochondria (Table 1).

Aas & Daae (1971) have observed a decrease in liver whole-homogenate GPAT activity after 48h of starvation. Microsomal GPAT specific activity per mg of protein has been reported as being unchanged (Fallon & Kemp, 1968; Wiegand *et al.*, 1973) or slightly decreased (Van Tol, 1974) after 48–72h of starvation. On the other hand, short periods of starvation (10–16h) have been reported to decrease liver microsomal GPAT activity considerably

Table 3. *Effect of starvation and adrenalectomy on GPAT and DHAPAT activities*

Liver samples were freeze-clamped and extracted as described in the Materials and Methods section. *N*-Ethylmaleimide-insensitive GPAT activity was measured in the presence of 10mM-*N*-ethylmaleimide (NEM). The values are means ±S.E.M.; the numbers of separate experiments are indicated in parentheses. *$P<0.05$, **$P<0.02$, ***$P<0.01$, ****$P<0.001$ respectively for the effects of starvation compared with appropriate fed controls; †$P<0.05$, ††$P<0.02$, †††$P<0.001$ respectively for effects of adrenalectomy compared with appropriate sham-operated controls.

Expt. no.	Status of animals	Liver weight (g)	Total liver protein (g)	Total GPAT activity		NEM-insensitive GPAT activity		NEM-sensitive GPAT activity		Ratio	DHAPAT activity	
				(nmol/min per mg of protein)	(nmol/min per liver)	(nmol/min per mg of protein)	(nmol/min per liver)	(nmol/min per mg of protein)	(nmol/min per liver)	MNE-insensitive / NEM-insensitive GPAT activities	(nmol/min per mg of protein)	(nmol/min per liver)
1	Fed (9)	7.3±0.2	1.15±0.05	0.85±0.10	982±139	0.65±0.08	755±107	0.20±0.02	227±33	3.39±0.19	—	—
	Starved (48h) (9)	4.6±0.2	0.90±0.02****	0.74±0.06	673±66	0.50±0.04	455±44*	0.24±0.03	218±27	2.26±0.23***	—	—
2	Fed (7)	7.4±0.2	1.26±0.03	—	—	—	—	—	—	—	0.14±0.003	180±8
	Starved (48h) (7)	4.5±0.1	1.00±0.03****	—	—	—	—	—	—	—	0.14±0.01	136±9***
3	Sham-adrenalectomized, fed (8)	—	—	0.93±0.04	—	0.74±0.03	—	0.18±0.02	—	4.32±0.54	—	—
	Adrenalectomized, fed (8)	—	—	0.80±0.03†	—	0.62±0.02††	—	0.18±0.02	—	3.63±0.33	—	—
	Sham-adrenalectomized, starved (48h) (6)	—	—	0.93±0.03	—	0.68±0.02	—	0.25±0.01**	—	2.69±0.1*	—	—
	Adrenalectomized, starved (48h) (6)	—	—	0.60±0.03****†††	—	0.47±0.03****†††	—	0.13±0.01*†††	—	3.81±0.34††	—	—

(Mangiapane *et al.*, 1973). With one exception (Van Tol, 1974) we are unaware of any previous studies of the effect of starvation of the mitochondrial activity of GPAT. As found here (Table 3), Van Tol (1974) observed a non-significant decrease in the mitochondrial specific activity per mg of protein. From the present study we conclude that relatively long periods of starvation lead to a divergence in the responses of mitochondrial and microsomal GPAT to this stress.

Table 3 shows that 48h of starvation significantly decreased the total liver activity of DHAPAT to 76% of the control value, whereas there was no change in the specific activity per mg of protein. Rao *et al.* (1971) have previously reported a 48% decrease in rat liver microsomal DHAPAT specific activity per mg of protein after 3 days of starvation.

The synthesis and export of hepatic triacylglycerol is decreased after adrenalectomy (Klausner & Heimberg, 1967; Kirk *et al.*, 1976) and increased by glucocorticoid therapy (Klausner & Heimberg, 1967; Reaven *et al.*, 1974). We are unaware of any previous studies of changes in hepatic triacylglycerol-synthesizing enzymes after adrenalectomy. In fed animals, adrenalectomy decreased the specific activity per mg of protein of *N*-ethylmaleimide-insensitive (mitochondrial) GPAT (Table 3). No change was seen in the *N*-ethylmaleimide-sensitive (microsomal) activity. Starved adrenalectomized rats showed a greater percentage decrease in the *N*-ethylmaleimide-insensitive (mitochondrial) GPAT activity compared with their starved, sham-operated controls. Also, after starvation *N*-ethylmaleimide-sensitive (microsomal) activity was considerably decreased by adrenalectomy, resulting in a significant increase in the mitochondrial/microsomal activity ratio. Concerning the effects of starvation, in sham-operated animals, a significant decrease in the mitochondrial/microsomal activity ratio was seen, as with the starvation of normal animals. However, this change in the relative proportions of the two forms of the enzyme was not seen after removal of the adrenals. These findings suggest that, in addition to starvation (Table 3) and streptozotocin-diabetes (Bates & Saggerson, 1977), adrenalectomy decreases mitochondrial GPAT activity. It is possible that these decreases are related to the decrease in insulin concentrations associated with all of these conditions (Van Lan *et al.*, 1974). In addition, adrenal secretions appear to play a role in the maintenance of the microsomal enzyme in longer-term periods of starvation.

In the experiments summarized in Table 4 and Fig. 5, rats were injected with anti-insulin serum under sodium pentobarbitone anaesthesia. GPAT and DHAPAT activities were then measured after 1h in samples of freeze-clamped liver. Treatment with the anti-insulin serum was effective in raising plasma glucose concentration (Fig. 5). There was no significant change in the liver content of glycogen or protein (Table 4). Treatment with anti-insulin serum slightly decreased the total GPAT specific activity. This decrease was confined to the *N*-ethylmaleimide-insensitive (mitochondrial) activity, which was significantly decreased by 14%. This would appear to be another manifestation of an acute control of this enzyme by insulin that is confined to the mitochondrial activity (Bates *et al.*, 1977). Treatment of the animals with anti-insulin serum had no effect on the specific activity of DHAPAT in the whole homogenate. The mechanism(s) underlying the relatively rapid changes in mitochondrial GPAT activity after insulin administration to perfused rat liver (Bates *et al.*, 1977) or after anti-insulin serum treatment *in vivo* (Table 4) are not understood at present. They are persistent enough to survive freeze-stopping and extraction of the tissue, suggesting enzyme covalent modification or changes in enzyme content rather than the reversible binding of effector molecules.

Measurements of GPAT and DHAPAT in parenchymal and non-parenchymal cells

Radioisotopic studies with incubated rat liver

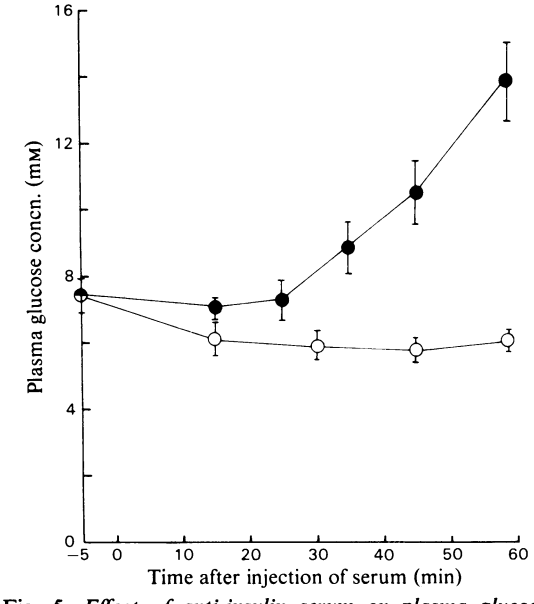

Fig. 5. *Effect of anti-insulin serum on plasma glucose concentration in vivo*

These measurements were made in the experiment summarized in Table 4. Blood was taken from the tail at the indicated times and glucose was measured enzymically in plasma. Anti-insulin serum (●) or control serum (○) was injected at zero time. The bars indicate S.E.M.

slices have suggested that the dihydroxyacetone phosphate pathway may be responsible for about half of the total glycerolipid synthesis (Manning & Brindley, 1972). On the other hand, studies with isolated rat liver parenchymal cells have only assigned a minor role to this pathway (Rognstad *et al.*, 1974). As much as 35% of the liver cell number is non-parenchymal, representing approx. 10% of the tissue mass (Weibel *et al.*, 1969). It was thought that the apparent discrepancy between the findings of Manning & Brindley (1972) and Rognstad *et al.*, (1974) might be resolved if the non-parenchymal cells from the liver contained a relative enrichment of DHAPAT over GPAT activity compared with whole liver. Table 5 shows that the specific activity of DHAPAT in parenchymal cells was 7 times that in non-parenchymal cells, whereas the specific activity of GPAT in parenchymal cells was 41 times that in the non-parenchymal cells. This is reflected in the considerable difference in the GPAT/DHAPAT activity ratios between the two cell preparations. However, because of the considerably higher specific activities of both enzymes in parenchymal cells it is unclear whether this might account for the discrepancies between studies with whole liver slices and parenchymal cells discussed above.

In extracts from non-parenchymal cells it was observed that 10mM-*N*-ethylmaleimide increased GPAT activity. This is at variance with the effect of this agent seen in extracts from parenchymal cells or whole liver. The reason for this is unknown at present.

Parenchymal cells contain the L-type isoenzyme of pyruvate kinase, whereas non-parenchymal cells contain the M_2-type enzyme (van Berkel *et al.*, 1972; van Berkel, 1974). The absence of stimulation of non-parenchymal cell pyruvate kinase by fructose 1,6-bisphosphate (Table 5) indicated that this preparation was essentially free of contamination by parenchymal cells. The pyruvate kinase specific activities per mg of protein shown in Table 5 for both types of cell preparation were very similar to those reported by van Berkel & Kruijt (1977). In the parenchymal cells the specific activity of glutamate dehydrogenase was also very similar to that reported by van Berkel & Kruijt (1977), but that in non-parenchymal cells was 16 times greater. At present we have no explanation for this considerable discrepancy.

General conclusions

The present findings appear to differentiate between at least two forms of both GPAT and DHAPAT. For both enzymes, microsomal fractions contain an activity that is inhibited to some extent by *N*-ethylmaleimide and has a lower affinity for the palmitoyl-CoA substrate under the chosen assay conditions. The other forms of these activities are found in fractions isolated at lower centrifugal fields. These are not inhibited by *N*-ethylmaleimide and have higher affinity for palmitoyl-CoA. At present it is unknown whether these differences reflect differences in membrane environment or the presence of more than one enzyme protein for each activity. The latter might be inferred for GPAT, since the regulation of the mitochondrial and the microsomal activities appears to be different in several circumstances.

In every case examined so far, i.e. in diabetes (Bates & Saggerson, 1977), after acute insulin administration *in vitro* (Bates *et al.*, 1977), in starvation (Table 3), after adrenalectomy (Table 3) and after anti-insulin serum treatment (Table 4), the mitochondrial GPAT activity is significantly changed. In every case this change is in the same direction as changes in triacylglycerol synthesis and export observed in perfused liver after these treatments (Heimberg *et al*, 1966; Klausner & Heimberg, 1967; Mayes, 1970; Topping & Mayes, 1972; Woodside & Heimberg, 1976). These previous studies have shown changes in the partitioning of a constant input of fatty acids between oxidation and esterification processes in the isolated liver. *In vivo*, of course,

Table 4. *Effect of anti-insulin serum on GPAT and DHAPAT activities in vivo*

As described in the Materials and Methods section, rats were treated with 0.5ml of control or anti-insulin serum (sufficient to bind 1.65 units of insulin) under sodium pentobarbitone anaesthesia. After 1h liver samples were freeze-clamped and extracted. *N*-Ethylmaleimide-insensitive GPAT activity was measured in the presence of 10mM-*N*-ethylmaleimide (NEM). The values are means ± S.E.M. The numbers of separate experiments are indicated in parentheses. Plasma glucose data from this experiment are shown in Fig. 5. *$P<0.05$ versus animals treated with control serum.

Treatment	Liver weight (g)	Liver protein content (mg/g wet weight)	Liver glycogen content (μmol of glucose/g wet weight)	GPAT activity (nmol/min per mg of protein)			DHAPAT activity (nmol/min per mg of protein)
				Total	NEM-insensitive	NEM-sensitive	
Control serum (6)	6.9 ± 0.3	158 ± 4	35.7 ± 4.3	0.95 ± 0.04	0.74 ± 0.03	0.21 ± 0.04	0.12 ± 0.01
Anti-insulin serum (5)	6.8 ± 0.2	160 ± 5	33.8 ± 4.5	0.90 ± 0.03	0.64 ± 0.02*	0.26 ± 0.03	0.12 ± 0.01

Table 5. *Enzyme activities in parenchymal and non-parenchymal cells*

Cells were isolated and extracted as described in the Materials and Methods section. Enzyme activities are expressed as nmol/min per mg of protein. *N*-Ethylmaleimide-insensitive GPAT activity was measured in the presence of 10mM-*N*-ethylmaleimide (NEM). The values for parenchymal cells are means ± S.E.M. from three separate preparations. The values for non-parenchymal cells are from two separate preparations and range of values on either side of the mean is indicated.

Cell type	DHAPAT activity	GPAT activity			GPAT/DHAPAT activity ratio	Pyruvate kinase activity			Glutamate dehydrogenase activity
		Total	NEM-insensitive	NEM-sensitive		Without fructose 1,6-bisphosphate	With fructose 1,6-bisphosphate (0.5mM)	Ratio with/without fructose 1,6-bisphosphate	
Parenchymal	0.184 ± 0.018	1.611 ± 0.196	1.176 ± 0.151	0.435 ± 0.059	8.72 ± 0.22	17.62 ± 1.28	104.2 ± 7.1	5.9 ± 0.3	1038 ± 95
Non-parenchymal	0.026 ± 0.002	0.039 ± 0.003	0.053 ± 0.003	—	1.54 ± 0.20	69.9 ± 2.4	72.2 ± 1.7	1.03 ± 0.01	915 ± 39

decreases in the absolute rate of esterification may not be observed in starvation or diabetes, since an increased supply of non-esterified fatty acids to the liver may compensate for the tendency to oxidize a larger proportion of incoming fatty acids. However, the physiological responses of the mitochondrial GPAT are noteworthy and merit further study, since they may participate in the metabolic switching of fatty acids between oxidation and esterification.

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