Exclusive localization in peroxisomes of dihydroxyacetone phosphate acyltransferase and alkyl-dihydroxyacetone phosphate synthase in rat liver

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Abstract Dihydroxyacetone phosphate acyl transferase (DHAP-AT), alkyl dihydroxyacetone phosphate synthase (alkyl-DHAP-synthase), and glycerol-3-phosphate acyltransferase (GPAT) activities were investigated under optimal assay conditions using highly purified organelle preparations. The data presented clearly indicate that GPAT activity was mainly localized in mitochondria and microsomes, whereas DHAP-AT and alkyl-DHAP-synthase activities were exclusively localized in peroxisomes. A small fraction of the total DHAP-AT and alkyl-DHAP-synthase activities observed in purified mitochondrial preparations was due to the presence of intact peroxisomes. DHAP-AT and alkyl-DHAP-synthase activities were very low in purified microsomes (< 1% compared to peroxisomes) and these activities are thought to be due to sedimentation of peroxisomal fragments (generated during homogenization of liver and processing of liver homogenate) with microsomes. The results indicate that the dihydroxyacetone phosphate pathway does not contribute to the synthesis of glycerolipids other than ether lipids in rat liver. The ether bond formation occurs exclusively in peroxisomes, and all the biosynthetic reactions for plasmalogen synthesis may also be operating within peroxisomes in rat liver.—**Singh, H., K. Beckman, and A. Poulos.** Exclusive localization in peroxisomes of dihydroxyacetone phosphate acyltransferase and alkyl-dihydroxyacetone phosphate synthase in rat liver. *J. Lipid Res.* 1993. **34:** 467–477.

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Dihydroxyacetone phosphate (DHAP) is formed from glucose during glycolysis. In addition, it can also be formed from glycerol-3-phosphate by the action of glycerol-3-phosphate dehydrogenase. DHAP is converted to acyl DHAP (a key intermediate in ether lipid biosynthesis) by transfer of fatty acid from fatty acyl-CoA catalyzed by an enzyme dihydroxyacetone phosphate acyl transferase (DHAP-AT) (EC 2.3.1.42) (1). The ester linkage of acyl DHAP is converted to an ether linkage by an exchange reaction with long chain alcohol (formed by reduction of fatty acid) catalyzed by alkyl-dihydroxyacetone phosphate synthase (alkyl-DHAP-synthase) (EC 2.5.1.26) (2).

DHAP-AT activity was first demonstrated in guinea pig liver mitochondria (1). Subsequently, the enzyme activity was reported in rat liver microsomes (3–5). Schlossman and Bell (3) and Coleman and Bell (6) claimed that the microsomal glycerol 3-phosphate acyltransferase (GPAT, EC 2.3.1.15) and DHAP-AT activities are due to a single enzyme protein. Alkyl-DHAP-synthase activity was initially described in microsomal preparations of mouse brain (2), Harderian glands, and Ehrlich ascites carcinoma (7, 8). During the past 13 years both DHAP-AT and alkyl-DHAP-synthase activities have been found to sediment with peroxisomal-enriched fractions of rat or guinea pig liver (9–15).

The discovery (16, 17) that ether lipid levels are deficient in peroxisome-deficient (Zellweger syndrome) patients suggests indirectly that peroxisomes play a significant role in ether lipid biosynthesis. DHAP-AT (15, 18–20) and alkyl-DHAP-synthase (15, 19–21) activities are deficient in Zellweger syndrome skin fibroblasts. Ether lipid biosynthesis is also reduced in cultured skin fibroblasts of Zellweger syndrome (22, 23). However, significant ether lipid biosynthesis is detected in Zellweger syndrome skin fibroblasts (22–24). Also, a significant level of ether lipids is present in liver and skin fibroblasts of Zellweger syndrome patients (16, 17, 24).

In view of the absence of normal peroxisomes in Zellweger patients, the above findings suggest that some ether

Abbreviations: DHAP, dihydroxyacetone phosphate; DHAP-AT, dihydroxyacetone phosphate acyltransferase; DHAP-synthase, alkyldihydroxyacetone phosphate synthase; GPAT, glycerol-3-phosphate acyltransferase; TCA, trichloroacetic acid; NEM, N-ethylmaleimide; INT, iodonitrotetrazolium.

lipid synthesis may take place in other subcellular organelles. This is supported by the reports that both DHAP-AT and alkyl-DHAP-synthase are present in purified mitochondrial and microsomal preparations of rat liver (11, 12, 14, 15, 25). In order to examine the possibility that some of the ether lipid biosynthesis might be extra-peroxisomal (see above), we set out to reinvestigate localization of DHAP-AT and alkyl-DHAP-synthase in rat liver. The data presented in the present paper provide first direct evidence that the first two ether lipid biosynthetic enzymes are localized only in peroxisomes.

MATERIALS

[1-^{14}C]palmitic acid (55 mCi/mmol), [U-^{14}C]glycerol-3-phosphate (155 mCi/mmol), and [1-^{14}C]glutamic acid (57 mCi/mmol) were purchased from Amersham Australia Pty Ltd., Sydney, New South Wales. Nycodenz, used for gradient centrifugation, was obtained from Nycodenz AS, Oslo, Norway. DEAE filters (DE-81) were supplied by Whatman International, England. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Analytical grade solvents were supplied by May and Baker Australia Pty Ltd., Melbourne, Victoria or Ajax Chemicals, Sydney, New South Wales.

METHODS

Adult Porton strain rats (purchased from Agricultural Experiment Station, Gilles Plains, South Australia) were killed by exposure to carbon dioxide in gas chambers; livers were excised and rinsed 2–3 times with 10–15 ml of ice-cold buffered sucrose (0.25 M sucrose–10 mM Tris-HCl buffer (pH 7.5)–1 mM EDTA). The washed livers (10–12 g) were finely minced and hand-homogenized in 5–6 vol of buffered sucrose using a glass homogenizer with a loose-fitting pestle. The resultant homogenate was centrifuged at 200 *g* for 5 min and the nuclear-free supernatant was designated as the homogenate. The entire subcellular fractionation procedure was carried out at 4°C using a fixed-angle Ti-70 rotor (Beckman Instruments, Palo Alto, CA). The homogenate was centrifuged at 2000 *g* for 10 min and the pellet was gently homogenized in 20–25 ml of buffered sucrose and centrifuged as above. The resultant pellet (crude mitochondria) was gently dispersed in 10–12 ml of buffered sucrose and used for the isolation of mitochondria, lysosomes, and plasma membranes (see below). The 2000 *g* supernatants were combined and centrifuged further at 7300 *g* for 10 min. The pellet was gently homogenized in 20–25 ml of buffered sucrose and centrifuged as above. The pellet (crude peroxisomes) was resuspended gently in 8–10 ml of buffered sucrose and used for the isolation of peroxisomes (see below). The 7300 *g* supernatants were combined, further centrifuged at 100,000 *g* for 60 min, and the clear supernatant was removed. The pellets (crude microsomes) were dispersed in 8–10 ml of buffered sucrose and used for the isolation of microsomes.

Three volumes of a stock solution of Percoll (100%) were diluted with one volume of 1 M sucrose containing 40 mM Tris-HCl buffer, pH 7.1, and 4 mM EDTA to obtain isotonic Percoll (75%) solution. The isotonic Percoll solution (75%) was further diluted with buffered sucrose (see above) to prepare isotonic 60%, 35%, and 25% Percoll. Discontinuous Percoll gradients were prepared by carefully layering step-wise 5 ml of 60%, 5 ml of 35%, and 8 ml of 25% Percoll solution in centrifuge tubes (22 ml). The crude mitochondrial fraction (2 ml) was layered onto the top of the 25% Percoll solution and the tubes were centrifuged at 16,500 *g* for 60 min. A minor protein band at the top of 25% Percoll was collected, diluted with an equal volume of buffered sucrose, and centrifuged at 2000 *g* for 10 min. This protein pellet (purified plasma membrane) was dispersed in buffered sucrose. The major protein band at the interphase of 35% and 60% Percoll was collected, diluted with three volumes of buffered sucrose, hand-homogenized, and centrifuged at 2000 *g* for 10 min. The pellet was gently dispersed in buffered sucrose, hand-homogenized, and designated as purified mitochondria. Lysosomes in 60% Percoll were collected, diluted with four volumes of buffered sucrose, centrifuged at 7300 *g* for 10 min, and the purified lysosomal pellet was dispersed in buffered sucrose. Microsomes were similarly purified from crude microsomal pellets on discontinuous Percoll density gradients (above). The major microsomal protein band in 25% Percoll was collected, diluted with two volumes of buffered sucrose, and centrifuged at 7300 *g* for 10 min. The pellet was discarded and the membranes in supernatants were described as purified microsomes.

Peroxisomes were purified from crude peroxisomal pellets by a combination of Nycodenz and Percoll density gradients. Nycodenz solution (35%) was prepared in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and further diluted with water to make 25% and 20% Nycodenz solutions. Discontinuous Nycodenz gradients were prepared in 22-ml centrifuge tubes by carefully layering step-wise 10 ml of 35%, 4 ml of 25%, and 4 ml of 20% Nycodenz solutions. Crude peroxisomal fractions (2 ml) were layered onto the top of the 20% Nycodenz gradient and the tubes were centrifuged at 45,800 *g* for 90 min. Peroxisomal pellets at the bottom of 35% Nycodenz gradient were collected, diluted gently in buffered sucrose, hand-homogenized, and centrifuged at 7300 *g* for 10 min. The pellet was hand-homogenized in buffered sucrose, loaded onto discontinuous Percoll density gradients (see above for isolation of mitochondria), and centrifuged at 16,500 *g* for 60 min. The major protein band at the interphase of 25% and 35% Percoll was collected, and the

proteins were diluted with three volumes of buffered sucrose and centrifuged at 7300 *g* for 10 min. The supernatants were discarded and the loose pellet of purified peroxisomes was gently dispersed in buffered sucrose.

Dihydroxyacetone phosphate acyltransferase assay

[U-^{14}C]glycerol-3-phosphate was enzymatically converted to [U-^{14}C]DHAP as described previously (15). The DHAP-AT assays were performed at pH 5.4 (unless indicated) in sodium acetate buffer. The optimal assay conditions for DHAP-AT were determined in the laboratory. The incubation mixture (0.1 ml) consisted of sodium acetate buffer, pH 5.4 (90 mM), sodium fluoride (50 mM), [U-^{14}C]DHAP (1–1.2 mM), palmitoyl coenzyme A (0.4 mM), and fatty acid-free bovine serum albumin (0.4 mM). The reaction was started with 2–50 μg of cellular protein and carried out at 37°C for 30 min. The reaction was stopped with 450 μl of chloroform–methanol 1:2 (by volume); 150 μl of chloroform and 150 μl of 2 M KCl containing 0.2 M H_3PO_4 were added, mixed, and centrifuged at 200 *g* for 5 min to separate the two phases. The radioactivity extracted in the chloroform phase (containing >98% of the radiolabeled product and < 1% of the radiolabeled substrate) was spotted onto a 15 × 15 mm filter paper strip. The filters were transferred to glass scintillation vials and washed three times with 4 ml of 1% ice-cold trichloroacetic acid (TCA) to remove any radiolabeled DHAP. The washed filters were dried at room temperature and the radioactivity retained onto the filters was determined. The product 1-O-acyldihydroxyacetone-3-phosphate was retained (> 95%) on the filters under these conditions. Control experiments, where the enzyme was omitted from the incubations, were included with each set of analyses. The control experiments indicate that < 0.01% of the radiolabeled substrate was retained on the filters using the above extraction and quantification procedure.

Glycerol-3-phosphate acyl transferase

The assay was conducted in Tris-HCl buffer, pH 8.0, (0.1 M). The assay conditions were the same as described for DHAP-AT but [U-^{14}C]glycerol-3-phosphate was used as a substrate.

Alkyldihydroxyacetone phosphate synthase assay

The substrate 1-O-palmitoyl dihydroxyacetone-3-phosphate was synthesized according to the method of Hajra, Saraswathi, and Das (26). The substrate 1-O-palmitoyl dihydroxyacetone-3-phosphate was dispersed by sonication in 20 mM α-cyclodextrin solution (27). [1-^{14}C]hexadecanol (cetyl alcohol, prepared from palmitic acid by lithium aluminium hydride reduction) and dipalmitoyl phosphatidylcholine (molar ratio 2:1) were dispersed in distilled water by sonication (27) and used for alkyl-DHAP-synthase assay. The optimal assay conditions for alkyl-DHAP-synthase were determined. The incubation mixture (0.1 ml) consisted of Tris-HCl buffer, pH 8.0, (100 mM), sodium fluoride (50 mM), fatty acid-free bovine serum albumin (0.1 mM), [1-^{14}C]hexadecanol (20–25 μM), dipalmitoyl phosphatidylcholine (10–12 μM), 1-O-palmitoyl-dihydroxyacetone-3-phosphate (0.4 mM), and α-cyclodextrin (4 mM). The reaction was started by adding 2–50 μg cellular protein, and incubated at 37°C for 20 min; the reaction was stopped by adding 3 ml of chloroform–methanol 2:1 (by volume). The two phases were separated by the addition of 0.6 ml water, and the chloroform phase was discarded. Using the above partitioning procedure, < 30% of the radiolabeled product and > 98% of the radiolabeled substrate was extracted into the chloroform phase. The aqueous phase (containing radiolabeled product) was acidified with 20 μl of 10 M HCl; 300 μl of fresh chloroform was added, and the contents were mixed and centrifuged. The radioactivity extracted in the chloroform phase (> 80% of the radiolabeled product from the aqueous phase was extracted into chloroform) was spotted onto 15 × 15 mm strips of DE-81 (ion-exchange) filters. The strips were transferred to glass scintillation vials and washed five times with 3 ml of methanol. The radiolabeled substrate spotted onto DE-81 filters was removed by organic solvent (methanol). The radioactivity retained onto DE-81 filters was determined. Appropriate controls, without enzyme proteins, were performed with each set of assays. The recovery of 1-O-palmitoyl dihydroxyacetone-3-phosphate using the above procedure was consistently found to be between 50 and 55%. The enzyme activities were corrected taking into account the recoveries of the product through the above analytical procedure. The control experiments indicated that < 0.1% of the radiolabeled substrate was retained on DE-81 filters using the above extraction and quantification procedure.

Marker enzyme assays

Succinate-dehydrogenase using iodonitrotetrazolium (INT) as an electron acceptor (succinate-INT-reductase), a mitochondrial inner membrane enzyme and α-ketoglutarate dehydrogenase, a mitochondrial matrix enzyme, activities were assayed as reported (28–30). Activities of catalase and palmitoyl CoA oxidase (acyl-CoA oxidase), peroxisomal matrix enzymes, were assayed as described (30–32). β-Hexosaminidase, a lysosomal matrix enzyme, activity was assayed fluorometrically (33). Activities of NADPH-cytochrome C reductase and arylsulfatase C, microsomal enzymes, were assayed spectrophotometrically and fluorometrically, respectively (30, 34). The activity of glucose-6-phosphatase, another microsomal enzyme, was assayed at pH 6.4. The incubations (total volume 0.2 ml) consisted of sodium cacodylate buffer, pH 6.4 (50 mM), glucose-6-phosphate (10 mM), EDTA (5 mM), and sodium potassium tartrate (10 mM).

The reaction was started by the addition of protein (2–50 μg); incubations were carried out at 37°C for 30 min and the reaction was terminated with 0.8 ml of 1% TCA. Inorganic phosphate released during incubations was measured by reacting with malachite green. Plasma membrane enzyme (5'-nucleotidase) assays were performed in Tris-HCl buffer, pH 7.5, (50 mM). Sodium potassium tartrate (10 mM), magnesium (10 mM), Triton X-100 (0.1%), and

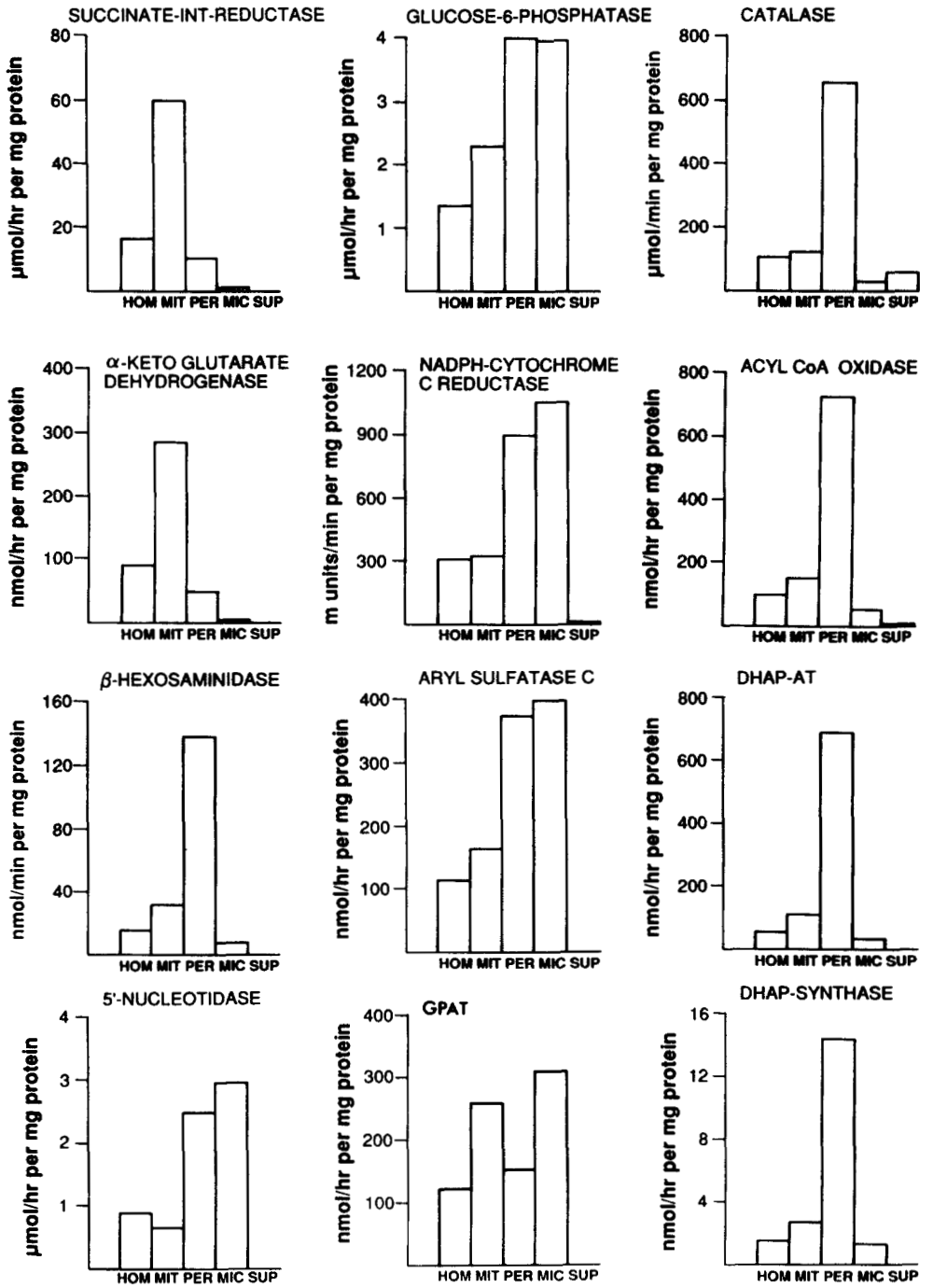

Fig. 1. Crude fractions from rat liver were prepared by differential centrifugation of liver homogenate and the enzyme activities were analyzed (see Methods). HOM, MIT, PER, MIC, and SUP represent homogenate, crude mitochondria, crude peroxisomes, crude microsomes, and supernatant fractions, respectively. DHAP-AT, DHAP-synthase, and GPAT represent dihydroxyacetone phosphate acyl transferase, alkyldihydroxyacetone phosphate synthase, and glycerol 3-phosphate acyl transferase, respectively. Five independent fractionation experiments were conducted and the results were similar. The data from a single experiment are presented as specific activities of the enzymes.

adenosine-5'-phosphate (1.5 mM) were included in the incubations (total volume, 0.2 ml). Assays were performed at 37°C for 30 min using 2–50 μg protein; the reaction was terminated with 0.8 ml of 1% TCA and the inorganic phosphate released was measured. Protein was assayed fluorometrically using human albumin as standard (35).

RESULTS

Mitochondrial marker enzyme activities, namely succinate-INT reductase and α-ketoglutarate dehydrogenase, were enriched in crude mitochondrial pellets obtained upon differential centrifugation of rat liver homogenate (**Fig. 1**). β-Hexosaminidase, a lysosomal enzyme, was enriched in crude peroxisomal fractions (Fig. 1). Mitochondrial marker enzyme activities mainly sedimented in crude mitochondrial pellets, and lysosomal marker enzyme sedimented equally in crude mitochondrial and crude peroxisomal pellets (**Table 1**). Some β-hexosaminidase activity also sedimented in crude microsomal pellets, and < 4% of the activity was detected in high speed supernatants (100,000 *g* supernatant) indicating minimal breakage of lysosomes (Table 1). Activities of glucose-6-phosphatase, NADPH-cytochrome C reductase, and arylsulfatase C (the three microsomal enzymes) were present in all of the crude membrane fractions, but the highest specific activities were observed in crude microsomal pellets (Fig. 1). Plasma membrane marker (5'-nucleotidase) mainly (> 95%) sedimented along with nuclei (data not shown). The small fraction of the total cellular plasma membrane enzyme activity that appeared in post-nuclear supernatants was found to be distributed in all of the three crude membrane pellets, but the highest specific activity was in crude microsomal pellets (Fig. 1).

Peroxisomal matrix enzymes (catalase and acyl CoA oxidase) were enriched in crude peroxisomal pellets (Fig. 1). Crude mitochondrial pellets also contained significant amounts of peroxisomal matrix enzymes (Table 1). Some activity of peroxisomal matrix enzymes was also detected in crude microsomal pellets (Table 1). High speed supernatants also contained acyl-CoA oxidase and catalase activity (Table 1). The presence of acyl-CoA oxidase activity in high-speed supernatant suggests some breakage or leakage of peroxisomes. A significant fraction of the cellular catalase was detected in high-speed supernatants, indicating that catalase is more readily released from peroxisomes than acyl-CoA oxidase, or some of the catalase activity in rat liver is present in the cytosolic compartment. Careful fractionation studies in rat liver indicate that at least 10% of cellular catalase activity is present in the cytosolic compartment. DHAP-AT and alkyl-DHAP-synthase activities were enriched in crude peroxisomal pellets, whereas GPAT was enriched in crude mitochondrial and microsomal pellets (Fig. 1). Crude mitochondrial and microsomal pellets also contained DHAP-AT and alkyl-DHAP-synthase activities (Table 1). In contrast to DHAP-AT and alkyl-DHAP-synthase, the bulk of GPAT activity sedimented in crude mitochondrial pellets (Table 1).

The mitochondrial marker enzyme activities were enriched in purified mitochondrial preparations. Relative specific activities of the mitochondrial markers in purified peroxisomal, microsomal, and lysosomal preparations were extremely low (**Table 2**). Purified plasma membranes isolated from crude mitochondrial pellets on Percoll density gradients contained significant mitochondrial

TABLE 1. Percent distribution of enzyme activities in rat liver fractions

	Crude Mitochondria	Crude Peroxisomes	Crude Microsomes	Supernatants
		%		
Succinate-INT-reductase	93.0 ± 3.4	5.8 ± 3.1	1.2 ± 0.4	0
α-Ketoglutarate dehydrogenase	94.4 ± 2.1	5.4 ± 1.7	0	0
β-Hexosaminidase	40.2 ± 7.1	48.8 ± 3.8	8.4 ± 2.4	2.4 ± 0.9
5'-Nucleotidase	28.0 ± 5.9	16.2 ± 3.9	55.6 ± 6.7	0
Glucose-6-phosphatase	42.8 ± 4.8	18.2 ± 1.6	39.2 ± 3.8	0
NADPH-cytochrome C-reductase	29.0 ± 4.6	19.8 ± 2.0	51.2 ± 3.1	0
Arylsulfatase C	36.2 ± 4.9	19.2 ± 1.6	44.6 ± 4.0	0
Catalase	25.0 ± 6.1	32.6 ± 8.7	3.4 ± 0.5	39.2 ± 12.7
Acyl-CoA oxidase	34.8 ± 4.3	42.0 ± 4.8	12.4 ± 4.9	10.8 ± 4.2
DHAP-AT	36.3 ± 2.1	55.7 ± 1.5	8.0 ± 2.0	0
Alkyl-DHAP-synthase	37.0 ± 4.6	51.3 ± 2.3	11.7 ± 2.5	0
GPAT	64.0 ± 7.9	14.3 ± 2.1	21.7 ± 6.4	0
Protein	25.8 ± 4.4	7.4 ± 0.5	14.0 ± 1.2	52.8 ± 4.4

Five separate rat liver fractionation experiments were conducted and the enzyme activities were assayed in duplicate in each fraction (see Methods). The recoveries of the enzymes were between 85 and 100%, except acyl-CoA oxidase where 65–85% recoveries were obtained. The data were corrected for recoveries and presented as mean ± SD in each fraction.

TABLE 2. Relative specific activities of marker enzymes in purified rat liver fractions

Marker Enzyme	Mitochondria	Peroxisomes	Microsomes	Plasma Membranes	Lysosomes
Succinate-INT-Reductase	6.34	0.13	0.05	0.54	0
α-Ketoglutarate dehydrogenase	5.65	0.02	0	0.42	0.36
β-Hexosaminidase	0.65	0.08	0.12	0.62	149.73
5'-Nucleotidase	0.16	0	4.92	8.17	0
Glucose-6-phosphatase	1.36	1.36	5.00	2.79	0
NADPH-cytochrome C-reductase	0.86	1.89	7.64	1.77	0
Arylsulfatase C	0.99	1.43	6.81	2.70	1.84
Catalase	0.65	79.44	0.02	0.46	0.06
Acyl-CoA oxidase	0.47	46.35	0.17	0.79	0

Marker enzyme activities were assayed in purified fractions as described under Methods, and the results are presented as specific activities relative to liver homogenate. Three independent fractionation experiments were conducted and similar results were obtained.

activity (up to 10% relative specific activity compared to purified mitochondria). Purified mitochondrial preparations contained some acyl-CoA oxidase and catalase (peroxisomal matrix enzymes) activities, indicating the presence of intact peroxisomes, but the relative specific activities were < 1% compared to purified peroxisomal preparations (Table 2). Purified plasma membrane fractions contained some catalase and acyl-CoA oxidase activity (Table 2), presumably due to low-density peroxisomes or nonspecific aggregation of some peroxisomes with plasma membranes. The relative specific activity of the plasma membrane marker (5'-nucleotidase) was < 2% in purified mitochondria compared to the activity in purified plasma membrane fractions. Plasma membrane marker enzyme activity could not be detected in purified lysosomes or peroxisomes (Table 2). Purified microsomes contained significant plasma membrane marker enzyme activity (Table 2). The reason for 5'-nucleotidase activity in microsomes is not clear at present. It is interesting to note that all of the purified organelle preparations, excepting lysosomes, contained microsomal marker enzyme activities, although the highest relative specific activities were present in purified microsomes (Table 2). The arylsulfatase C activity detected in purified lysosomes may be due to highly active arylsulfatase A and B activities in this organelle that were not completely inhibited by 0.3 M sodium sulfate in the incubations. The activities of microsomal marker enzymes in highly purified peroxisomal preparations are not due to microsomal contamination. We believe that glucose-6-phosphatase, NADPH-cytochrome C reductase, and arylsulfatase C activities are also present in peroxisomes. The role of these enzymes in peroxisomes is not clear at present but it requires further investigations.

Activities of DHAP-AT and alkyl-DHAP-synthase, the two key enzymes involved in ether lipid biosynthesis, were enriched in peroxisomes (**Table 3**). The relative specific activities of DHAP-AT and alkyl-DHAP-synthase in purified mitochondrial and microsomal preparations were < 1%, and in purified plasma membrane fractions were 3–5%, compared to peroxisomes. In contrast, GPAT activity was present in all of the purified organelles, but the specific activity was greater in mitochondria and microsomes (Table 3). Purified lysosomes contained low but measurable GPAT activity (Table 3). DHAP-AT activity (measured at pH 5.4) in peroxisomes was 18- to 33-fold greater compared to GPAT activity measured at pH 8.0 (Table 3). At pH 8.0 (optimal pH) the DHAP-AT activity in peroxisomes was at least 50-fold greater than GPAT activity (data not given).

The optimal pH of peroxisomal DHAP-AT was 8.0–9.0 and that of peroxisomal alkyl-DHAP-synthase was around 7.0–8.0 (**Fig. 2**). The optimal pH of mitochondrial GPAT was 8.5–9.0 and of microsomal GPAT was 8.0–8.5 (Fig. 2). The results described in Fig. 2 for the optimal pH of the enzymes were conducted using Tris-maleate buffer (for the entire range). The final pH values of the incubations were checked in the presence of cofactors and substrates (see Methods) and were found to be the same as that of the Tris-maleate buffer because we used 100 mM buffer (final concentration) for the entire pH range. The optimal pH of the enzymes (DHAP-AT, alkyl-DHAP-synthase, and GPAT) was also checked using potassium phosphate buffer (100 mM) and Tris-HCl buffer (100 mM) and the results were similar to those described in Fig. 2. However, in potassium phosphate buffer the specific activities of DHAP-AT were slightly lower than those described in Fig. 2. Comparison of DHAP-AT activity in peroxisomes at pH 5.4 using sodium acetate buffer or Tris-maleate buffer indicated that the activity in sodium acetate buffer was always 5- to 10-fold greater (data not given). GPAT activity could not be demonstrated at pH 5.4 in purified mitochondrial, microsomal, and peroxisomal preparations irrespective of whether sodium acetate buffer or Tris-maleate buffer was employed (Fig. 2). Measurement of DHAP-AT activity in

TABLE 3. Activities of glycerolipid biosynthetic enzymes in purified rat liver fractions

Experiment	Marker Enzyme	Specific Activities				
		Mitochondria	Peroxisomes	Microsomes	Plasma Membranes	Lysosomes
		nmol product formed /h/ mg protein				
I	DHAP-AT	37.5	3677.4	26.7	131.5	19.5
		(0.6)	(63.8)	(0.5)	(2.3)	(0.3)
II	DHAP-AT	40.9	5254.9	27.7	144.1	0
		(0.6)	(83.4)	(0.4)	(2.3)	(0)
III	DHAP-AT	46.8	5128.2	31.7	110.0	0
		(0.9)	(93.9)	(0.6)	(2.0)	(0)
I	Alkyl-DHAP-synthase	1.1	106.6	0.9	3.6	0
		(0.8)	(79.0)	(0.7)	(2.7)	(0)
II	Alkyl-DHAP-synthase	0.8	116.7	1.0	3.9	0
		(0.5)	(75.8)	(0.6)	(2.5)	(0)
III	Alkyl-DHAP-synthase	1.2	152.9	1.5	5.4	0
		(0.7)	(93.8)	(0.9)	(3.3)	(0)
I	GPAT	394.2	203.2	525.8	183.5	11.0
		(3.7)	(1.9)	(4.9)	(1.7)	(0.1)
II	GPAT	521.5	204.7	584.8	468.5	14.7
		(5.6)	(2.2)	(6.3)	(5.1)	(0.2)
III	GPAT	561.7	153.8	794.2	316.7	48.1
		(5.1)	(1.4)	(7.2)	(2.9)	(0.4)

The enzyme activities were measured in duplicate (see under Methods) and the data from three separate experiments are presented. The results in parentheses represent relative specific activities of the enzymes in purified fractions compared to liver homogenate.

purified rat liver fractions indicated that purified microsomes had < 1% relative specific activity compared to peroxisomes irrespective of whether the activity measurements were conducted at pH 5.4 or 7.5 (**Table 4**). As expected, the specific activities were higher at pH 7.5 than at pH 5.4 (Table 4).

DISCUSSION

On the basis of relative specific activities of marker enzymes, we calculated that mitochondrial preparations had < 1% peroxisomal proteins and purified microsomal preparations were essentially free from intact peroxisomes. Thus, the DHAP-AT and alkyl-DHAP-synthase activities detected in purified mitochondrial preparations could be due to the presence of peroxisomes. As the relative specific activities of DHAP-AT and alkyl-DHAP-synthase in purified mitochondrial preparations were < 1% compared to purified peroxisomes, it indicates that liver mitochondria lack both of the enzymes involved in ether lipid biosynthesis. In contrast, purified mitochondrial preparations were enriched in GPAT activity, indicating that mitochondria are equipped for biosynthesis of glycerolipids other than ether lipids. The mitochondrial localization of GPAT has also been described by other investigators (11, 36, 37).

The observations reported herein confirm the earlier findings from our group and others that DHAP-AT and alkyl-DHAP-synthase are enriched in peroxisomes (9, 15, 38, 39). We and others reported previously that DHAP-AT and alkyl-DHAP-synthase activities are present in purified mitochondrial preparations (11, 15). The small amount of enzyme activity in purified mitochondrial preparations reported by us (15) was due to underestimation of the activities in purified peroxisomal preparations. The specific activities of DHAP-AT and alkyl-DHAP-synthase in purified mitochondrial preparations that we reported were similar to those found in the present study, but the activities in purified peroxisomal preparations were several-fold lower (compare Table 2 of ref. 15 with Table 3 of present paper). Declercq et al. (11) observed that DHAP-AT activity in purified mitochondrial preparations was slightly inhibited by pre-incubation with N-ethylmaleimide (NEM) and totally inhibited by glycerol-3-phosphate. In contrast, the peroxisomal DHAP-AT activity was slightly stimulated by pre-incubation with NEM and the activity was not affected by the inclusion of glycerol-3-phosphate in the incubations. These differences and the different pH optima of the mitochondrial and peroxisomal enzymes led Declercq et al. (11) to believe that the activities were due to separate proteins. They provided some evidence that DHAP was not converted to glycerol-3-phosphate under their assay conditions. They reported at least 50-fold and 10-fold lower specific activities of DHAP-AT in their purified mitochondrial preparations compared to peroxisomes at pH 5.7 and 7.5, respectively (compare Fig. 2 and Fig. 7

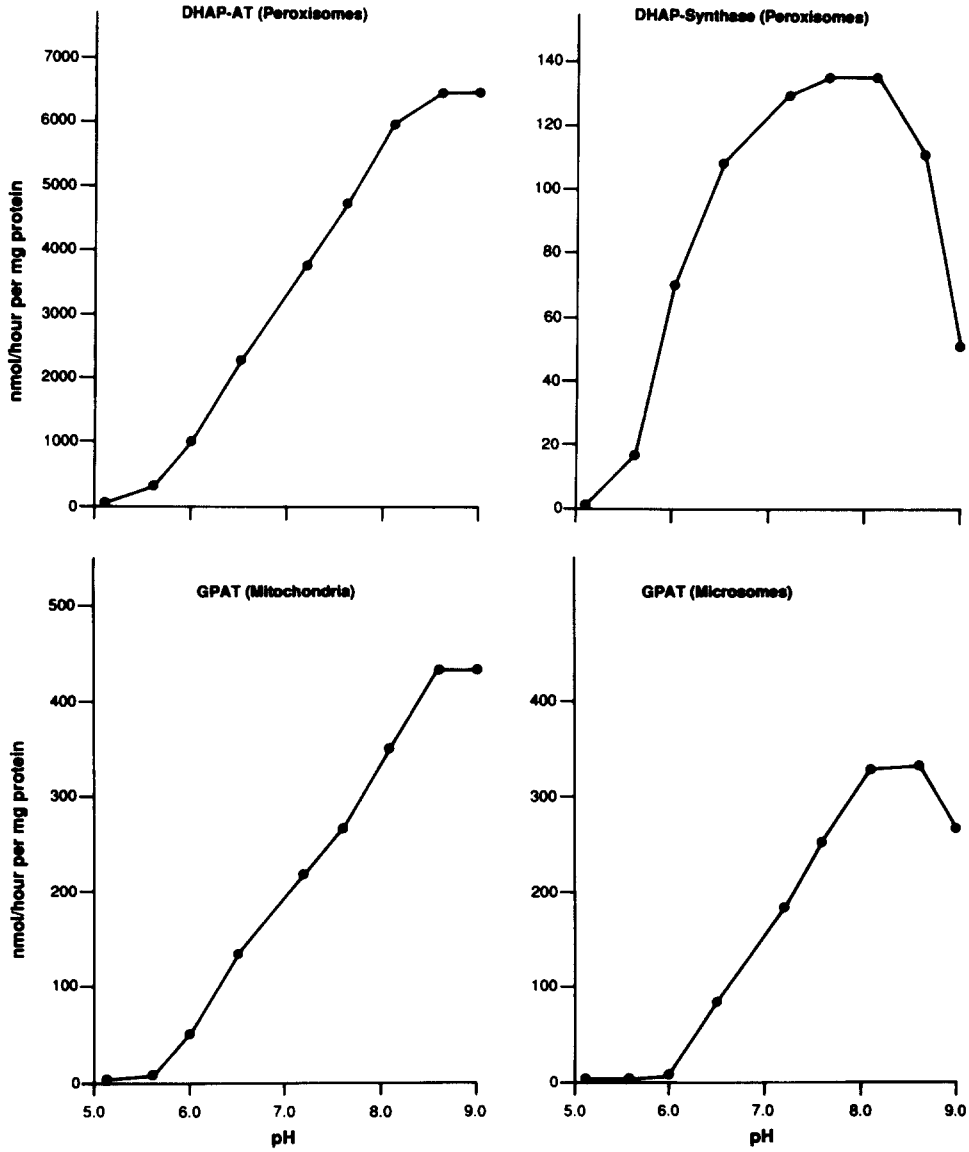

Fig. 2. The optimal pH values of dihydroxyacetone phosphate acyl transferase (DHAP-AT) and alkyldihydroxyacetone phosphate synthase (alkyl-DHAP-synthase) in purified peroxisomes and of glycerol-3-phosphate acyltransferase (GPAT) in purified mitochondria and microsomes were determined as described under Methods. The enzyme assays were performed in duplicate, in 100 mM Tris-maleate buffer (in the entire pH range) at indicated pH using 0.8 μg peroxisomal protein for DHAP-AT, 4 μg peroxisomal protein for alkyl-DHAP-synthase, 11 μg mitochondrial protein and 10 μg of microsomal protein for GPAT assays (for assay conditions see Methods). The specific activities of the enzymes at the indicated pH represent an average of two observations at each point on the graph. The activity of DHAP-AT at pH 5.4 was 5- to 10-fold greater in sodium acetate buffer than in Tris-maleate buffer.

of ref. 11). In contrast, we observed 104-fold and 148-fold lower DHAP-AT activities in purified mitochondrial preparations compared to peroxisomes at pH 5.4 and 7.5, respectively (Table 4). These differences may be due to differences in the assay conditions. We used higher molar ratios of palmitoyl CoA/BSA and higher sodium fluoride concentrations in the incubations compared to Declercq et al. (11). We observed that the molar ratio of palmitoyl CoA/BSA (1:1) and 50 mM sodium fluoride were optimal for measurement of enzyme activities in liver homogenate. We find that AMP, KCN, and Mg^{2+} were not required under our assay conditions. Both at pH 5.4 and 7.5, we measured several-fold higher DHAP-AT activities in purified peroxisomal preparations than reported by Declercq et al. (compare Fig. 2 and 3 of ref. 11 with Table 4 of present paper). Also, at pH 5.4 and 7.4 we find higher specific activities of DHAP-AT in purified mitochondria than reported by Declercq et al. (compare Fig. 7 of ref. 11 with Table 4 of present paper).

DHAP-AT and alkyl-DHAP-synthase activities ob-

TABLE 4. DHAP-AT activity in purified rat liver fractions

Assayed at	Specific Activities				
	Mitochondria	Peroxisomes	Microsomes	Plasma Membranes	Lysosomes
	nmol product formed /h/ mg protein				
pH 5.4	46.8 (0.9)	5128.2 (93.9)	31.7 (0.6)	110.0 (2.0)	0 (0)
pH 7.5	107.1 (0.7)	16779.5 (103.6)	106.7 (0.7)	310.0 (1.9)	25.9 (0.2)

The activity was assayed at pH 5.4 in 90 mM sodium acetate buffer and at pH 7.5 in 100 mM Tris-HCl buffer. The assays were carried out in duplicate (see under Methods) and the results are presented as mean values. The data in parentheses represent the relative specific activity of the enzyme compared to liver homogenate.

served in purified microsomal preparations (Tables 3 and 4) are unlikely to be due to contamination with intact peroxisomes because the activities of peroxisomal matrix enzymes (acyl-CoA oxidase and catalase) are very low. Acyl-CoA oxidase is known to be located only in the peroxisomal matrix, whereas catalase is present in the peroxisomal matrix as well as in the cytosolic compartment in rat liver. As 10% of cellular acyl-CoA oxidase was detected in high speed supernatant fractions (Table 1), it is reasonable to speculate that approximately 10% of the liver peroxisomes were lysed during homogenization and fractionation of rat liver. Thus, if peroxisomal membranes were not separated from microsomal membranes during the isolation procedure, we would expect some peroxisomal membrane activity in purified microsomes. On the basis of relative specific activities of peroxisomal and microsomal markers in the purified organelles (Table 2), we calculate that up to 1% of the peroxisomal membrane marker activities could be expected to sediment along with purified microsomes. Both DHAP-AT and alkyl-DHAP-synthase activities are known to be associated with peroxisomal membranes (10–14). Thus, we believe that the activities detected in purified microsomes are due to sedimentation of peroxisomal membrane fragments with purified microsomes. Our observations suggest that microsomes (endoplasmic reticulum) and mitochondria lack both DHAP-AT and alkyl-DHAP-synthase activities. We previously reported that microsomes lack DHAP-AT activity, but contain some (< 3%) alkyl-DHAP-synthase activity. Using a newly developed assay procedure (see Methods) we find a 2-fold higher alkyl-DHAP-synthase activity in purified peroxisomes and a 2-fold lower activity in purified microsomes than that described in our earlier report (compare Table 2 of ref. 15 with Table 3 of present paper). In our earlier studies (15) we reported alkyl-DHAP-synthase activities in crude microsomes, which are known to contain peroxisomes (Table 1, Fig. 1). In previous studies (15) we dispersed the lipid substrates, hexadecanol and 1-O-palmitoyl dihydroxyacetone-3-phosphate, in Triton X-100. Using purified peroxisomes as an enzyme source, we find that Triton X-100 is inhibitory. Thus, in the new assay procedure (see Methods) we have eliminated Triton X-100 from the incubations. As Triton X-100 was previously included in the incubations, we possibly underestimated alkyl-DHAP-synthase activity in purified peroxisomal preparations (15). Declercq et al. (11) reported that microsomal DHAP-AT activity was inactivated by pre-incubation with NEM and inhibited by glycerol-3-phosphate. It is possible that some of the DHAP was converted to glycerol-3-phosphate and microsomal GPAT was measured under their assay conditions. The data presented (Table 3) clearly indicate that GPAT is enriched in purified microsomal preparations. Declercq et al. (11) reported 3- to 6-fold lower DHAP-AT activity in their purified microsomal preparations than that described in the present paper (compare Fig. 4 of ref. 11 with Tables 3 and 4 of present paper). Previously, Bell and associates (3, 6) presented evidence to support that GPAT and DHAP-AT are microsomal enzymes and both of the reactions are carried out by a single enzyme protein. Subcellular localization studies described herein do not support these findings. Our findings are in agreement with the observations of Datta and Hajra (25), suggesting that DHAP-AT and GPAT activities are due to separate proteins.

The optimal pH of DHAP-AT described in the present paper is in agreement with the previous report on partially purified enzyme from guinea pig liver (40). However, others (10, 11) reported optimal pH of peroxisomal DHAP-AT around 5.4–6.0. Jones and Hajra (10) reported that the optimal pH of peroxisomal DHAP-AT was shifted from 5.4 to 7.5 after pre-incubation with sodium cholate. Declercq et al. (11) reported that when glycerol-3-phosphate was omitted from the incubations the optimal pH of peroxisomal DHAP-AT was 5.4 and the pH optima shifted to 6.0 when glycerol-3-phosphate was included in the incubations. The optimal pH of microsomal DHAP-AT was reported to be 7.0 (3, 11, 25)

and 8.0 (6). Microsomal DHAP-AT has been suggested to have optimal pH of 7.4 and when DHAP-AT assays are conducted at pH 5.4, peroxisomal DHAP-AT activity is mainly measured (5). In contrast to the above belief, we find that GPAT is not detected at pH 5.4, irrespective of whether Tris-maleate or sodium acetate buffer is used (see Fig. 2). The substrate for DHAP-AT, namely, dihydroxyacetone phosphate, can be easily converted to glycerol-3-phosphate by crude preparations. Thus, when the DHAP-AT assays are conducted at acidic pH (pH 5.4) there is no interference from GPAT. We investigated DHAP-AT activities at pH 5.4 and 7.5 using purified preparations from rat liver (Table 4). We do not find any evidence to suggest the presence of microsomal DHAP-AT as reported by others (3, 5, 6, 11, 25). We believe that the microsomal DHAP-AT activity detected by others (3, 5, 6, 11, 25) is due to sedimentation of peroxisomal membrane fragments with microsomes.

Microsomal localization of alkyl-DHAP-synthase activity has been described by other investigators (12, 14). Rabert, Volkl, and Debuch (12) reported that the major reaction product of microsomal alkyl-DHAP-synthase reaction was 1-O-alkyl dihydroxyacetone not 1-O-alkyldihydroxyacetone phosphate. We and others did not find any evidence to support these observations (14, 15). We report herein activities of alkyl-DHAP-synthase in purified peroxisomal preparations 50- to 60-fold higher than those described by Rabert et al. (12). Hardeman and van den Bosch (14) reported 5- to 8-fold lower alkyl-DHAP-synthase activities in their purified peroxisomal preparations and 2- to 3-fold higher activities in microsomal preparations (compare Table 1 of ref. 14 with Table 3 of present paper). It is clear that Hardeman and van den Bosch (14) underestimated alkyl-DHAP-synthase activities in their purified peroxisomal preparations leading them to believe that alkyl-DHAP-synthase activity was also present in microsomes. Why they observed different distribution patterns of DHAP-AT and alkyl-DHAP-synthase activities in their linear metrizamide density gradients (14) is not clear. Using the new assay procedure (see Methods) we do not find any alkyl-DHAP-synthase activity in high speed supernatants, which disagrees with our previous observations (15) but is in agreement with the report of Hardeman and van den Bosch (14).

The exclusive localization of DHAP-AT activity in peroxisomes strongly suggests that this enzyme is involved only in ether lipid biosynthesis. The organelles (mitochondria and microsomes) that are responsible for glycerolipid biosynthesis lack DHAP-AT activity, suggesting that the dihydroxyacetone phosphate pathway is not involved in the synthesis of glycerolipids other than ether lipids. The exclusive localization of DHAP-AT and alkyl-DHAP-synthase in peroxisomes in rat liver further suggests that the same may be true in human tissues and this is supported by reports that ether lipids and DHAP-AT and alkyl-DHAP-synthase activities are deficient in patients lacking peroxisomes (15–24). We postulate that the residual DHAP-AT and alkyl-DHAP-synthase activities observed in Zellweger syndrome are due to "peroxisomal ghosts" (15, 18, 19, 24). The third enzyme involved in ether lipid biosynthesis, namely alkyl-dihydroxyacetone phosphate reductase, has been shown to be present also in peroxisomes (41, 42). We speculate that plasmalogen biosynthesis can occur in peroxisomes. Further studies on peroxisomal localization of the remaining biosynthetic enzymes namely, 1-alkyl-glycerophosphate acyltransferase, CDP-ethanolamine transferase, and 1-alkyl-2-acylglycerophosphoethanolamine desaturase, will provide evidence for such a hypothesis.

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