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Phosphatidate Biosynthesis in Mitochondrial Subfractions of Rat Liver

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1. After conventional fractionation of rat liver homogenates in 0·88M-sucrose the mitochondrial fraction was subjected to short-term water lysis followed by separation of the resulting membrane preparations. 2. Phosphatidate formation was measured in all subcellular fractions and subfractions and was compared with the distribution of succinate dehydrogenase, monoamine oxidase, rotenone-insensitive NADH cytochrome *c* reductase, arylsulphatase, urate oxidase, aryl-esterase and glucose 6-phosphatase. 3. The results obtained indicated that mitochondria were capable of synthesizing phosphatidate, though this activity was only about one-third of the total homogenate activity. 4. Mitochondrial phosphatidate formation was located predominantly in the outer mitochondrial membrane. Although this membrane preparation was found to be significantly contaminated by the microsomal fraction, this contamination was estimated to account for not more than about 20% of the total phosphatidate formation observed in preparations of outer mitochondrial membrane.

In previous studies of the biosynthesis of glycerides in the mitochondrial fraction of rat liver (Smith & Hübscher, 1966) and in the microsomal fraction of the intestinal mucosa (Hübscher, Smith & Gurr, 1964) a requirement for the particle-free supernatant was reported. This was subsequently found to be mainly due to the presence in the particle-free supernatant of a specific L-α-phosphatidate phosphohydrolase (EC 3.1.3.4) acting on either mitochondrial or microsomal membrane-bound phosphatidate formed as an intermediate in the biosynthesis of glycerides (Smith, Sedgwick, Brindley & Hübscher, 1967).

The interaction of the phosphatidate phosphohydrolase in the cell sap with the phosphatidate formed in the membranes of the endoplasmic reticulum might be more readily accomplished than an interaction with the mitochondrial phosphatidate if the latter were formed in the inner membrane. A preliminary report had indicated that phosphatidate formation might be located in the outer mitochondrial membrane (Wojtcak & Zborowski, 1967). In the present study, the distribution of the enzymes catalysing phosphatidate formation in fractions containing inner and outer mitochondrial membranes was compared with that of a number of marker enzymes. It was hoped that thereby contaminations of preparations of inner and outer mitochondrial membranes could be evaluated and that possible erroneous interpretations might be avoided.

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MATERIALS AND METHODS

Preparation of subcellular fractions. These were prepared in 0·88M-sucrose containing 2mM-EDTA and 5mM-tris–HCl (final pH 7·4) and all operations were carried out at 0–4°. Six rat livers were homogenized in this medium (1:3, w/v) by six up-and-down strokes of a Teflon pestle–glass homogenizer (radius clearance 0·0055–0·0075in.; 600rev./min.), filtered through muslin and the volume was made up to 7·5ml. of homogenizing medium/g. of liver.

The homogenate was centrifuged at $1000g_{av.}$ for 10min. and two washes of the nuclei-plus-debris fraction were given by resuspension of the pellets in 5ml. of homogenizing medium/g. of liver, with three up-and-down strokes of the pestle homogenizer and subsequent centrifugation at 1000*g* for 10min. The final pellets were suspended in homogenizing medium (nuclei-plus-debris fraction).

The combined supernatants (nuclei-free homogenate) were centrifuged at 17000*g* for 15min. followed by three washes in which the mitochondrial pellets were homogenized as indicated above in 3ml. of homogenizing medium/g. of liver followed by centrifugation as before. The final pellets were suspended in 0·4–0·6ml. of homogenizing medium/g. of liver (mitochondrial fraction; Mit. 1).

In Expts. 1 and 2 the combined supernatants (mitochondria-free homogenate) were centrifuged at 104000*g* for 90min. The pellets, a combined lysosomal and microsomal fraction, were suspended in homogenizing medium and the supernatant (particle-free supernatant) was retained.

In Expts. 3 and 4 a portion of the mitochondria-free homogenate was centrifuged at 59000*g* for 15min. and the

supernatant carefully decanted. The latter was then centrifuged at 104000*g* for 90 or 120 min. The pellets were suspended in homogenizing medium (microsomal fraction).

Preparation of mitochondrial fractions and subfractions. A 5–8 ml. portion of fraction Mit. 1 was layered over a discontinuous sucrose density gradient consisting of 5 ml. of 1·6 M-sucrose (bottom) and 5 ml. of 1·4 M-sucrose (top). Both sucrose solutions contained 2 mM-EDTA and 5 mM-tris–HCl (final pH 7·4). After centrifugation for 45 min. at 98000*g* in a swing-out rotor, three fractions were obtained: a band at each interphase and a pellet. Most of the mitochondrial activity as indicated by succinate dehydrogenase recovery was obtained in the band at the interphase between 1·4 M- and 1·6 M-sucrose and the lysosomal and microsomal contamination of this fraction was diminished compared with fraction Mit. 1. After collection of this band from the density gradient it was suspended in a small volume of 0·88 M-sucrose (fraction Mit. 2).

A portion of fraction Mit. 2 was centrifuged for 15 min. at 21000*g* and the pellet subjected to water lysis by using the method of Caplan & Greenawalt (1966) as described by Thompson, Coleman & Finean (1967) with slight modifications. Between 29 mg. and 46 mg. of protein of fraction Mit. 2/ml. of demineralized water, previously adjusted to pH 7·4 with $NaHCO_3$, was dispersed with a glass rod. After 5–6 min. at 0° the suspension was centrifuged for 30 min. at 59000*g*. This procedure was repeated twice. The final pellet was suspended in 0·3 M-sucrose (fraction Mit. 3), giving a protein concentration of between 19 mg. and 34 mg./ml.

A 5 ml. portion of fraction Mit. 3 was layered over a discontinuous sucrose density gradient consisting of 5 ml. of 1·6 M-sucrose (bottom) and 5 ml. of 1·37 M-sucrose (top). Both sucrose solutions contained 2 mM-EDTA and 5 mM-tris–HCl, pH 7·4. After centrifugation for 2 hr. at 98000*g* in a swing-out rotor three fractions were obtained and these were collected as indicated in Fig. 1(*a*). They were diluted with water to give a final concentration of approx. 0·3 M-sucrose and designated subfractions O_1, O_2 and O_3 respectively.

In some experiments subfraction O_1 was centrifuged at 104000*g* for 60 min. and the pellet (subfraction O_{1p}) suspended in 5 ml. of 0·3 M-sucrose. This was layered over 10 ml. of a continuous linear sucrose density gradient (0·88–1·4 M) and centrifuged for 2 hr. at 98000*g*. Two diffuse bands and a pellet were obtained and these were collected as indicated in Fig. 1(*b*), giving subfractions A_1–A_4.

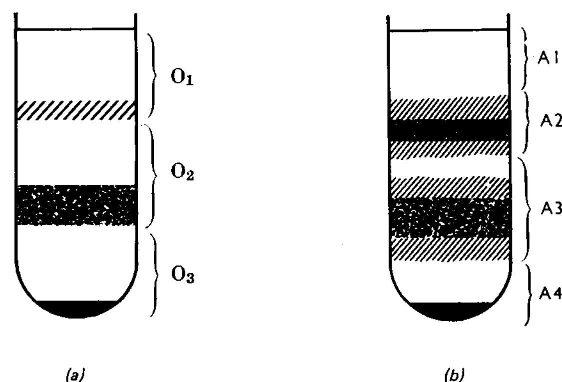


Fig. 1. Diagrammatic representation of the separation achieved after centrifugation of (*a*) fraction Mit. 3 layered over a discontinuous sucrose density gradient and (*b*) subfraction O_{1p} layered over a continuous sucrose density gradient.

Subfractionation of microsomal fraction. A microsomal fraction and two subfractions were prepared as described by Dallner, Orrenius & Bergstrand (1963) except that the pellets were washed only once.

Preparation of samples for electron microscopy. Tissue samples were fixed in 25% glutaraldehyde in 0·2 M-cacodylate buffer, pH 7·2, washed with this buffer, transferred to 1% OsO_4 (Zetterquist), dehydrated in ethanol solutions and embedded in Araldite. Ultra-thin sections were examined with an A.E.I. EM6 electron microscope.

Preparation of [^{14}C]*palmitate suspensions.* In Expt. 1 an aqueous suspension of palmitate, prepared as described by Clark & Hübscher (1961), was employed. An albumin complex of palmitate prepared as described below was used in Expts. 2–4. [1-^{14}C]Palmitic acid (1 μc/6 μmoles) dissolved in ether was titrated to neutrality with potassium ethoxide. After evaporation of the organic solvents the potassium palmitate was dissolved in a small amount of water and enough of an aqueous solution of fatty acid-poor serum albumin (previously adjusted to pH 7·4 by the addition of $NaHCO_3$) was added to give a final concentration of 45 mg. of albumin and 4·8 μmoles of palmitate/ml.

Measurement of phosphatidate and glyceride biosynthesis. This was determined by measuring the incorporation of [^{14}C]palmitate into phosphatidate and glycerides. The assay system was that described by Smith *et al.* (1967) for the mitochondrial fraction from rat liver except that fluoride was omitted, that less than 2 mg. of protein was added per assay and that in Expts. 2–4 0·8 mM-palmitate was replaced by 0·8 mM-palmitate added as an albumin complex. This system was found to be optimum also for the assay of the homogenate of rat liver (Vavrečka, Mitchell & Hübscher, 1968). Subcellular fractions were assayed at the same time and mitochondrial fractions and subfractions and the microsomal fraction were assayed together as soon as possible after preparation of the final subfraction.

The reaction was stopped with 11·25 ml. of chloroform–methanol (1:2, v/v) and to each tube was added 0·3 ml. of a carrier lipid solution containing total rat liver phospholipids (equivalent to 375 μg. of lipid P), phosphatidate (equivalent to 100 μg. of lipid P) and 27 mg. of olive oil. This was followed by 3·6 ml. of chloroform and 3·75 ml. of 10 mM-$CaCl_2$.

The collection of reaction products and their separation into phosphatidate and glycerides by column chromatography on silicic acid and alumina was carried out as described by Brindley, Smith, Sedgwick & Hübscher (1967).

Enzyme assays. These were carried out in duplicate on fractions that had been stored at −20° and thawed usually only once. Incubations were at 37°, but monoamine oxidase (with kynuramine as substrate) and rotenone-insensitive NADH cytochrome *c* reductase were assayed at 30° and urate oxidase at room temperature.

(1) Succinate dehydrogenase (EC 1.3.99.1). The assay system was that described by Pennington (1961) except that EDTA and albumin were added to increase the reaction rate and ensure linearity between protein concentration and product formed. The assay system contained (final vol. 1 ml.) the following final concentrations: 50 mM-

potassium phosphate buffer, pH7·4, 0·1% of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride, 2mM-EDTA, 0·1mg. of bovine serum albumin (fatty acid-poor), 50mM-sodium succinate, pH7·4, and 0·04–0·4mg. of protein of the fraction to be assayed. A molar extinction coefficient for the formazan formed of $\epsilon_{490}^{1\,cm.}$ 20·1 × 10³ was used.

(2) Monoamine oxidase (EC 1.4.3.4). This enzyme was assayed with either benzylamine or kynuramine as substrate. With benzylamine as substrate the method of Schnaitman, Erwin & Greenawalt (1967) was used. A molar extinction coefficient for benzaldehyde of $\epsilon_{250}^{1\,cm.}$ 1·32 × 10⁴ was used (Tabor, Tabor & Rosenthal, 1954). The method of Wiessbach, Smith, Daly, Witkop & Udenfriend (1960) was employed when kynuramine was used as substrate. A molar extinction coefficient of $\epsilon_{360}^{1\,cm.}$ 4·35 × 10³ for kynuramine was obtained from the substrate solution. In the first method the appearance of product and in the second method the disappearance of substrate is measured. Thus in the latter method monoamine oxidase activity is determined free from interference of aldehyde oxidase (EC 1.2.3.1), which might act on the product formed in the reaction catalysed by monoamine oxidase.

(3) Arylesterase (EC 3.1.1.2). This was assayed by a modification of the method described by Easter (1967), which was adapted from that of Underhay, Holt, Beaufay & de Duve (1956). The assay system contained (final vol. 2·6ml.) 50mM-potassium phosphate buffer, pH6·8 (final concentration) and 0·02–0·1mg. of protein of the fraction to be assayed. The reaction was started by adding 0·1ml. of 25mM-indoxyl acetate in 50% ethanol. The formation of indoxyl was followed spectrophotometrically at 386nm. and a molar extinction coefficient of $\epsilon_{386}^{1\,cm.}$ 3·0 × 10³ (Underhay et al. 1956) was used.

(4) Glucose 6-phosphatase (EC 3.1.3.9). The enzyme was assayed in the presence of 4mM-EDTA and 2mM-KF as described by Hübscher & West (1965). Increase in activity of this enzyme by exposure to aq. NH_3 (Stetten & Burnett, 1966) was achieved by preincubation for 1hr. at room temperature in a final concentration of 0·2M-NH_3.

(5) Rotenone-insensitive NADH cytochrome c reductase (EC 1.6.99.?). The method of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967) was employed. The change in E_{550} accompanying the reduction of cytochrome c was measured by using the molar extinction coefficient of $\epsilon_{550}^{1\,cm.}$ 1·96 × 10⁴ (Horecker & Heppel, 1949).

(6) Urate oxidase (EC 1.7.3.3). This was assayed as described by Mahler, Hübscher & Baum (1955).

(7) Arylsulphatase (EC 3.1.6.1). This was assayed by a modification of the method described by Roy (1958) for arylsulphatases A and B. The fractions were preincubated for 15min. at 0° with Triton X-100 (final concn. 0·1%, v/v). The assay system contained (final vol. 0·8ml.) the following final concentrations: 0·125M-sodium acetate buffer, pH5·4, 30mM-nitrocatechol sulphate and 0·4ml. of the preincubated preparation. After incubation for 20min. at 37°, the reaction was stopped with 0·7ml. of 4% phosphotungstic acid in 0·2M-HCl. The mixture was centrifuged and 1·2ml. of the supernatant was added to 2ml. of quinol reagent prepared by the method of Roy (1953). A molar extinction coefficient of $\epsilon_{520}^{1\,cm.}$ 11·3 × 10³ for the nitrocatechol complex was used (Robinson, Smith & Williams, 1951).

(8) β-Glucuronidase (EC 3.2.1.31). This was assayed by a modification of the method of Hübscher, West & Brindley (1965). The assay system contained (final vol. 2ml.) the following final concentrations: 0·625mM-phenolphthalein β-glucuronide, 75mM-sodium acetate buffer, pH5·2, and 0·2–1·5mg. of protein of the subcellular fraction. After incubation for 30min. the reaction was stopped with 0·2ml. of 50% (w/v) trichloroacetic acid and the precipitate removed by centrifugation. To 2ml. of the supernatant 0·1ml. of 40% (w/v) KOH was added followed by 2ml. of glycine–NaOH–NaCl buffer, pH10·7 (106mM-glycine, 94mM-NaOH, 106mM-NaCl). The phenolphthalein released was determined colorimetrically at 550nm. and a molar extinction coefficient of $\epsilon_{550}^{1\,cm.}$ 2·26 × 10⁴ used (Talalay, Fishman & Huggins, 1946).

Other determinations. Protein, RNA and inorganic phosphate were determined as described by Hübscher et al. (1965).

Materials. CoA (from yeast, grade I), ATP (disodium salt), cytochrome c (from horse heart, type II), DL-α-glycerol phosphate (disodium salt), glucose 6-phosphate (disodium salt), NADH, phenolphthalein mono-β-glucuronide (sodium salt) and kynuramine hydrobromide were obtained from Sigma (London) Chemical Co., London S.W.6. 2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride and indoxyl acetate were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Bovine serum albumin (fraction V, fatty acid-poor) was obtained from Pentex Inc., Kankakee, Ill., U.S.A., [1-¹⁴C]-palmitic acid from The Radiochemical Centre, Amersham, Bucks., silicic acid (Silicic AR CC-7; 100–200 mesh) from Camlab, Cambridge, and alumina (Camag, 5016-A-1) from Ralph N. Emanuel, London S.E.1.

Animals. Female rats (CFE strain) weighing 200–250g. were used. They were purchased from Carworth Europe, Alconbury, Hunts.

EXPERIMENTAL AND RESULTS

Demonstration of mitochondrial glyceride biosynthesis. A study of the submitochondrial site of formation of phosphatidate and glycerides requires initially experimental evidence that rat liver mitochondria do in fact contain the enzymes responsible for this synthesis. This is of particular importance in view of the microsomal localization of some of the enzymes catalysing the biosynthesis of complex lipids (Wilgram & Kennedy, 1963). In initial experiments evidence was therefore sought for a mitochondrial localization of the enzyme system synthesizing phosphatidate and glycerides from 3-glycerophosphate and labelled palmitate (Table 1).

As the incorporation of labelled palmitate into glycerides in particulate subcellular fractions is dependent on the phosphatidate phosphohydrolase present in the particle-free supernatant (Smith & Hübscher, 1966; Smith et al. 1967), the incorporation of labelled palmitate into glycerides and phosphatidate was determined by assaying homogenate, nuclei-free homogenate, nuclei- and mitochondria-free homogenate and particle-free supernatant. The relative activities present in nuclear,

Table 1. *Percentage recovery of homogenate activity in subcellular fractions*

Incorporation of palmitate into glycerides and phosphatidate was determined in the homogenate, nuclei-free homogenate, mitochondria-free homogenate and particle-free supernatant, and the percentage recovery in subcellular fractions with respect to homogenate was found by difference. The total incorporation of palmitate into phosphatidate includes that palmitate incorporated into phosphatidate that was subsequently converted into glycerides. The latter was obtained by multiplying the amount of palmitate found in glycerides by the factor $\frac{5}{6}$, which was derived from the relative distribution of palmitate in di- and tri-glycerides.

	Expt. no.	Arylesterase	β-Glucuronidase	Succinate dehydrogenase	Incorporation of palmitate into glycerides	Incorporation of palmitate into phosphatidate	Total incorporation of palmitate into phosphatidate
Nuclei + debris fraction	1	17·2	9·7	12·8	27·9	19·8	21·6
	2	9·9	2·4	8·8	11·8	25·3	21·5
Mitochondrial fraction	1	10·6	20·7	56·8	26·4	36·3	34·3
	2	9·5	22·0	78·7	22·1	36·1	32·2
Lysosomal + microsomal fraction	1	54·1	58·1	17·1	36·2	39·8	39·1
	2	43·5	41·6	16·7	44·7	32·7	36·0
Particle-free supernatant	1	12·0	9·1	0·5	15·3	4·1	5·0
	2	15·8	7·4	1·0	21·4	5·9	10·2

mitochondrial and lysosomal plus microsomal fractions were obtained by difference. Marker enzymes succinate dehydrogenase, β-glucuronidase and arylesterase for mitochondrial, lysosomal and microsomal activities respectively were assayed directly on the subcellular fractions isolated. The enzyme system responsible for the formation of phosphatidate from 3-glycerophosphate and labelled palmitate appeared to be equally distributed between mitochondrial and microsomal fractions. However, there was a definite mitochondrial biosynthetic activity, as not all of the activity found in the mitochondrial fraction could be accounted for by microsomal contamination, as indicated by the recovery of arylesterase. The microsomal fraction contained the largest proportion of the homogenate activity with respect to the incorporation of labelled palmitate into glycerides. Again, the biosynthetic activity found in the mitochondrial fraction could not be accounted for by microsomal contamination.

The different distribution of phosphatidate and of glyceride biosynthesis among the subcellular fractions may reflect a difference in behaviour of the supernatant phosphohydrolase on mitochondrial and microsomal phosphatidate. The presence of biosynthetic activity in the nuclei-plus-debris fraction and particle-free supernatant was probably due to microsomal and mitochondrial contamination of these fractions.

Results of experiments designed to provide further evidence for a mitochondrial localization of phosphatidate biosynthesis are summarized in Table 2. In these experiments the retention of this biosynthetic activity in mitochondrial fractions during their purification by density-gradient centrifugation (fraction Mit. 2) and water lysis (fraction Mit. 3) was compared with the retention of a number of marker enzymes. Succinate dehydrogenase and monoamine oxidase were retained in a similar manner in fractions Mit. 1, Mit. 2 and Mit. 3 and in most instances their specific activities increased successively in the two steps of preparation. This result is consistent with the design of the experiment to purify mitochondria. Arylesterase and glucose 6-phosphatase were removed from fractions Mit. 1 and Mit. 2 in an almost parallel manner. The specific activities of these two enzymes relative to homogenate activity decreased to well below 1·0 in fraction Mit. 3, indicating that the microsomal contamination was gradually decreased during the preparation. Lysosomal arylsulphatase, which was assayed under conditions that do not measure microsomal arylsulphatase (see the Materials and Methods section), was removed to the greatest extent. This enzyme rather than β-glucuronidase was used as a measure of lysosomal contamination as β-glucuronidase has been reported to occur partially in the microsomal fraction of rat liver (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). However, the percentage of lysosomal arylsulphatases in fractions Mit. 1, Mit. 2 and Mit. 3 relative to homogenate activity may be artificially high, as it has been observed that the enzymes are difficult to assay in homogenates of rat liver (Roy, 1958). Support for this may be obtained from the distribution of β-glucuronidase in the subcellular fractions (see Table 1). Only about 20% of the homogenate activity was recovered in the mitochondrial fraction, even though this would include some microsomal β-glucuronidase. Thus it would appear that the lysosomal contamination of

Table 2. *Percentage recovery of homogenate activity and specific activity at various stages of the preparation*

Enzyme assayed	Expt. no.	Mit. 1 fraction: Recovery (% of homogenate)	Mit. 1 fraction: Sp. activity (relative to homogenate)	Mit. 2 fraction: Recovery (% of homogenate)	Mit. 2 fraction: Sp. activity (relative to homogenate)	Mit. 3 fraction: Recovery (% of homogenate)	Mit. 3 fraction: Sp. activity (relative to homogenate)	Microsomal fraction sp. activity (relative to homogenate)	Homogenate sp. activity (nmoles/min./mg. of protein)
Succinate dehydrogenase	2	78·7	5·3	37·2	3·2	28·7	3·5	Not determined	$1·48\times10^4$
	3	62·7	2·7	41·5	4·9	34·5	6·4	0·28	$2·10\times10^4$
	4	84·6	4·2	52·0	4·4	37·8	5·2	0·19	$2·23\times10^4$
Monoamine oxidase	2	51·2	3·4	Not determined		15·4	4·0	Not determined	(4·9) Benzylamine method
	3	66·0	2·7	46·9	5·5	38·6	7·2	0·74	1·21
	4	81·3	3·7	36·2	2·8	29·3	3·7	Not detected	1·55
Rotenone-insensitive NADH cytochrome *c* reductase	3	22·6	0·96	11·5	1·4	7·0	1·3	6·8	0·32
	4	29·8	1·4	15·0	1·2	6·8	0·85	4·2	0·26
Incorporation of palmitate into phosphatidate observed	3		5·11†	14·7	5·27†	8·4	4·72†	12·8	
	4	(36·2)*	6·33	15·6	4·60	8·2	3·56	13·1	
Total incorporation of palmitate into phosphatidate	3		5·07†	13·8	5·88†	7·9	5·34†	14·5	
	4	(33·1)*	6·71	13·4	4·35	7·4	4·03	18·4	
Arylesterase	3	7·12	0·3	3·1	0·36	1·3	0·25	4·1	$8·1\times10^2$
	4	20·2	1·0	5·9	0·50	2·1	0·28	5·4	$7·2\times10^2$
Glucose 6-phosphatase	3	13·2	0·56	5·5	0·65	2·8	0·52	6·5	57·0
	4	19·4	0·81	4·1	0·31	1·2	0·15	2·6	85·0
Urate oxidase	3	17·6	0·90	Not determined		5·1	0·94	0·75	5·9
	4	24·8	1·1	Not determined		6·0	0·75	0·63	6·1
Arylsulphatase	3	98·6	4·2	18·3	2·2	4·8	0·89	2·2	4·1
	4	62·8	2·9	16·5	1·3	3·1	0·39	0·94	10·8

* The average values of the observed and total incorporation of palmitate into phosphatidate for Expts. 1 and 2 (see Table 1) were used to calculate the percentage of the respective homogenate activities recovered in fractions Mit. 2 and Mit. 3, e.g. the observed incorporation of palmitate into phosphatidate observed in fraction Mit. 1 was called 36·2% of homogenate activity and subsequent observed incorporation in fractions Mit. 2 and Mit. 3 related to this.

† Specific activities are expressed in absolute terms (nmoles/min./mg. of protein) rather than relative to homogenate as fractions Mit. 1, Mit. 2 and Mit. 3 were assayed in the absence of supernatant and in separate experiments from the determination of homogenate activity.

fraction Mit. 3 is considerably less than that indicated by arylsulphatase activity. However, arylsulphatase is a useful guide for the assessment of the removal of lysosomal contamination in fractions Mit. 2 and Mit. 3 relative to fraction Mit. 1.

The percentage recovery in fractions Mit. 1, Mit. 2 and Mit. 3 of the enzyme system responsible for the formation of phosphatidate was similar to those found for rotenone-insensitive NADH cytochrome *c* reductase and urate oxidase. Further, the percentage recovery obtained for these three enzymic activities in fractions Mit. 1, Mit. 2 and Mit. 3 was intermediate between those recorded for the mitochondrial enzymes succinate dehydrogenase and monoamine oxidase and the microsomal enzymes arylesterase and glucose 6-phosphatase. However, in terms of specific activity relative to fraction Mit. 1, the distribution of phosphatidate formation resembled more closely that of the microsomal enzymes than that of the mitochondrial enzymes. The same observation applied to the relative specific activities of rotenone-insensitive NADH cytochrome *c* reductase and urate oxidase.

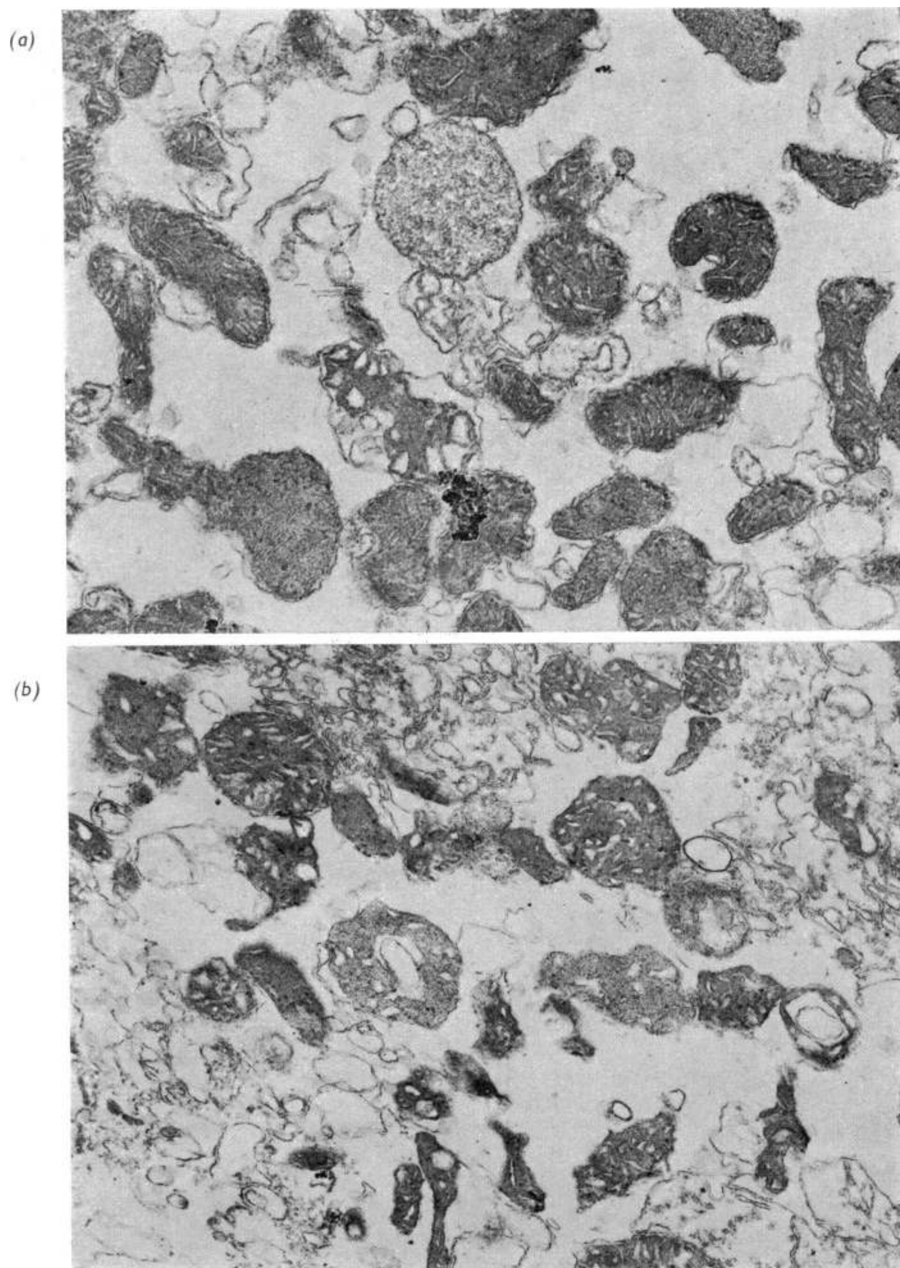
The conclusions that might be drawn from the results summarized in Table 2 are as follows. The activity of the three mitochondrial fractions with respect to phosphatidate formation cannot be solely accounted for by microsomal contamination, as shown by the relative recoveries of arylesterase and glucose 6-phosphatase. Nor is the biosynthetic activity solely located in the mitochondria, as indicated by a comparison with the distribution of succinate dehydrogenase and monoamine oxidase. Support for the conclusion that phosphatidate biosynthesis occurs in both mitochondrial and microsomal fraction is obtained from the parallel distribution of phosphatidate biosynthesis and rotenone-insensitive NADH cytochrome *c* reductase in Mit. 1, Mit. 2 and Mit. 3 because the latter enzyme has been reported to occur, not only in the microsomal fraction, but also in the outer mitochondrial membrane (see the Discussion section). Unfortunately, this argument is weakened by the fact that urate oxidase, present in neither mitochondrial nor microsomal fractions but in microbodies, had a distribution closely resembling that of rotenone-insensitive NADH cytochrome *c* reductase and phosphatidate biosynthesis. However, the results given in Table 3 show that urate oxidase and the enzymes catalysing the biosynthesis of phosphatidate do not reside in the same subcellular structure. Thus the apparently contradictory results given in Tables 2 and 3 with respect to the distribution of urate oxidase and phosphatidate biosynthesis demonstrate the necessity to examine the distribution of a given enzyme under as many conditions of separation at the subcellular level as possible before a valid interpretation can be obtained about the location of the enzyme.

Enzyme activities in mitochondrial subfractions. It was hoped that a comparison of the distribution of various enzymes within mitochondrial subfractions might give an answer to the question whether the proportion of phosphatidate biosynthesis that is of truly mitochondrial origin is located in the inner or the outer mitochondrial membrane. The enzymic composition of the mitochondrial preparations (fraction Mit. 3) that were used for the preparation of mitochondrial subfractions is given in Table 2. The recovery of enzymic activity is expressed relative to fraction Mit. 3 (see Table 3). Succinate dehydrogenase and monoamine oxidase differ significantly in their distribution among the subfractions. The distribution of phosphatidate formation is not similar to that of succinate dehydrogenase, but rather to that of monoamine oxidase. It should be noted that most of the arylesterase, rotenone-insensitive NADH cytochrome *c* reductase and arylsulphatase present in fraction Mit. 3 was also recovered in subfraction O_1.

Electron micrographs of the three subfractions are shown in Plates 1–3. These, together with the distribution of the various enzymic activities among the three subfractions, allow the following interpretation. Subfraction O_1 contained outer mitochondrial membranes, but was contaminated with some intact mitochondria. It also contained most of the microsomal and lysosomal contamination that was present in the original preparation (fraction Mit. 3). Subfraction O_2 contained mainly unbroken mitochondria and some inner membranes, and subfraction O_3 contained mainly swollen inner-membrane vesicles with a few intact mitochondria. The double membranes apparent in the micrograph of subfraction O_3 are predominantly paired membranes of adjacent vesicles.

From the results shown in Table 3 it may be concluded that the biosynthetic activity was not localized to any significant extent in the inner mitochondrial membranes or in micro-bodies and that, under the conditions of procedure used to fractionate fraction Mit. 3, the membrane bearing the biosynthetic activity had properties similar to those membranes bearing monoamine oxidase, rotenone-insensitive NADH cytochrome *c* reductase, arylesterase and arylsulphatase.

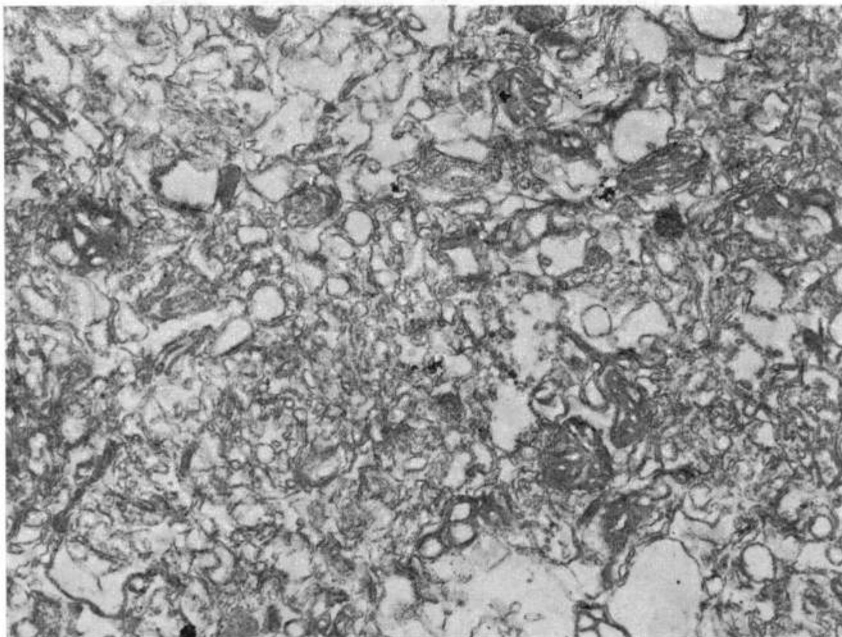
A conclusion on the localization of the enzymes synthesizing phosphatidate can only be drawn in conjunction with the results quoted in Table 2. This has been done in Fig. 2, where the data shown in Table 3 are expressed in terms of percentage recovery relative to homogenate activity. In general, the four enzymic activities mentioned above as being recovered mainly in subfraction O_1



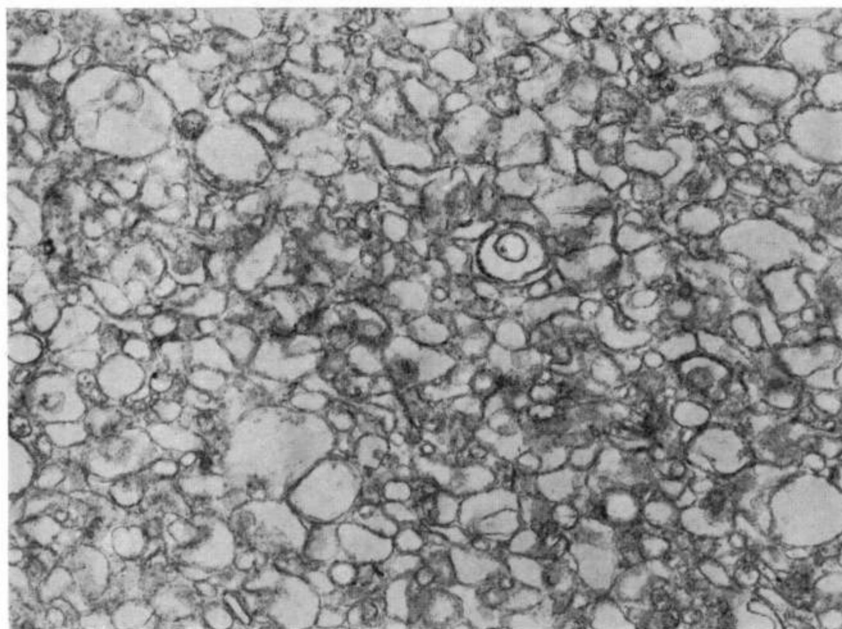
EXPLANATION OF PLATE I

Electron micrographs of (*a*) fraction Mit. 2 and (*b*) fraction Mit. 3. Magnification ×20000.

(a)



(b)



EXPLANATION OF PLATE 2

Electron micrographs of (*a*) fraction O_{1p} and (*b*) fraction obtained from supernatant above O_{1p} after centrifugation of O_1. Magnification $\times 20000$.

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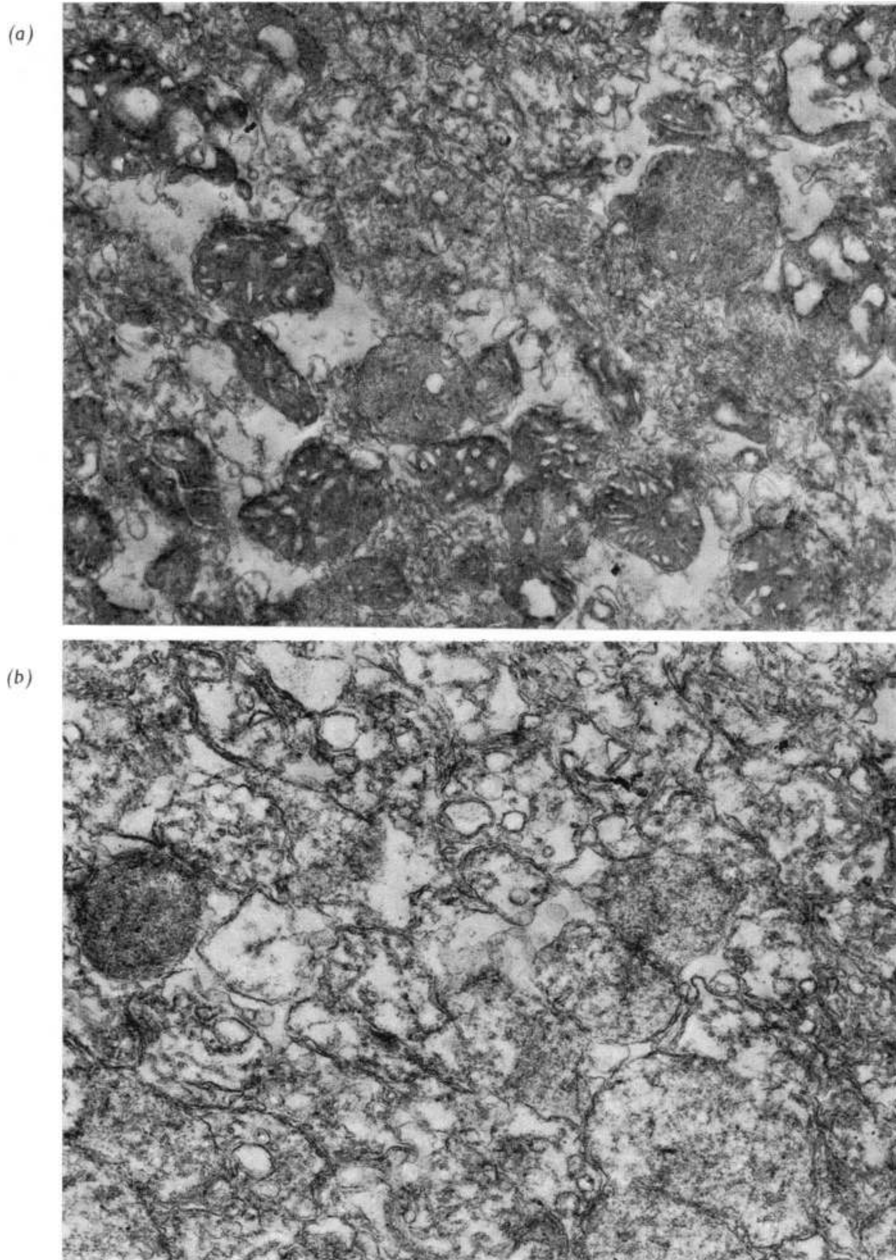

EXPLANATION OF PLATE 3

Electron micrographs of (*a*) fraction O_2 and (*b*) fraction O_3. Magnification $\times 20000$.

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Table 3. *Recovery of fraction Mit. 3 activity in subfractions and specific activity of enzymes in these subfractions relative to fraction Mit. 3*

Enzyme assayed	Expt. no.	Subfraction O_1		Subfraction O_2		Subfraction O_3		Recovery (% of fraction Mit. 3 activity)
		Activity (% of fraction Mit. 3)	Sp. activity (relative to fraction Mit. 3)	Activity (% of fraction Mit. 3)	Sp. activity (relative to fraction Mit. 3)	Activity (% of fraction Mit. 3)	Sp. activity (relative to fraction Mit. 3)	
Succinate dehydrogenase	2	9·7	0·37	58·7	0·98	11·2	0·59	78·2
	3	12·8	0·54	58·9	0·83	12·0	0·74	83·4
	4	11·1	0·58	56·6	1·40	29·8	1·12	97·3
Monoamine oxidase*	2	(43·7)	1·63	(29·4)	0·48	(3·4)	0·18	(76·5)
	3	63·5 (66·3)	2·68	28·5 (36·7)	0·40	2·2 (2·8)	0·14	94·2 (105·8)
	4	63·9	3·30	30·3	0·75	9·1	0·34	103·3
Rotenone-insensitive NADH	3	65·8	2·80	34·7	0·49	2·6	0·16	106·2
cytochrome *c* reductase	4	60·3	3·13	34·3	0·85	5·1	0·19	99·7
Incorporation of palmitate	2	70·7	2·68	11·1	0·19	7·3	0·39	89·1
into phosphatidate	3	82·8	3·45	19·7	0·28	1·5	0·09	104·0
	4	67·2	3·50	19·8	0·49	4·4	0·16	91·8
Arylesterase	3	104·2	4·40	2·9	0·04	1·9	0·12	109·0
	4	58·5	3·04	18·3	0·46	8·4	0·32	85·2
Urate oxidase	3	17·1	0·72	48·8	0·68	32·2	1·99	98·1
	4	10·7	0·56	57·4	1·43	60·6	2·30	128·7
Arylsulphatase	3	68·0	2·87	29·6	0·41	7·2	0·44	104·8
	4	104·4	5·41	17·0	0·42	6·4	0·24	129·8

* Values are given for the determination of the enzyme with benzylamine (values in parenthesis) or kynuramine as substrate.

have the same trend of distribution among the three subfractions of fraction Mit. 3 as was observed in Table 3, but the actual percentages obtained for each enzymic activity were significantly different. In fraction Mit. 3 and subfractions of fraction Mit. 3 the relative percentage of arylesterase and arylsulphatase in no case exceeded that of the biosynthetic activity.

Thus on the basis of percentage recovery relative to homogenate activity it was unlikely that microsomal contamination could account for all the biosynthetic activity observed in fraction Mit. 3 and subfraction O_3. However, an accurate assessment of the percentage recovery of phosphatidate biosynthesis in mitochondrial fractions relative to homogenate is complicated by the fact that homogenates contain the soluble phosphatidate phosphohydrolase. For this reason, an alternative approach was sought for an assessment of the contribution of microsomal phosphatidate biosynthesis to that observed in mitochondrial fractions and subfractions. A microsomal fraction was further fractionated on a sucrose density gradient by the procedure of Dallner *et al.* (1963). As arylesterase and the enzymes synthesizing phosphatidate were found to have similar distributions among smooth-vesicle and rough-vesicle fractions it was assumed that the ratio of arylesterase activity and phosphatidate formation in the microsomal fraction might be the same as that in the microsomal membranes that contaminated the mitochondrial subfraction O_1. On this basis the contribution of the microsomal contamination to the total incorporation of labelled palmitate into phosphatidate observed in subfraction O_1 could then be calculated. All relevant data are given in Tables 2 and 3 except the amount of protein in subfraction O_1, which was 82mg. and 66mg. in Expts. 3 and 4 respectively. It was found that the amount of incorporation due to microsomal contamination of subfraction O_1 was 21% and 15% of the total observed in the two preparations.

An attempt was made to remove microsomal contamination from outer mitochondrial membranes. Subfraction O_{1p} was prepared from subfraction O_1 and further fractionated by density gradient centrifugation (see the Materials and Methods section). The distribution of various enzymic activities in the subfractions of subfraction

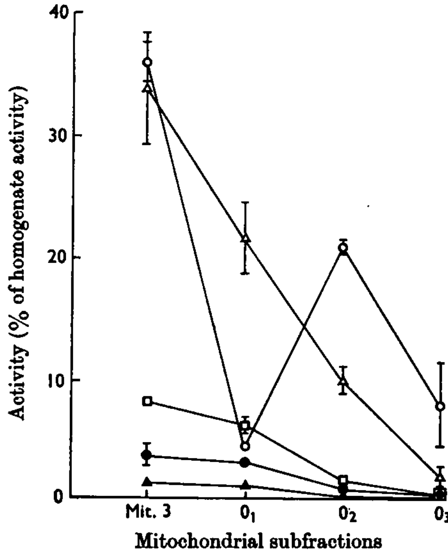

Fig. 2. Percentage recovery of homogenate activity of various enzymes in mitochondrial subfractions (average of Expts. 3 and 4 given). ○, Succinate dehydrogenase; △, monoamine oxidase; □, incorporation of palmitate into phosphatidate; ●, arylsulphatase; ▲, arylesterase.

Table 4. *Distribution of the enzyme system responsible for incorporation of palmitate into phosphatidate in submicrosomal fractions compared with various enzyme activities*

	Total microsomal preparation		Smooth-vesicle fraction		Rough-vesicle fraction		
	Recovery (% of total microsomal prep.)	Sp. activity (nmoles/min./mg. of protein)	Recovery (% of total microsomal prep.)	Sp. activity (nmoles/min./mg. of protein)	Recovery (% of total microsomal prep.)	Sp. activity (nmoles/min./mg. of protein)	Total recovery (%)
Protein	100		30·1		62·8		92·9
RNA	100		23·6		89·9		113·5
Arylesterase	100	$2·10\times10^3$	15·4	$1·07\times10^3$	90·5	$3·03\times10^3$	105·9
Glucose 6-phosphatase	100	$2·81\times10^2$	15·5	$1·45\times10^2$	64·8	$2·91\times10^2$	80·3
Incorporation of palmitate into phosphatidate	100	15·6	13·9	7·18	89·6	22·2	103·5
Incorporation of palmitate into glycerides	100	2·60	15·4	1·32	88·6	3·67	104·0

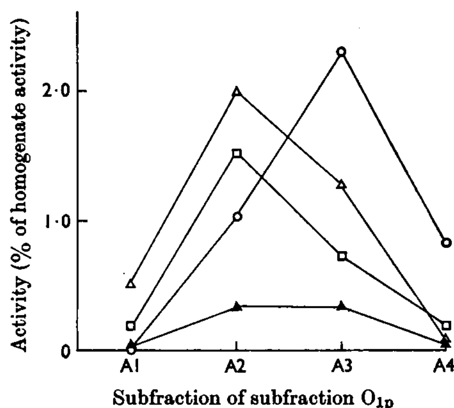

Fig. 3. Percentage recovery of homogenate activity of various enzymes in subfractions of subfraction O_{1p}. ○, Succinate dehydrogenase; △, monoamine oxidase; □, incorporation of palmitate into phosphatidate; ▲, arylesterase.

O_{1p} relative to homogenate activity are shown in Fig. 3. A separation of monoamine oxidase and succinate dehydrogenase activities was achieved and the distribution of the enzyme system responsible for incorporating palmitate into phosphatidate paralleled rather closely that of monoamine oxidase. Arylesterase activity was rather broadly spread among the four fractions and its distribution was different from that of the biosynthetic activity.

DISCUSSION

In the experiments on the submitochondrial localization of phosphatidate biosynthesis, a palmitoyl-CoA-generating system rather than palmitoyl-CoA was employed for the following reasons. In view of the presence of a long-chain acyl-CoA deacylase in liver preparations (Lands, 1960; Lands & Merkl, 1963) and the inhibitory effect of high concentrations of palmitoyl-CoA (Lands & Merkl, 1963; Lands & Hart, 1965; Kuhn & Lynen, 1965; Kuhn, 1967) it is impossible to measure phosphatidate formation under conditions that would give optimum reaction rates (see Lands & Hart, 1965; Kuhn, 1967). This is a serious limitation if an accurate assessment of the distribution of phosphatidate-synthesizing enzymes is sought in mitochondrial fractions and subfractions. On the other hand, long-chain acyl-CoA synthetase did not seem to be the rate-limiting enzyme as the formation of 26·6nmoles/min./mg. of mitochondrial protein (calculated from the data of Pande & Mead, 1968) was far in excess of the rates of palmitate incorporation obtained in the present investigation, which varied from 3·8 to 6·3nmoles/min./mg. of protein of preparations Mit. 1, Mit. 2 and Mit. 3. A truly mitochondrial long-chain acyl-CoA synthetase has been reported for rat liver (Norum, Farstad & Bremer, 1966; Farstad, Bremer & Norum, 1967). Although the mitochondrial acyl-CoA synthetase was found to be located mainly in the fraction containing outer mitochondrial membrane (Norum *et al.* 1966) the amount of this enzyme activity remaining in the inner-membrane preparation would still have been in excess of the low rate of palmitate incorporation observed in the present investigation (0·7–1·0nmole/min./mg. of protein of inner-membrane preparation). Further, recoveries of between 89 and 104% (see Table 3) were obtained for phosphatidate biosynthesis when fraction Mit. 3 was separated into subfractions. This result could not have been obtained if acyl-CoA synthetase had become rate-limiting in one of the mitochondrial subfractions.

In an assessment of the localization of phosphatidate biosynthesis in inner or outer mitochondrial membranes, several criteria have to be considered: first, that mitochondria in fact have the capacity to catalyse this biosynthesis; secondly, that the marker enzymes employed for the characterization of inner and outer mitochondrial membranes are representative of the respective membranes; thirdly, that, in view of the known microsomal biosynthesis of phosphatidate, the slight but inevitable microsomal contamination of the mitochondrial fraction does not lead to erroneous interpretations.

A truly mitochondrial localization of phosphatidate biosynthesis is indicated by the results quoted in Table 1. From the relative distribution of glucose 6-phosphatase, succinate dehydrogenase and phosphatidate plus glyceride formation it may be estimated that about 34% of the total homogenate activity with respect to lipid biosynthesis was localized in the mitochondrial fraction.

In this study, succinate dehydrogenase, monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase were used as marker enzymes for submitochondrial fractions. Although succinate dehydrogenase is generally accepted as being solely located in the inner membrane of mitochondria, the distribution of monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase has been and to some extent still is a matter of discussion. Monoamine oxidase was reported to be localized mainly in the mitochondrial fraction of rat liver, although a small portion of this enzyme was usually detected in the microsomal fraction (Cotzias & Dole, 1951; Oswald & Strittmatter, 1963; Baudhuin *et al.* 1964). Baudhuin *et al.* (1964) stated that the mitochondrial fraction contained approx. 70% and the microsomal

fraction 24% of the monoamine oxidase activity present in rat liver homogenates. In the present study, only very low activities of monoamine oxidase or none at all were detected in the microsomal fraction when the enzyme was assayed with kynuramine as substrate. The results expressed in Table 2 suggest that the enzyme was predominantly mitochondrial, as its recovery in fractions Mit. 1, Mit. 2 and Mit. 3 paralleled very closely that of succinate dehydrogenase. If monoamine oxidase is localized in the outer mitochondrial membrane, it is conceivable that microsomal monoamine oxidase in fact represents fragments of the outer mitochondrial membrane that were recovered in the microsomal fraction.

There is strong experimental evidence that, after fractionation of rat liver mitochondria into inner and outer membranes, most of the monoamine oxidase is recovered in the outer-membrane fraction (Tipton, 1967; Schnaitman *et al.* 1967; Schnaitman & Greenawalt, 1968; Beattie, 1968), although there are also some reports stating that this enzyme is localized in the inner membrane (Ragland & Mitchell, 1967; Green, Allman, Harris & Tan, 1968). In the present study, benzylamine and kynuramine were used as substrates in parallel assays to eliminate possible interference by aldehyde oxidase. With both substrates similar distributions were observed in mitochondrial subfractions and in each case monoamine oxidase was recovered in a membrane fraction that contained relatively little succinate dehydrogenase activity. From consideration of Table 3, Plates 1–3 and Fig. 2 it appears that a fair degree of separation of inner and outer mitochondrial membranes was achieved and that the results are consistent with the view that monoamine oxidase occurs in the outer mitochondrial membrane.

It has been suggested that rotenone-insensitive NADH cytochrome *c* reductase is localized solely in the microsomal fraction of rat liver (Green *et al.* 1968). However, evidence from several laboratories shows that a small percentage of the total homogenate activity of this enzyme is in mitochondria and the mitochondrial enzyme resides predominantly in the outer mitochondrial membrane (Parsons, Williams & Chance, 1966; Sottocasa *et al.* 1967; Sottocasa, Ernster, Kuylenstierna & Bergstrand, 1967; Lévy, Toury & André, 1967; Okamato, Yamamato, Nozaki & Hayaishi, 1967; Schnaitman & Greenawalt, 1968; Beattie, 1968). In the present study, a comparison of the relative distribution of arylesterase, glucose 6-phosphatase and rotenone-insensitive NADH cytochrome *c* reductase in mitochondrial fractions showed that the last-named enzyme was retained to a significantly higher degree than the former two enzymes (see Table 2). The ratio of specific activity in the microsomal fraction to specific activity in fraction Mit. 3 for arylesterase and glucose 6-phosphatase was four to six times that of rotenone-insensitive NADH cytochrome *c* reductase. After water lysis and separation of submitochondrial fractions, the rotenone-insensitive NADH cytochrome *c* reductase was recovered in the membrane fraction that was enriched with respect to monoamine oxidase and contained relatively little succinate dehydrogenase activity. These results support the view that there is a mitochondrial rotenone-insensitive NADH cytochrome *c* reductase and that the mitochondrial reductase is predominantly in the outer membrane.

The distribution of phosphatidate biosynthesis among submitochondrial fractions followed closely that of monoamine oxidase and of rotenone-insensitive NADH cytochrome *c* reductase rather than that of succinate dehydrogenase. From this it may be concluded that the enzymes catalysing the synthesis of phosphatidate are located in the outer mitochondrial membrane or in a membrane that, under the methods of separation employed, had similar properties to the outer membrane.

An assessment of the extent of microsomal contamination was of obvious importance in view of the microsomal biosynthesis of phosphatidate. Arylesterase (Underhay *et al.* 1956) and glucose 6-phosphatase (Hers, Berthet, Berthet & de Duve, 1951) were used as microsomal marker enzymes, although in mitochondrial subfractions only the former was assayed. The degree of contamination by the microsomal fraction is difficult to assess, as this fraction contains several types of membranes that do not have the same enzymic composition (Dallner *et al.* 1963). Further, it has been shown that, if a microsomal preparation was applied to the same density gradient as that used to purify the outer mitochondrial membrane, the smooth-vesicle fraction was obtained in the same region of the gradient as the outer mitochondrial membranes (Parsons, Williams, Thompson, Wilson & Chance, 1967). In view of these observations, the phosphatidate biosynthesis measured in subfraction O_1 might merely represent activity present in the smooth endoplasmic reticulum. The experiment summarized in Table 4 is therefore of importance, because it showed that arylesterase and phosphatidate formation had similar distributions between rough-membrane and smooth-membrane fractions. Thus arylesterase seemed to be a useful indicator of contaminating microsomal phosphatidate-forming enzymes and it was therefore taken as a basis for the calculation made to assess the microsomal contribution to the phosphatidate formation observed in mitochondrial fractions and subfractions. Provided that this argument is valid, only 15–21% of the total biosynthetic activity observed in fractions from outer-mitochondrial

membranes was found to be due to microsomal contamination.

At some stages of this investigation arylsulphatase (Viala & Gianetto, 1955) and urate oxidase (Beaufay *et al.* 1964) were also assayed to obtain some indication of contamination by lysosomes and micro-bodies respectively. The lysosomal contamination of fraction Mit. 1 was greatly decreased after density-gradient centrifugation and water lysis. This may be explained by the fact that hypo-osmotic conditions activate lysosomal enzymes and this is accompanied by solubilization. The solubilization of arylsulphatase by hypo-osmotic conditions has been reported (Viala & Gianetto, 1955). Nevertheless, the arylsulphatase activity that had remained particulate after water lysis was still a useful guide in that it gave some indication of the degree of contamination of the fraction containing outer mitochondrial membranes by lysosomal membranes. That lysosomal membranes may behave in sucrose density gradients similarly to outer mitochondrial membranes was demonstrated by Vignais & Nachbaur (1968), who assayed arylsulphatase and acid phosphatase. On the basis of arylsulphatase activity actually measured, the specific activity of incorporation of palmitate into phosphatidate in lysosomal membranes would have to be about twice that of subfraction O_1 for the lysosomal contamination present in subfraction O_1 to account entirely for the phosphatidate formation observed in subfraction O_1. It is not known whether lysosomal membranes have the capacity to synthesize phosphatidate. It is also questionable how representative arylsulphatase activity is for lysosomal membranes obtained after water lysis. The experiments summarized in Tables 2 and 3 certainly indicate that lysosomal contamination could contribute to the phosphatidate biosynthesis observed in mitochondrial subfractions.

Thus the degree of contamination of mitochondrial fractions and subfractions by lysosomal and microsomal membranes and their possible contribution to phosphatidate biosynthesis may be taken as an illustration of the difficulties encountered in interpreting the submitochondrial distribution of metabolic activities that are known to occur also outside mitochondria.

Stoffel & Schiefer (1968) reported that five enzymes involved in the biosynthesis of phosphatidylcholine, including those catalysing the formation of phosphatidate, were located in the outer mitochondrial membrane of rat liver mitochondria. However, three of these enzymes had a higher specific activity in the microsomal fraction than in the outer-membrane fraction and the enrichment factors of the five enzymes in the outer-membrane fraction relative to whole mitochondria varied from 27 to 0·3. As no information was given for recoveries of enzyme activity or for the degree of microsomal contamination of the outer-membrane fraction it is difficult to draw valid conclusions. Further, the outer-membrane fraction was, relative to whole mitochondria, not only enriched ninefold with respect to monoamine oxidase, but also 1·2-fold with respect to succinate dehydrogenase. These findings throw doubt on the nature of the outer-membrane fraction employed by Stoffel & Schiefer (1968).

Evidence has been provided that intact rat liver mitochondria incorporate labelled choline and serine into the endogenous phosphatidylcholine (Kaiser & Bygrave, 1968) and phosphatidylserine (Bygrave & Bücher, 1968) of the outer mitochondrial membrane. Nachbaur & Vignais (1968) reported that rat liver mitochondria, said to be devoid of lysosomal and microsomal contamination, incorporated labelled oleate into endogenous phosphatidylcholine and phosphatidylethanolamine. However, in none of the experiments mentioned above was there an indication of a new synthesis of phospholipids in the outer mitochondrial membrane.

The present results provide an indication that phosphatidate is synthesized in the fraction containing outer mitochondrial membranes. If this process were to occur at the outer side of the outer membrane then the phosphatidate phosphohydrolase present in the cell sap could act on the phosphatidate formed and thus diglyceride could be formed. The question of synthesis of triglyceride may be answered when the submitochondrial localization of diglyceride transacylase has been studied, although this enzyme has been reported to be located solely in the microsomal fraction (Wilgram & Kennedy, 1963).

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