Characteristics of Mitochondrial and Microsomal Monoacyl- and Diacylglycerol 3-Phosphate Biosynthesis in Rabbit Heart

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Acylation of *sn*-glycerol 3-phosphate by heart subcellular fractions was characterized. The enzyme kinetics revealed that the rate of reaction of acylation by mitochondria was slower, but constant for a longer period (up to 20 min), than that by the microsomal fraction. The range of palmitate, oleate and linoleate concentrations yielding optimal *sn*-glycerol 3-phosphate acylation was broader for mitochondria than for the microsomal fraction, the latter showing a preference for linoleate. The mitochondrial fraction synthesized a relatively large quantity of monoacyl-*sn*-glycerol 3-phosphate, reaching 135% of the microsomal biosynthesis during an assay period of 15 min. By contrast, the microsomal fraction formed considerably more diacyl- than monoacyl-*sn*-glycerol 3-phosphate, except with linoleate as the acyl donor, in which case approximately equal quantities of the two products were produced. The biosynthesis of monoacyl-*sn*-glycerol 3-phosphate was also observed in experiments in which hepatic subcellular fractions were used to provide supporting evidence. Cardiac mitochondrial diacyl-*sn*-glycerol 3-phosphate formation was less than 17% of the microsomal formation. However, evidence is presented to exclude the possibility that monoacyl-*sn*-glycerol 3-phosphate in the mitochondrial fraction is formed by deacylation of the contaminating microsomal diacyl-*sn*-glycerol 3-phosphate. The participation of the dihydroxyacetone phosphate pathway in the biosynthesis of these substances was minimal. The addition of CTP and the fatty acid specificity of the reaction both provided results that reinforced the postulate that mitochondrial differs from microsomal acylation. Thus our findings demonstrate that the characteristics of acyl-CoA-*sn*-glycerol 3-phosphate *O*-acyltransferase (EC 2.3.1.15) in rabbit heart mitochondria are distinct from those of cardiac microsomal enzyme and hepatic enzymes.

Kornberg & Pricer (1953) demonstrated that the acylation of *sn*-glycerol 3-phosphate (glycerol 3-phosphate) is catalysed by guinea-pig liver microsomal fractions and produces diacyl-*sn*-glycerol 3-phosphate (diacylglycerol 3-phosphate). Since then, a number of workers have confirmed that diacylglycerol 3-phosphate plays a key role as intermediate in the biosynthesis of glycerolipids (Stein *et al.*, 1957; Buell & Reiser, 1959; Kennedy, 1961). Although there are numerous studies on this reaction in the liver, there is very little information available on it in the heart. Further, the results reported vary widely with respect to subcellular localization, reaction products, activities and fatty acid specificity of glycerol 3-phosphate acyltransferase (acyl-CoA-*sn*-glycerol 3-phosphate *O*-acyltransferase, EC 2.3.1.15). Thus Daae & Bremer (1970) provided evidence supporting the relatively high activity of glycerol 3-phosphate esterification by mitochondrial as compared with microsomal fractions. This conclusion agrees with that of earlier papers, which reported

considerably lower activities (Stoffel & Schiefer, 1968; McMurray & Dawson, 1969; Shephard & Hübscher, 1969; Zborowski & Wojtczak, 1969; Sarzala *et al.*, 1970). By contrast, Eibl *et al.* (1969) and Davidson & Stanacev (1972) attributed the mitochondrial activity entirely to contamination by microsomal enzymes.

There has also been disagreement regarding the product of acylation. In contrast with studies with *Escherichia coli* (Pieringer *et al.*, 1967; Ray *et al.*, 1970), diacylglycerol 3-phosphate has been identified in mammalian systems as the sole product of acylation. However, Fallon & Lamb (1968) and Daae & Bremer (1970) were able to detect monoacyl-*sn*-glycerol 3-phosphate (monoacylglycerol 3-phosphate) as one of the products formed by liver microsomal and mitochondrial fractions, respectively. The latter finding has been confirmed more recently (Monroy *et al.*, 1972; Sánchez *et al.*, 1973). The fatty acid specificity of the glycerol 3-phosphate acylation reaction has also been a controversial subject; some studies indicate a random acylation process (Lands & Hart, 1965; Abou-Issa & Cleland, 1969; Eibl *et al.*, 1969;

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Yamashita & Numa, 1972) whereas others point to a high degree of positional and fatty acid specificity in diacylglycerol 3-phosphate biosynthesis (Possmayer *et al.*, 1969; Zborowski & Wojtczak, 1969; Lamb & Fallon, 1970; Daee, 1972).

All these studies have used subcellular fractions prepared from rat or guinea-pig liver and have not been extended to subcellular particles from cardiac tissue. It is well known that some 60% of the total energy consumed by the heart is derived from the oxidation of exogenous fatty acids (Bing, 1965), and there are numerous conditions resulting in an increase in the lipid content of the myocardium (Kako & Dubuc, 1968; Opie, 1969*a,b*; Kikuchi & Kako, 1970). Therefore, we investigated the acylation of glycerol 3-phosphate by subcellular fractions of rabbit hearts. Our results show that both mitochondrial and microsomal acylation reactions of the heart differ from those of the liver. A preliminary account of this work has appeared (Kako & Liu, 1973).

Experimental

Materials

ATP (disodium salt), CoA, palmitoyl-CoA, palmitic acid, oleic acid and linoleic acid, 1-palmitoyl-*sn*-glycerol, 1,3-dipalmitoylglycerol, 1,3-dioleoylglycerol, 1,2-dioleoyl-*sn*-glycerol and tripalmitoylglycerol were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); potassium salts of fatty acids were prepared by titrating fatty acids with 1M-KOH; L-carnitine chloride and sodium phosphatide were obtained from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.) and Pierce Chemical Co. (Rockford, Ill., U.S.A.), respectively. CTP, dihydroxyacetone phosphate, NADH and NADPH were products of C. F. Boehringer Corp (New York, N.Y., U.S.A.); cyclohexylammonium salt of dimethylketal dihydroxyacetone phosphate was converted into the sodium salt by washing it with Dowex 50 (H⁺ form) (Ballou & Tomita, 1960). The monoacylglycerol 3-phosphate was a gift from Dr. M. Kates (University of Ottawa, Ont., Canada), acyldihydroxyacetone phosphate was from Dr. A. K. Hajra (Ann Arbor, Mich., U.S.A.) and L-palmitoylcarnitine was kindly given by Otsuka Pharmaceutical Co., Osaka, Japan. Bovine serum albumin (fraction V) was treated with charcoal to remove fatty acids (Chen, 1967). In most of the experiments, *sn*-[U-¹⁴C]glycerol 3-phosphate (New England Nuclear Corp., Boston, Mass., U.S.A.) was diluted with *rac*-glycerol 3-phosphate (Sigma Chemical Co.) to a specific radioactivity of approx. 350000 d.p.m./ μ mol of glycerol 3-phosphate. The radiochemical purity of the ¹⁴C-labelled glycerol 3-phosphate was verified by paper chromatography with phenol-water (5:2, w/v) (Benns & Proulx, 1971) and was found to be free of cyclic gly-

cerol 3-phosphate and other labelled impurities. [1-¹⁴C]Palmitic acid was purchased from New England Nuclear Corp. and was converted into its potassium salt; ¹⁴C-labelled monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate were synthesized enzymically by incubating rabbit liver microsomal fractions with [¹⁴C]glycerol 3-phosphate and isolating the products by t.l.c. [¹⁴C]Phosphatidylethanolamine and [¹⁴C]phosphatidylcholine were generously supplied by Dr. P. R. Proulx (University of Ottawa). Carnitine palmitoyltransferase (EC 2.3.1.23) was prepared by the method of Kopec & Fritz (1971). T.l.c. plates coated with silica gel G (E. Merck, Darmstadt, Germany), either prepared by our laboratory or purchased from Brinkmann Co. (Toronto, Ont., Canada), were activated at 150°C for 1 h and then stored in a desiccator. All other reagents were of analytical grade.

Preparation of subcellular fractions

Albino male rabbits (2–3 kg) were anaesthetized by an intraperitoneal injection of 2.5 ml of 2% (w/v) α -chloralose (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) in 20% (w/v) urethane/kg body wt. The hearts were excised, trimmed to remove fat, then weighed and chopped into small pieces. The tissue was then homogenized with 4 vol. of 0.25M-sucrose containing 0.02M-Tris-HCl, pH 7.4, in a Potter-Elvehjem-type homogenizer with a Teflon pestle rotated at 1000 rev./min. The homogenization was standardized by performing two strokes up and down with a loose-fitting pestle and then one stroke with a tight-fitting pestle (Kako, 1969). The homogenate was centrifuged at 800g (*r*_{av}, 7.5 cm) for 15 min to remove nuclei and cell debris, and the supernatant carefully decanted and centrifuged at 10000g (*r*_{av}, 7.5 cm) for 15 min. The resulting supernatant was removed and further centrifuged at 100000g (*r*_{av}, 5.74 cm) for 60 min. The pellet obtained was used as the microsomal fraction after being suspended in the homogenizing medium to give 2–5 mg of protein/ml. The 10000g pellet was resuspended and centrifuged at 8000g for 15 min, yielding the mitochondrial pellet, which was suspended in the homogenizing medium giving 8–10 mg of protein/ml. Although the yield of mitochondria can be improved by the use of a proteinase (Nagarse) this step was omitted since acyl-CoA synthetase [acid-CoA ligase (AMP), EC 6.2.1.3] is easily destroyed by this procedure (De Jong & Hülsmann, 1970; Pande & Blanchaer, 1970). Electron-microscopic examination revealed that the mitochondrial preparations were little contaminated by lysosomes. Hepatic subcellular particles were isolated and compared with those from the heart organelles. The liver microsomal fraction which was isolated also served as the source of the enzyme used to synthesize labelled diacylglycerol 3-phosphate, as stated above.

Assay of glycerol 3-phosphate acyltransferase

Unless otherwise stated, the reaction mixture contained, in a final vol. of 2.0 ml: 100 μ mol of Tris-phosphate buffer, pH 7.4; 2.0 μ mol of potassium palmitate, oleate or linoleate; 0.8 μ mol of CoA; 12.0 μ mol of ATP; 6.0 μ mol of $MgCl_2$; 1% (w/v) of fatty acid-poor bovine serum albumin; 6.0 μ mol of [^{14}C]glycerol 3-phosphate; fresh enzyme preparation (approx. 2.5 mg of mitochondrial protein or 0.8 mg of microsomal protein). With liver fractions, the amounts of mitochondrial and microsomal proteins were decreased to approx. 1.5 and 0.5 mg respectively, because of their higher enzyme activity. In some experiments, 0.8 μ mol of palmitoyl-CoA was used instead of CoA, ATP, palmitate and $MgCl_2$. When L-palmitoylcarnitine served as the acyl donor (Daae & Bremer, 1970), CoA, ATP, palmitate and $MgCl_2$ were replaced with 0.8 μ mol of L-palmitoylcarnitine, 1.6 μ mol of CoA and purified carnitine palmitoyl-transferase (10.3 μ g of protein with a specific activity of 3.7 μ mol/min per mg of protein at 25°C). These concentrations of acyl donors were chosen from the substrate-product relationships obtained in a preliminary experiment. Additions of some other factors were carried out as specified in the legends to the figures and tables.

The reaction commenced with the addition of the enzyme preparations and the incubation was carried out in a metabolic shaker at 37°C in air. The duration of incubation is stated for individual experiments. The reaction was stopped by the addition of 3 ml of water-saturated butan-1-ol, followed by the addition of 4 ml of butan-1-ol-saturated water (Daae & Bremer, 1970). After separation of the two phases, the upper phase was washed again with butan-1-ol-saturated water and the lower phase with water-saturated butan-1-ol. This modified procedure gave very low control values for zero-time incubations, which improved the accuracy and reproducibility of the assay. A portion of the combined butan-1-ol extracts was suspended in 10 ml of Bray's (1960) solution for radioactivity determination. The remainder was used for product analysis.

Chloroform-methanol extraction procedures (Folch *et al.*, 1957; Bligh & Dyer, 1959; Hajra *et al.*, 1968; Eibl *et al.*, 1969; Huang & Kako, 1970) constantly produced a high control value in the presence of subcellular fractions before incubation. Radioactivity was high and variable, even when the tissue fraction was omitted from the incubation mixture, which was probably due to non-specific binding of albumin (Barden & Cleland, 1969). Constant and low control values are essential to a successful assay since the radioactivity found in the products is 0.1–0.01% of the precursor radioactivity (0.7×10^6 – 17×10^6 d.p.m./tube) in most studies with liver particulate fractions (e.g., Abou-Issa & Cleland, 1969;

Davidson & Stanacev, 1972; Monroy *et al.*, 1972), which possess higher activities than do the heart particulate fractions. With the modified version of the butan-1-ol-extraction procedure, more than 90% of the radioactivity was recovered when ^{14}C -labelled diacylglycerol 3-phosphate, monoacylglycerol 3-phosphate, phosphatidylcholine or phosphatidylethanolamine were tested individually.

When [$1-^{14}C$]palmitate was used as a tracer, the specific radioactivity was adjusted to approx. 500 000 d.p.m./ μ mol, and the assay was carried out as described, except that the unused labelled palmitate was first removed by t.l.c. with a solvent system of light petroleum (b.p. 30–60°C)–diethyl ether–acetic acid (40:10:1, by vol.) (Possmayer & Mudd, 1971; Huang & Kako, 1970). The phospholipid and the neutral lipid fractions were eluted with chloroform-methanol (1:1, v/v); the eluates were evaporated to a small volume under N_2 , and re-chromatographed in another system for further identification (see below). Between 98 and 104% of the radioactivity of the mono-, di- and tri-glycerides was recovered after elution, evaporation and the second chromatography procedure, but the calculated amounts of the reaction products, monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate, had to be corrected by their recovery values, which were approx. 70%.

Identification of reaction products

The butan-1-ol extract was evaporated to dryness under N_2 . The residue was dissolved in chloroform-methanol (1:1, v/v) and then chromatographed on silica gel G plates. The quantities of phospholipids, neutral lipids and fatty acids were determined by developing the plate with light petroleum–diethyl ether–acetic acid (40:10:1, by vol.) as the solvent system. The quantities of monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate were determined by subsequently developing the plate with chloroform-methanol–acetic acid–water (65:25:8:4, by vol.) (Skipski *et al.*, 1962). In general, the spots were identified by exposure to I_2 vapour and their R_F values compared with authentic standards. The appropriate bands were scraped off and suspended in either Aquasol (New England Nuclear Corp.) or Cab-O-Sil (Packard Instruments Co., Downers Grove, Ill., U.S.A.) for radioactivity determination. More than 90% of the radioactivity was recovered after t.l.c. To verify that the products were monoacyl- and diacyl-glycerol 3-phosphate, the developed chromatograms were scanned with an Actigraph III (Nuclear-Chicago Corp.) to localize the peaks of radioactivity, which were then compared with those of authentic standards. In addition, they were detected with three reagents, namely 0.3% (w/v) ninhydrin in ethanol for amino groups (Jatzkewitz & Mehl, 1960), Schiff-periodate spray for vicinal

hydroxy groups (Waldi, 1965), and Dittmer & Lester's (1964) reagent to detect phosphorus.

Radioactivity counting and determination of protein

A Nuclear-Chicago liquid-scintillation spectrometer, mark I, was used. The counting efficiency was calculated by the channels-ratio method and was above 70%. The calibration graph for this method was constructed by using variously quenched standards in the presence of Cab-O-Sil.

The protein concentration of the subcellular fractions was measured either by the method of Lowry *et al.* (1951), after having dissolved the sample in 1M-NaOH and diluting it, or by the biuret method of Lane & Mavrides (1969).

Results and Discussion

Characteristics of glycerol 3-phosphate acylation

The incorporation of glycerol 3-phosphate into mitochondrial lipids was studied in the presence or the absence of various cofactors (Table 1). The addition of CoA, ATP and MgCl₂ was essential. The requirement for Mg²⁺ ions could be one of the characteristics of the glycerol 3-phosphate acylating reaction (Yamashita & Numa, 1972; Monroy *et al.*, 1973) or could be that of acyl-CoA synthetase (Sánchez *et al.*, 1973). Doubling the ATP or CoA concentrations did not alter the reaction rate, but the omission of albumin produced a sigmoid-shaped kinetic curve during an incubation period of 20 min. Therefore albumin was always added to the incubation mixture. Approximately 29% of the activity remained in the absence of added palmitate, suggesting that there was a fairly large pool of endogenous fatty acids in the rabbit heart mitochondrial fractions. This result points to a possible error involved in assaying fatty acid esterification by using radioactive fatty acids or fatty acyl-CoA esters. Since the N₂ atmosphere and NaF had no effect on the activity, incubations were carried out in air in the studies reported. CTP (5 mM) failed to show a regulatory effect on the biosynthesis of diacylglycerol 3-phosphate, which disagrees with the inhibitory effect (Possmayer & Mudd, 1971) or stimulatory effect (Zborowski & Wojtczak, 1969) reported. L-Carnitine depressed the activity, presumably by enhancing the oxidation of activated fatty acids (Table 1).

The acyltransferase reaction was proportional to the amount of mitochondrial protein (up to 4 mg) added. A similar linear relationship between added protein (up to 2 mg) and glycerol 3-phosphate acylation was also obtained with microsomal preparations.

Nature of the acyl donors

The extent of glycerol 3-phosphate acylation obtained at the optimum concentration (0.4 mM) of

Table 1. *Effects of substances omitted from or added to the incubation mixture on the activities of acyl-CoA-sn-glycerol 3-phosphate O-acyltransferase of heart mitochondrial fractions*

Heart mitochondrial fractions were isolated by differential centrifugation. The incubation mixture of the control experiments contained 100 μ mol of Tris base adjusted to pH 7.4 with phosphoric acid, 2.0 μ mol of potassium palmitate, 0.8 μ mol of CoA, 12.0 μ mol of ATP, 6.0 μ mol of MgCl₂, 1% (w/v) of fatty acid-poor bovine serum albumin, 6.0 μ mol of *sn*-[U-¹⁴C]glycerol 3-phosphate (350000 d.p.m./ μ mol) (12.0 μ mol of *rac*-glycerol 3-phosphate) and water to a final volume of 2.0 ml. Each value represents the average of three experiments, in which mitochondrial preparations contained 2.42, 2.50 and 2.61 mg of protein respectively. After a 10-min incubation, the reaction was stopped and lipids were extracted with butan-1-ol as described in the Experimental section. A portion of the butan-1-ol phase was used to determine the radioactivity. The rate of acylation is expressed as nmol of glycerol 3-phosphate incorporated/min per mg of protein. All experiments were carried out in air except for one indicated as 'N₂', in which the incubation flask was flushed with N₂ gas (99.7%) throughout the incubation period.

Condition	<i>sn</i> -Glycerol 3-phosphate incorporated (nmol/min per mg of protein)
Expt. 1 (omissions)	
None	0.52
CoA	0.07
ATP	0
MgCl ₂	0.33
Albumin	0.50
Palmitate	0.15
Expt. 2 (additions)	
None	0.53
None + N ₂	0.52
NaF (3 mM)	0.52
CTP (5 mM)	0.52
L-Carnitine chloride (2 mM)	0.40
NADPH (5 mM) + NaCN (3 mM)	0.54
NADH (3 mM)	0.48

palmitoyl-CoA as an acyl donor was similar to that with the system using an optimum concentration of palmitate (1 mM), CoA, ATP and MgCl₂, indicating that acyl-CoA synthetase does not limit glycerol 3-phosphate acylation in heart mitochondria. The same conclusion was reached by Davidson & Stanacev (1972), Smith & Hübscher (1966), Shephard & Hübscher (1969), Brindley (1973) and Sánchez *et al.* (1973).

The microsomal glycerol 3-phosphate acyltransferase activity measured by using L-palmitoylcarnitine was compared with that for palmitoyl-CoA, since a number of investigators have used these acyl donors in their assays. It was found that the rate of glycerol 3-phosphate acylation was similar when a concentration of 0.4 mM of either substance was used, although

inhibition of the activity at a concentration above 0.4mM was more pronounced with palmitoylcarnitine, resulting in an extremely narrow optimum range of concentrations.

Microsomal acyl-CoA synthetase activity has been reported to be 38–100nmol/min per mg of protein (Abou-Issa & Cleland, 1969; De Jong & Hülsmann, 1970; Aas, 1971; Lippel, 1971) and showed some specificity towards individual long-chain fatty acids (Hübscher, 1970). Therefore it is improbable that the reaction rate and fatty acid specificity of the glycerol 3-phosphate acyltransferase observed in our present study reflect the characteristics of acyl-CoA synthetase. Further, the detergent property and complex physicochemical state in an aqueous solution (Barden & Cleland, 1969) of palmitoyl-CoA and palmitoylcarnitine produce an anomalous kinetic behaviour of the enzyme reaction (Smith & Hübscher, 1966; Abou-Issa & Cleland, 1969; Zahler & Cleland, 1969; Lamb & Fallon, 1970). In addition, owing to the action of palmitoyl-CoA hydrolase (EC 3.1.2.2), a high concentration of acyl-CoA, which inhibits glycerol 3-phosphate acyltransferase activities, may be required for the assay (Shephard & Hübscher, 1969; Hübscher, 1970). These considerations, together with the known subcellular localization of glycerol 3-phosphate acyltransferase (Daae & Bremer, 1970; Monroy *et al.*, 1972), did not justify the adoption of more elaborate assay methods, and hence the remaining experiments were performed with fatty acids.

Identification of reaction products

The major reaction products were scanned on the developed t.l.c. plate to localize the radioactive peaks,

the R_F values of which correspond to the authentic standards; 0.63 for monoacylglycerol 3-phosphate and 0.94 for diacylglycerol 3-phosphate. The staining test demonstrated the existence of phosphorus and the absence of amino groups and vicinal hydroxyl groups from both peaks. These results indicated that monoacyl- and diacyl-glycerol 3-phosphate were the two major products of acylation.

To characterize these reaction products further, [^{14}C]palmitate and [^{14}C]glycerol 3-phosphate were used individually as tracers in separate experiments under otherwise identical assay conditions (Table 2). The molar ratios of fatty acid/glycerol 3-phosphate in both monoacyl- and diacyl-glycerol 3-phosphate were almost identical with the theoretical values. In addition, a significantly greater quantity of glycerides was produced when [^{14}C]palmitate served as the labelled precursor, whereas only a negligible amount of neutral lipids was formed from [^{14}C]glycerol 3-phosphate, indicating that the bulk of the glycerides was produced from palmitate through pathways not involving glycerol 3-phosphate, but perhaps other acyltransferases (Elovson, 1965; McMurray & Magee, 1972). A relatively high rate of incorporation of fatty acids into glycerides has previously been reported (Scherphof & Van Deenen, 1966; Monroy *et al.*, 1972).

Distinction between mitochondrial and microsomal glycerol 3-phosphate acylation

Under standard assay conditions, the mitochondrial acylation yielded 32–42% monoacylglycerol 3-phosphate, 58–68% diacylglycerol 3-phosphate and less than 2% neutral lipids after 10min of incubation. However, the products of microsomal acylation were

Table 2. Analysis of products synthesized from two different radioactive precursors

In experiments with [$1\text{-}^{14}\text{C}$]palmitate (approx. 500000 d.p.m./ μmol) the total non-radioactive palmitate concentration was 2.0 μmol . The assay procedure is described in the legend to Table 1 and in the Experimental section. The incubation was for 10min and contained 2.67mg of mitochondrial protein. The unreacted, radioactive palmitate was separated by t.l.c., and the lipids were eluted with chloroform–methanol (1:1, v/v). The neutral lipid fraction was further separated into mono-, di- and tri-glycerides with the solvent system light petroleum (b.p. 30–60°C)–acetone–formic acid (38:12:1, by vol.). The phospholipid fraction was further separated into monoacyl- and diacyl-glycerol 3-phosphate by developing the plate with chloroform–methanol–acetic acid–water (85:25:8:4, by vol.). The assay with [$\text{U-}^{14}\text{C}$]glycerol 3-phosphate (350000 d.p.m./ μmol) was carried out in the same manner as the experiments described above, except that part of the butan-1-ol extract was used directly for the isolation of monoacyl- and diacyl-glycerol 3-phosphate. The remainder was used for the separation of mono-, di- and tri-glycerides. The recovery of phosphatides in these procedures was estimated and the values obtained were taken into account in the calculation of the products formed.

Radioactive precursor ...	[$1\text{-}^{14}\text{C}$]Palmitate incorporated (nmol/min per mg of protein)	<i>sn</i> -[$\text{U-}^{14}\text{C}$]Glycerol 3-phosphate incorporated (nmol/min per mg of protein)	Ratio of [^{14}C]palmitate/ [^{14}C]glycerol 3-phosphate
Product			
Monoacylglycerol 3-phosphate	0.16	0.15	1.06
Diacylglycerol 3-phosphate	0.70	0.33	2.12
Monoglyceride	0.05	0.01	—
Diglyceride	0.06		
Triglyceride	0.04		

70–89% diacylglycerol 3-phosphate with the remainder being 11–20% monoacylglycerol 3-phosphate and 2% neutral lipids after 5 min of incubation with palmitate as an acyl donor.

The time-courses of mitochondrial and microsomal glycerol 3-phosphate acylation were compared in Fig. 1. The rate of glycerol 3-phosphate acylation in the mitochondrial fraction was linear for up to 20 min of incubation (not shown), whereas the linear relationship in the microsomal fraction was maintained for only 5 min, illustrating the different kinetics of the acylating systems in each fraction. The rate of formation of diacylglycerol 3-phosphate from the radioactive precursor by the mitochondrial fraction was much slower than that by the microsomal fraction (Fig. 1b). Consequently, the ratio of mitochondrial/microsomal monoacylglycerol 3-phosphate increased linearly to 135% during 15 min incubation, whereas the ratio of mitochondrial/microsomal diacylglycerol 3-phosphate formation remained at

Fig. 1. Time-course of *sn*-[U-¹⁴C]glycerol 3-phosphate incorporation into monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate in mitochondrial and microsomal fractions

The assay procedure and product analysis were described in the legend to Tables 1 and 2, and in the Experimental section; 2.6 and 2.9 mg of mitochondrial protein and 0.9 and 1.0 mg of microsomal protein were used in both (a) and (b); two experiments were carried out for each subcellular fraction. (a), Formation of monoacylglycerol 3-phosphate; (b), diacylglycerol 3-phosphate (nmol/mg of protein). ●, Mitochondrial monoacylglycerol 3-phosphate formation; ■, mitochondrial diacylglycerol 3-phosphate formation; □, microsomal monoacylglycerol 3-phosphate; ○, microsomal diacylglycerol 3-phosphate.

11–17% (Fig. 2). These findings clearly demonstrate that the mitochondrial fraction is capable of synthesizing monoacylglycerol 3-phosphate from glycerol 3-phosphate and fatty acids, and that the activity of the enzyme catalysing this reaction differs from that existing in the microsomal fraction.

The results obtained from rabbit liver experiments (Table 3) supported those from heart mitochondria and showed in addition that the liver mitochondrial fraction incorporated [¹⁴C]glycerol 3-phosphate into monoacylglycerol 3-phosphate to an even greater extent (0.48 nmol/min per mg of protein) than did the heart mitochondrial fraction (0.16 nmol/min per mg of protein). In agreement with the results obtained by previous workers (Daae & Bremer, 1970; Monroy *et al.*, 1972; Sánchez *et al.*, 1973), the activities of hepatic enzymes were extremely high in comparison with those of heart enzymes.

There are less probable possibilities that could account for the observed difference in the products of acylation. First, acyl-CoA–monoacylglycerol 3-phosphate acyltransferase might be inhibited by lysosomes (Waite *et al.*, 1970), which could be contaminating the mitochondrial preparations (Eibl *et al.*, 1969). On the other hand, microsomal diacylglycerol 3-phosphate formation can be explained by

Fig. 2. Ratio of mitochondrial to microsomal monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate

The assays were carried out as in Fig. 1 with palmitate as the acyl donor. The ordinate indicates the ratio of mitochondrial/microsomal acylation products in percentages; each point represents the average of three experiments. ▲, Ratio of monoacylglycerol 3-phosphate synthesized by the mitochondrial fraction to that synthesized by the microsomal fraction; △, ratio of mitochondrial/microsomal diacylglycerol 3-phosphate.

Table 3. *Acylation of sn-[U-¹⁴C]glycerol 3-phosphate by rabbit liver subcellular fractions*

Rabbit liver subcellular fractions were prepared in the same manner as heart fractions by differential centrifugation. Mitochondrial (1.5 mg of protein) and microsomal (0.5 mg of protein) fractions were incubated separately in the presence of potassium palmitate, and the enzyme activity was assayed by the method used for heart particulate fractions (see the Experimental section in Tables 1 and 2). The reaction was stopped, lipids were extracted by butan-1-ol and the products were analysed as described in the Experimental section.

Subcellular fraction	Products	Incubation time ...	Acylation (nmol/mg of protein)		
			5 min	10 min	15 min
Mitochondrial	Monoacylglycerol 3-phosphate		2.2	5.2	7.4
	Diacylglycerol 3-phosphate		8.5	20.0	28.6
Microsomal	Monoacylglycerol 3-phosphate		2.9	3.5	3.6
	Diacylglycerol 3-phosphate		36.2	68.5	91.2
			Product ratio (%)		
Ratio: Mitochondrial/ microsomal acylation	Monoacylglycerol 3-phosphate		75.8	148.6	205.5
	Diacylglycerol 3-phosphate		23.5	29.2	31.4

the high activity of acyl-CoA-monoacylglycerol 3-phosphate acyltransferase (Eibl *et al.*, 1969; Barden & Cleland, 1969). Secondly, some investigators attribute the loss of lipids, notably monoacylglycerol 3-phosphate, to the chloroform-extraction procedure (Daae & Bremer, 1970; Sánchez *et al.*, 1973), whereas others do not (Pieringer *et al.*, 1967; Yamashita & Numa, 1972; De La Roche *et al.*, 1973).

The slow biosynthesis of diacylglycerol 3-phosphate by the mitochondrial fraction and the relatively constant ratio of mitochondrial/microsomal diacylglycerol 3-phosphate formation can be taken to indicate, at least in part, an effect of microsomal contamination of the mitochondrial preparations, as has been inferred by some investigators (Davidson & Stanacev, 1972). However, no attempt was made to investigate this postulate further by measuring the activities of microsomal marker enzymes, since marker enzymes of cardiac tissue are not necessarily exclusively localized in one subcellular compartment (Hulsmans, 1961; Sottocasa *et al.*, 1967; Brunner & Bygrave, 1969; Shephard & Hübscher, 1969; Sarzala *et al.*, 1970). Further, the evidence given below provided more definitive information regarding this problem than that which can be obtained with enzyme markers.

Cardiac versus hepatic enzymes: fatty acid specificity

The kinetics of monoacylglycerol 3-phosphate and diacylglycerol 3-phosphate formation by cardiac mitochondrial preparations differed greatly from those reported by Monroy *et al.* (1972) with hepatic mitochondrial preparations. Cardiac mitochondrial fractions synthesized monoacylglycerol 3-phosphate linearly with time for up to 15 min of incubation (Fig. 1), thereby continuously increasing the ratio of monoacyl-/diacyl-glycerol 3-phosphate (Fig. 2). By contrast, monoacylglycerol 3-phosphate appeared to be further acylated to form diacylglycerol 3-phos-

phate in hepatic mitochondrial fractions (Fig. 7 of Monroy *et al.*, 1972), resulting in a dramatic decrease in the monoacyl-/diacyl-glycerol 3-phosphate ratio during the 5-min period. Part of this difference could be due to such factors as the sonication or digitonin treatment of the mitochondrial fraction and the effects of aging or thiol inhibitors (Lands & Hart, 1965; Monroy *et al.*, 1972). In contrast with the enzyme from the liver, the cardiac mitochondrial glycerol 3-phosphate acyltransferase was more sensitive to storage (50% loss of activity in 3 weeks) and *N*-ethylmaleimide (complete inhibition at 1 mM).

The shapes of the activity-substrate concentration curve of the mitochondrial and microsomal enzymes are not identical (Fig. 3); the mitochondrial enzyme exhibits a broad optimum, whereas the microsomal enzyme shows a sharp peak at 1 mM. Although the concentration of albumin was constant in these experiments, the peak observed was not due to the effect of different molar ratios of fatty acid/albumin at various substrate concentrations, since similar patterns were recorded when the molar ratio of fatty acid/albumin was kept constant at each different substrate concentration.

Fatty acid analysis of glycerolipids in mammalian organs has shown unequivocally that the distribution of fatty acids is stereospecific (Van Deenen, 1965). Indeed, the glycerol 3-phosphate acyltransferase reaction of heart mitochondrial and microsomal fractions demonstrated substrate specificity (Fig. 3); microsomal monoacylglycerol 3-phosphate formation showed a strong affinity towards linoleic acid, whereas mitochondrial monoacylglycerol 3-phosphate had a preference for oleic acid (Figs. 3a and 3b). By comparison, the specificity for linoleic acid was more striking with mitochondrial diacylglycerol 3-phosphate biosynthesis than with microsomal diacylglycerol 3-phosphate formation (Figs. 3c and 3d). As summarized in Table 4 (Expt. 1), the biosynthesis of monoacylglycerol 3-phosphate was a

Fig. 3. Substrate specificity of acyl-CoA-*sn*-glycerol 3-phosphate *O*-acyltransferase of the mitochondrial (a) and (c) and microsomal (b) and (d) fractions

The assays were carried out as described in the legend to Tables 1 and 2, but the amounts of acyl donors were varied as indicated on the abscissa (mM). (a) and (b), Amounts of monoacylglycerol 3-phosphate formed; (c) and (d), amounts of diacylglycerol 3-phosphate (nmol/min per mg of protein). Incubation was 10 min for the mitochondrial fraction and 5 min for the microsomal fraction, each point representing the average of two experiments. ▲, Palmitate; ○, oleate; ●, linoleate.

Table 4. Rates of *sn*-[14 C]glycerol 3-phosphate acylation and 14 C-labelled diacylglycerol 3-phosphate deacylation by rabbit heart mitochondrial fraction

The assay of acyl-CoA-*sn*-glycerol 3-phosphate *O*-acyltransferase activity (Expt. 1a and b) is described in the legends to Tables 1 and 2. The concentration of palmitate and oleate was 1.0 mM, and mitochondrial protein contents were 2.4–2.9 mg. Expt. 2 was carried out under experimental conditions similar to those of Expt. 1, except that unlabelled glycerol 3-phosphate was used together with 14 C-labelled diacylglycerol 3-phosphate. The latter substrate was isolated from the products of liver microsomal acylation when palmitate (Expt. 2a) or oleate (Expt. 2b) served as an acyl donor in the presence of [14 C]glycerol 3-phosphate. Each sample of all the experiments was incubated for 10 min. The results of Expt. 1 were averages of six experiments, and those of Expt. 2 averages of three.

Expt.	Labelled precursor	Acyl donor	Radioactivity in phosphatides			
			Monoacylglycerol 3-phosphate		Diacylglycerol 3-phosphate	
			nmol/min per mg of protein	d.p.m.	nmol/min per mg of protein	d.p.m.
1 { (a) (b)	[14 C]Glycerol 3-phosphate	Palmitate	0.16	1490	0.34	3167
		Oleate	0.25	2340	0.35	3241
2 { (a) (b)	[14 C]Diacylglycerol 3-phosphate	Palmitate		164		3316
		Oleate		230		3080

little greater when oleate was used rather than palmitate. Since some other investigators, working with liver mitochondrial preparations, observed more prominent substrate preference in monoacylglycerol 3-phosphate production (Monroy *et al.*, 1972; Daae, 1972), the biosynthesis of both monoacyl- and diacyl-

glycerol 3-phosphate by cardiac mitochondrial preparations with either palmitate or oleate as substrate was examined at various time-intervals in another experiment (Table 5). The results confirm our other data (Table 4 and Fig. 3). Therefore preference for individual fatty acids by heart mitochondrial and

Table 5. Comparison of palmitate and oleate as acyl donors for acyl-CoA-sn-glycerol 3-phosphate O-acyltransferase of cardiac mitochondrial preparations from rabbits

The final concentration of both palmitate and oleate was 1.0 mM and the enzyme source was 2.1 mg of mitochondrial protein. For further details of the assay procedure see the legends to Tables 1 and 2.

Time (min)	Incorporation of palmitate (nmol/min per mg of protein)		Incorporation of oleate (nmol/min per mg of protein)	
	Monoacylglycerol 3-phosphate	Diacylglycerol 3-phosphate	Monoacylglycerol 3-phosphate	Diacylglycerol 3-phosphate
3	0.6	1.1	0.7	1.0
6	1.1	2.3	1.5	2.4
9	2.0	3.5	2.2	3.4
15	3.2	5.8	3.2	6.0
20	4.0	6.8	3.9	7.0

microsomal monoacyl- and diacyl-glycerol 3-phosphate formation is quite different from that reported for liver particulate fractions.

The problem of substrate specificity is a complex one, however, and some investigators have observed the preferential acylation of saturated fatty acids at position 1 of glycerol 3-phosphate (Possmayer *et al.*, 1969; Lamb & Fallon, 1970; Monroy *et al.*, 1972; Yamashita & Numa, 1972), whereas others have presented evidence against substrate specificity of the glycerol 3-phosphate acyltransferase reaction (Lands & Hart, 1965; Eibl *et al.*, 1969). In a recent publication, dipalmitoylglycerol 3-phosphate was found to be a predominant product after a 5-min incubation, suggesting that the acylation at C-2 of glycerol is less specific than the first acylation at C-1 (Monroy *et al.*, 1972), in spite of the well-known discrimination towards individual fatty acids by acyl-CoA-monoacylglycerol 3-phosphate acyltransferase (Barden & Cleland, 1969).

Our results with heart mitochondrial preparations may therefore be interpreted in three ways; (i) C-1 or C-2 of the glycerol 3-phosphate molecule is initially acylated according to the availability of particular fatty acids, (ii) the fatty acids are randomly acylated to C-1, or (iii) desaturation (Elovson, 1965), rearrangement (Zborowski & Wojtczak, 1969; McMurray & Magee, 1972) or migration (Daae, 1972) of the acyl moiety takes place after the formation of monoacylglycerol 3-phosphate.

Relationship between monoacyl- and diacyl-glycerol 3-phosphate

To test the effect of microsomal contamination on mitochondrial glycerol 3-phosphate acyltransferase, the mitochondrial fraction (4.0 mg of protein) was incubated with various amounts of microsomal protein (0–2.0 mg). The microsomal acylation was rather suppressed by the addition of the mitochondrial fraction in these experiments (see also

McMurray & Dawson, 1969; Davidson & Stanacev, 1972). Since the formation of neutral lipids was unaltered, it was unlikely that this effect was related to phosphatidate phosphohydrolase (EC 3.1.3.4). These results raised the question of whether or not phospholipases of albumin (Elsbach & Pettis, 1973) or those of mitochondrial preparations play a role in deacylating the diacylglycerol 3-phosphate formed by the contaminating microsomal fraction. Consequently, the mitochondrial fraction was incubated under standard conditions but in the presence of ^{14}C -labelled diacylglycerol 3-phosphate. The results (Table 4, Expt. 2) demonstrated that only one-tenth of the monoacylglycerol 3-phosphate was formed by the action of mitochondrial phospholipases, thus excluding the possibility that the formation of monoacylglycerol 3-phosphate by the cardiac mitochondria was a result of diacylglycerol 3-phosphate degradation. The same conclusion could be drawn from the substrate specificity shown in Fig. 3.

The dihydroxyacetone phosphate pathway

It is possible that the dihydroxyacetone phosphate pathway (Hajra *et al.*, 1968) may be operative in rabbit heart mitochondrial preparation, and that some monoacylglycerol 3-phosphate is formed via this pathway, since the acylated dihydroxyacetone phosphate is reduced to monoacylglycerol 3-phosphate in the presence of NADPH (La Belle & Hajra, 1972). However, the addition of NaCN (Table 1), or NaCN plus dihydroxyacetone phosphate and either NADH or NADPH (Table 6) all showed no change in the rate of glycerol 3-phosphate acylation, suggesting that the dihydroxyacetone phosphate pathway does not play a major role in mitochondrial monoacylglycerol 3-phosphate production in the heart. A directly measured amount of acyldihydroxyacetone phosphate formation reported recently (5.28 nmol/h per mg of protein; La Belle & Hajra, 1972) supports this conclusion. Since dihydroxyacetone phosphate is extremely labile (Hajra *et al.*,

Table 6. *Effect of dihydroxyacetone phosphate addition on the acylation of sn-[U-¹⁴C]glycerol 3-phosphate by rabbit heart mitochondrial preparations*

Sonicated mitochondrial preparations (2.55 mg of protein) were used for all the experiments, except Expt. 1 (2.58 mg of protein). For the disruption of isolated mitochondrial fractions, a Biosonik (Bronwill Scientific; Model B 10-II), operating at half of the maximum intensity (20 kHz, 150 W) for 15 s at 4°C, was used for a total period of 45 s. All assays were performed as stated in the legends to Tables 1 and 2.

Expt.	Addition	Incorporation of <i>sn</i> -glycerol 3-phosphate (nmol/min per mg of protein)	
		Monoacylglycerol 3-phosphate	Diacylglycerol 3-phosphate
1	None	0.21	0.30
2	None	0.17	0.25
3	Dihydroxyacetone phosphate (4 mM) NADPH (5 mM) NaCN (3 mM)	0.16	0.27
4	Dihydroxyacetone phosphate (8 mM) NADPH (5 mM) NaCN (3 mM)	0.15	0.24
5	Dihydroxyacetone phosphate (4 mM) NADH (5 mM) NaCN (3 mM)	0.15	0.26
6	Dihydroxyacetone phosphate (8 mM) NADH (5 mM) NaCN (3 mM)	0.15	0.27

1968; Bennis & Proulx, 1971) and hence easily destroyed during mild alkaline hydrolysis, this procedure was not used as a method for confirming the non-existence of acyldihydroxyacetone phosphate before hydrolysis or during the glycerol 3-phosphate acyltransferase assay period. Although the glycerol 3-phosphate-shuttle pathway, glycerol 3-phosphate oxidase (EC 1.1.1.8) and glycerol kinase (EC 2.7.1.30) in the cardiac mitochondria are of minor importance (Safer *et al.*, 1971), an elegant experimental approach utilizing 2-³H-labelled glycerol 3-phosphate or glycerol with liver slices was recently proposed (Manning & Brindley, 1972), which might produce a different result regarding the dihydroxyacetone phosphate pathway also in heart tissue.

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