Phospholipid Exchange Reactions within the Liver Cell

By W. C. McMURRAY* AND R. M. C. DAWSON

Department of Biochemistry, Agricultural Research Council, Institute of Animal Physiology,
Babraham, Cambridge

(Received 14 October 1968)

1. Isolated rat liver mitochondria do not synthesize labelled phosphatidylcholine from CDP-[^{14}C]choline or any phospholipid other than phosphatidic acid from [^{32}P]phosphate. The minimal labelling of phosphatidylcholine and other phosphoglycerides can be attributed to microsomal contamination. However, when mitochondria and microsomes are incubated together with [^{32}P]phosphate, the phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine of the reisolated mitochondria become labelled, suggesting a transfer of phospholipids between the two fractions. 2. When liver microsomes or mitochondria containing labelled phosphatidylcholine are independently incubated with the opposite unlabelled fraction, there is a substantial and rapid exchange of the phospholipid between the two membranes. Exchange of phosphatidylinositol also occurs rapidly, whereas phosphatidylethanolamine and phosphatidic acid exchange only slowly. There is no corresponding transfer of marker enzymes. The transfer of phosphatidylcholine does not occur at 0° , and there is no requirement for added substrate, ATP or Mg^{2+} , but the omission of a heat-labile supernatant fraction markedly decreases the exchange. 3. After intravenous injection of [^{32}P]phosphate, short-period labelling experiments of the individual phospholipids of rat liver microsomes and mitochondria *in vivo* give no evidence for a similar exchange process. However, the incubation of isolated microsomes and mitochondria with [^{32}P]phosphate also fails on reisolation of the fractions to demonstrate a precursor-product relationship between the individual phospholipids of the two membranes. 4. The intraperitoneal injection of [^{32}P]phosphate results in a far greater proportion of the dose entering the liver than does intravenous administration. After intraperitoneal administration of [^{32}P]phosphate the specific radioactivities of the individual phospholipids are in the order microsomes > outer mitochondrial membrane > inner mitochondrial membrane. 5. The incorporation of ^{32}P into cardiolipin is very slow both *in vivo* and *in vitro*. After labelling *in vivo* the radioactivity in the cardiolipin persists compared with that of the other phospholipids, whose specific radioactivities in the microsomes and mitochondrial fragments decay at a similar rate to that of the acid-soluble phosphate pool. 6. The possibility of phospholipid exchange processes occurring in the liver cell *in vivo* is discussed, and it is suggested that only a small but highly labelled part of the endoplasmic-reticulum lipoprotein pool is involved in the transfer.

There is now abundant experimental evidence that when radioactive phospholipid precursors are injected into animals the phospholipids of all the subcellular fractions of the liver cell become rapidly labelled, although at somewhat variable rates. The question therefore arises whether each type of membrane in the cell is capable of synthesizing its own phospholipid, or whether intracellular transport and exchange of newly synthesized phospholipid molecules can occur (Dawson, 1966).

The final stage of the synthesis of many phospho-

* Present address: Department of Biochemistry, University of Western Ontario, London, Ont., Canada.

lipids is known to be carried out by an isolated liver microsomal fraction, but evidence on the ability of mitochondria to synthesize phospholipids is conflicting. When mitochondria are incubated with inorganic [^{32}P]phosphate or [γ - ^{32}P]ATP only three phospholipids are appreciably labelled, phosphatidic acid, diphosphoinositide and acyl dihydroxyacetone phosphate (Garbus, DeLuca, Loomans & Strong, 1963; Galliard, Mitchell & Hawthorne, 1965; Hajra & Agranoff, 1967). Liver mitochondria appear not to contain significant amounts of the phosphatidylcholine-synthesizing enzymes CDP-choline-1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2)

and CTP-choline phosphate cytidyltransferase (EC 2.7.7.15) (Wilgram & Kennedy, 1963; Schneider, 1963), which are mainly localized in the microsomes. On the other hand, McCaman & Cook (1966) reported that CDP-choline-1,2-diglyceride choline-phosphotransferase is present to a limited but significant extent in mitochondria isolated from rat brain and bovine heart muscle, and Stoffel & Schiefer (1968) obtained evidence that some of the enzyme is contained in an outer-membrane fraction from rat liver mitochondria. Bygrave & Kaiser (1968) have reported that choline can be incorporated into the phospholipids of isolated rat liver mitochondria by a mechanism that appears to be energy-dependent and that is stimulated by the addition of CTP.

We therefore carried out experiments in an attempt to clarify the situation with regard to the synthesis of the nitrogen-containing phosphoglycerides by rat liver mitochondria. These suggested that the small mitochondrial synthesis of such phospholipids at least from [32 P]phosphate and [14 C]CDP-choline *in vitro* can be ascribed to contamination by microsomes. At the same time a rapid and reversible exchange of phosphatidylcholine between isolated microsomes and mitochondria was demonstrated. In addition, experiments were performed *in vivo* in an attempt to obtain evidence on whether a similar intracellular exchange of phospholipid occurs in the intact liver cell.

METHODS

Animals. The rats used were males (females in the experiments of Fig. 3) of the Wistar albino strain, 200–250 g. in weight. They were kept without food overnight before being killed in the studies *in vivo* to avoid glycogen complicating the fractionation, but were fed normally for experiments *in vitro*. The metabolism of phospholipids *in vivo* was followed after the injection of inorganic [32 P]-phosphate. For short-term studies (0–60 min.) the [32 P]phosphate (carrier-free) was injected intravenously by the femoral vein (Griffith & Jeffers, 1949). For longer studies (1 hr.–22 days) the [32 P]phosphate in the presence of carrier inorganic phosphate (1 mg. of P/mc of [32 P]) was injected intraperitoneally. In both instances the [32 P]-phosphate was in sterile aq. 0.9% NaCl solution; the animals were each injected with $2\ \mu\text{C}$ of [32 P]phosphate/g. body wt. while under light ether anaesthesia.

Isolation procedures. Animals were killed by decapitation and the livers rapidly excised and chilled. All subsequent operations were performed at 0°. The livers (pooled from three animals in studies involving the isolation of mitochondrial membranes) were minced with scissors and homogenized in a motor-driven homogenizer with 0.008 in. clearance (Aldridge, Emery & Street, 1960), for 1 min. in 10 vol. of cold 0.25 M-sucrose–0.1 mM-EDTA, pH 7.4. The homogenate was centrifuged at 2000 rev./min. for 10 min. in a Servall RC-2 centrifuge (SS-34 rotor), and the supernatant obtained was centrifuged at 6000 rev./min. for 10 min. and the speed increased to 9300 rev./min. for 2 min. The

mitochondrial pellet obtained was resuspended in the same sucrose–EDTA solution in a hand-operated homogenizer and resedimented as described above. This washing procedure, adapted from that of Parsons, Williams, Thompson, Wilson & Chance (1967), was repeated twice more to minimize the microsomal contamination of the fraction. In general, the washed mitochondrial fraction contained 4–9% contamination with microsomes, based on NADPH-cytochrome *c* reductase activity (Table 1). The supernatant decanted from the first mitochondrial pellet was centrifuged at 10 000 rev./min. (Servall SS-34 rotor) for 10 min. to remove lysosomes, and the pellet was discarded. The microsomal fraction was sedimented by centrifuging this supernatant at 36 000 rev./min. (Spinco 40 rotor) for 90 min. Although it is undoubtedly heterogeneous, this fraction is referred to for convenience throughout this paper as ‘microsomes’.

Mitochondrial membrane fractions. The procedure was based on the experiments of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967), with osmotic swelling and ultrasonic treatment being used to disrupt the mitochondria, and the discontinuous-gradient fractionation described by Parsons *et al.* (1967) to isolate the membrane fractions. The washed mitochondrial pellet was suspended in 0.01 M- K_2HPO_4 – KH_2PO_4 buffer–0.1 mM-EDTA, pH 7.5 (1 ml./g. of original tissue), in a hand-operated homogenizer and kept at 0° for 5 min. An equal volume of 17% (w/v) sucrose in 0.01 M-potassium phosphate buffer–0.1 mM-EDTA, pH 7.5, was added, and the suspension was thoroughly mixed. The suspension was subjected to mild ultrasonic treatment to remove outer membranes by immersion of the tube (to the depth of the liquid in the tube) for 5 min. in a 80 kcy./sec. 40 W ultrasonic cleaning bath (KG 80/1; Kerry's, Basildon, Essex). The ultrasonically treated suspension was centrifuged at 10 000 rev./min. in the Servall SS-34 rotor for 10 min. and the supernatant (S_1) decanted. Trial experiments showed that 85–95% of the mitochondrial monoamine oxidase activity, considered to be a marker enzyme for the outer membrane (Schnaitman, Erwin & Greenawalt, 1967; Schnaitman & Greenawalt, 1968), was released in the S_1 fraction, whereas 80–90% of the cytochrome *c* oxidase, a marker enzyme for the inner membrane, sedimented with the pellet. The pellet was resuspended (0.5 ml./g. of original tissue) in 0.01 M-potassium phosphate buffer–0.1 mM-EDTA, pH 7.5, and after 5 min. the suspension was mixed with an equal volume of 17% sucrose. To fragment the inner membranes the suspension was subjected to strong ultrasonic treatment three times for 1 min. each time, at 1 min. intervals to prevent overheating, in an ice bath with an ultrasonic drill probe (60 W; Mullard–MSE). The ultrasonically treated suspension was centrifuged at 10 000 rev./min. for 10 min. in the Servall SS-34 rotor. The supernatant fraction (S_2) contained 30–40% of the total cytochrome *c* oxidase activity. The S_1 and S_2 fractions in portions (15–20 ml.) were layered separately on 5–10 ml. of 37.7% (w/v) sucrose in 0.01 M- K_2HPO_4 – KH_2PO_4 buffer–0.1 mM-EDTA, pH 7.5, in a Spinco SW 25 tube, and centrifuged at 22 500 rev./min. for 120 min. in the Spinco SW 25 rotor. The material at the interface from the S_1 fraction (S_{1I}) was collected carefully by aspiration with a Pasteur pipette with a bent tip. The corresponding interfacial fraction from the S_2 supernatant (S_{2I}) contained some additional monoamine oxidase activity, but was heavily contaminated with cytochrome *c* oxidase and appeared to

Table 1. Distribution and recovery of 'marker' enzymes in isolated inner and outer membranes from rat liver mitochondria

Expt. no.	Cytochrome c oxidase						Monoamine oxidase						NADPH-cytochrome c reductase					
	Activity (% of that present in original mitochondrial fraction)			Specific activity (μmoles of reduced cytochrome c oxidized/min./mg. of protein)			Activity (% of that present in original mitochondrial fraction)			Specific activity (μmoles of benzaldehyde produced/min./mg. of protein)			Activity (% of that present in original mitochondrial fraction)			10 ³ × Specific activity (μmoles of cytochrome c reduced/min./mg. of protein)		
	Inner membrane		Outer membrane	Inner membrane	Outer membrane	Whole mitochondria	Inner membrane		Outer membrane	Inner membrane	Outer membrane	Whole mitochondria	Inner membrane		Outer membrane	Inner membrane		Outer membrane
	13.4	25.0	2.0	1.38	0.01	0.66	0.0	13.1	0	33.7	6.0	6.4	—	—	—	—	—	—
6	13.4	25.0	2.0	1.38	0.01	0.66	0.0	13.1	0	33.7	6.0	6.4	—	—	—	—	—	—
7	16.0	2.0	4.01	3.19	0.49	0.71	2.0	28.6	2.3	63.2	5.7	5.7	—	—	—	—	—	—
8	16.1	2.5	3.43	3.43	0.38	0.88	0.7	29.3	1.7	92.5	6.8	6.8	—	—	—	—	—	—
9	15.8	1.0	3.25	3.25	0.38	0.65	0.6	27.6	1.2	51.8	6.2	6.2	1.8	18.2	34	1.2	0.9	5.8
10	6.7	0.2	2.42	2.42	0.25	1.07	0.5	13.7	0.7	98.0	4.4	4.4	0.5	10.0	28	1.1	0.2	6.7
12	8.9	0.4	2.74	2.74	0.23	0.82	0.4	23.8	0.8	60.5	5.6	5.6	0.8	8.5	37	1.4	0.4	1.2
14	10.2	1.0	2.50	2.50	0.38	0.73	1.2	25.3	1.5	98.2	3.8	3.8	2.3	3.3	32	2.8	2.5	6.9
15										47.5	3.8	3.8	3.6	8.9	22	2.4	2.8	10.8

be a mixture of inner and outer membranes that was difficult to resolve by further fractionation; it was therefore discarded. The S₁I suspension was diluted severalfold with 0.01 M-K₂HPO₄-KH₂PO₄ buffer-0.1 mM-EDTA, pH 7.5, and centrifuged at 36 000 rev./min. for 60 min. in the Spinco 40 rotor. This pellet from the S₁I fraction, and the material that sedimented through the 37.7% (w/v) sucrose layer from both S₁ and S₂ fractions (S₁₊₂P), were each suspended with homogenization in 5 ml. of 25.5% (w/v) sucrose. The fractions (5 ml.) were layered separately on discontinuous gradients consisting of 5 ml. of each of 32.5% (w/v) sucrose, 37.7% (w/v) sucrose and 51.3% (w/v) sucrose (all in 0.01 M-K₂HPO₄-KH₂PO₄ buffer-0.1 mM-EDTA, pH 7.5) in Spinco SW 25 tubes. The tubes were centrifuged at 22500 rev./min. in the Spinco SW 25 rotor for 2 hr. The bulk of the protein and monoamine oxidase activity from the S₁I fraction accumulated at the interface between the 25.2% and the 32.5% sucrose layers; this interfacial fraction was collected and designated the outer-membrane fraction. The bulk of the protein and cytochrome c oxidase activity from the S₁₊₂P fraction sedimented at the interface between the 37.7% and 51.3% sucrose layers; this fraction was collected and designated the inner-membrane fraction.

An analysis of the membrane fractions, in comparison with the original mitochondria, and the microsomal fractions showed a considerable enrichment with respect to marker-enzyme activities (Table 1). Thus the purified outer-membrane fraction contained 13.48% of the total mitochondrial monoamine oxidase, and showed a six- to 18-fold enrichment based on specific activity. The purified inner-membrane fraction accounted for 10–15% of the cytochrome c oxidase activity from the mitochondria, and showed a three- to seven-fold enrichment, based on specific activity. Despite the double gradient fractionation the outer-membrane fraction showed some cross-contamination with inner-membrane fragments, as revealed by both the activity of cytochrome c oxidase and the presence of inner-membrane sub-units on some of the membranes as seen by electron microscopy after negative staining. The cross-contamination of the inner-membrane fraction with outer membranes was less extensive. The major problem in interpretation of ³²P-labelling experiments was the rather high contamination of the outer-membrane fractions with microsomal membranes, as indicated by NADPH-cytochrome c reductase activity (Table 1). A high proportion (10–15%) of the total NADPH-cytochrome c reductase activity of the mitochondrial fraction sedimented with the outer-membrane fraction, and the specific activity of this presumably microsomal marker enzyme in the outer-membrane fraction was greater than that for the total mitochondrial fraction. It is difficult to resolve mitochondrial outer membranes and smooth membranes from the microsomal fraction owing to their similar densities and sedimentation behaviour (Parsons *et al.* 1967). In the experiments reported in Fig. 2 the specific radioactivities of each of the phospholipids of the outer-mitochondrial-membrane fraction were corrected for the contamination due to microsomes, by using the values obtained for the NADPH-cytochrome c reductase activity, and assuming that the total-microsome fraction and the contaminating microsomes in the outer-membrane fraction had similar specific activities (O'Brien & Kalf, 1967).

Marker-enzyme assays. Cytochrome c oxidase activity was determined by measuring the decrease in *E*₅₅₀ of

cytochrome *c* that had previously been reduced by NaBH_4 (Sottocasa *et al.* 1967). The enzyme was assayed in very dilute solution so that the initial rate was linear with respect to enzyme concentration. Monoamine oxidase activity was determined by following the increase of E_{250} due to the formation of benzaldehyde from the substrate, benzylamine (Tabor, Tabor & Rosenthal, 1954). NADPH-cytochrome *c* reductase activity was determined spectrophotometrically by the procedure described by Sottocasa *et al.* (1967). Its activity did not change on incubation. This was in contrast with glucose 6-phosphatase, whose activity in both mitochondria and microsomes fell off rapidly on incubation at 37° . However, initial glucose 6-phosphatase assays showed the same microsomal contamination of mitochondria as was indicated by NADPH-cytochrome *c* reductase determinations. The spectrophotometric assays of both monoamine oxidase and NADPH-cytochrome *c* reductase were complicated by the high turbidity of concentrated microsomal and mitochondrial suspensions, and by non-specific changes in extinction due to swelling of the particles. In the early experiments these non-specific changes in extinction were minimized by subjecting all fractions to ultrasonic treatment under hypo-osmotic conditions before the addition of substrate. Later it was found that improved optical clearing of turbid suspensions was obtained by adding a non-ionic detergent, 0.015 ml. of 1% (w/v) Nonidet P40 (Shell Chemical Co. Ltd., London, W.C.2), to 0.05–0.10 ml. of the enzyme suspension. The final concentration of Nonidet P40 in the assay medium was 0.005%. Control studies showed that Nonidet P40 at this concentration, or the initially used procedure of ultrasonic treatment, did not affect the activity of the enzymes measured. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with a serum albumin standard.

Incubation conditions. The labelling of phospholipids in subcellular fractions *in vitro* was measured in either a tris-buffered or a phosphate-buffered medium. The tris-buffered medium contained 37.5 mM-tris-HCl buffer, pH 7.4, 10 mM-cysteine hydrochloride adjusted to pH 7.4, 20 mM- MgCl_2 and 0.17 M-sucrose, and was used where indicated in some experiments on the incorporation of CDP-[1,2- ^{14}C]choline (0.2 μmole , 0.028 μC in final volume 0.5 ml.). Although optimum incorporation of radioactivity from CDP-[1,2- ^{14}C]choline was obtained with this system it produced aggregation of particles, and the phosphate-buffered system was used in experiments where mitochondrial and microsomal fractions were isolated after the incubation. The phosphate-buffered medium contained 20 mM- KH_2PO_4 - K_2HPO_4 buffer, pH 7.4, 20 mM-sodium pyruvate, 1.3 mM-sodium malate, 5 mM- MgCl_2 , 1.7 mM-ATP, 0.25 mM-CTP, 0.1 mM- CoA , 1.7 mM-CMP, 0.17 M-sucrose and [*Me*- ^{14}C]choline (5 μmoles , 0.5 μC), [2- ^{14}C]ethanolamine (5 μmoles , 0.5 μC), or [^{14}C]inositol (2.5 μmoles , 0.25 μC), in final volume 1.5 ml. In the experiments with inorganic [^{32}P]phosphate (15 μmoles , 50 μC in final volume 1.5 ml.) the CMP was omitted, and 1.7 mM-choline, 1.7 mM-ethanolamine, 1.7 mM-inositol, 2 mM- NaF and 2 mM-EDTA were added. Samples were incubated at 37° with shaking for the times indicated in the text.

Fractionation after incubation. When the incubated samples were separated into mitochondrial and microsomal fractions the reaction was stopped by immersing the samples in an ice bath and adding 5–10 vol. of cold 0.25 M-sucrose–

0.1 mM-EDTA, pH 7.4. The samples were centrifuged immediately at 6000 rev./min. for 10 min. followed by 9300 rev./min. for 2 min. in the Servall SS-34 rotor to sediment the mitochondria. The supernatant was centrifuged at 36000 rev./min. for 90 min. in the Spinco 40 rotor to yield the microsomal pellet. The mitochondrial pellet was washed by resuspension in 0.25 M-sucrose–0.1 mM-EDTA, pH 7.4, and centrifugation at 6000 rev./min. for 10 min. The washing procedure was repeated twice with the centrifugation time decreased to 5 min.

Phospholipid analyses. A sample of each of the fractions from the experiments *in vivo* was extracted overnight at 0° with 20 vol. of chloroform-methanol (2:1, v/v) and the extract filtered. The tissue residue was re-extracted for 30 min. at room temperature with 10 vol. of chloroform-methanol (2:1, v/v) and filtered. The pooled filtrate was evaporated almost to dryness, and the residue was extracted with 20 ml. of chloroform-methanol (2:1, v/v) + 4 ml. of aq. 0.9% NaCl. The biphasic system was warmed, shaken vigorously and transferred to a centrifuge tube. After centrifugation, the upper phase was removed and discarded, and the lower phase was washed by shaking it three times with 8 ml. of chloroform-methanol-aq. 0.9% NaCl (3:48:47, by vol.). In the experiments *in vitro* the entire sample (1.5 ml.) was extracted overnight at 0° with 12 ml. of chloroform-methanol (1:1, v/v) and filtered through glass wool into a centrifuge tube. The residue was re-extracted for 30 min. at room temperature with 6 ml. of chloroform and filtered, and 2.1 ml. of aq. 0.9% NaCl was added to the combined filtrates. After being mixed thoroughly the two phases were separated by centrifuging, and the upper phase was discarded. The lower phase was washed three times with upper-phase solvent containing carrier chloroform-methanol-aq. 0.9% NaCl (3:48:47 by vol.) containing 2 mM-choline, ethanolamine or inositol, or 10 mM- KH_2PO_4 , depending on the labelled precursor used. This was followed by three washes with chloroform-methanol-aq. 0.9% NaCl (3:48:47, by vol.) to remove the carrier. Samples of the washed lipid extracts from experiments with ^{14}C -labelled precursors were evaporated to dryness in scintillation vials, and suspended with shaking in 10 ml. of scintillator solution containing 4 g. of 2,5-diphenyloxazole and 0.1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of toluene. Samples were counted in a Packard automatic liquid-scintillation spectrometer (model 2311). The lipid extracts from ^{32}P -labelling experiments were evaporated to dryness and the residue was digested with 1 ml. of 72% (w/v) HClO_4 (with caution). The digest was made up to 10 ml. and counted in an M-6 Geiger-Müller liquid-counting tube. Inorganic phosphate was determined on the same samples by using the procedure of Bartlett (1959). For the determination of radioactivity in individual phospholipids the samples were treated with ethanolic NaOH , and the ethanolsate was subjected to paper chromatography followed by ionophoresis (Dawson, Hemington & Davenport, 1962). The areas on the paper containing water-soluble degradation products were detected by radioautography and by ninhydrin and phosphate spray reagents. The spots were cut out and digested with 72% HClO_4 , and the radioactivity and inorganic phosphate were determined as described above.

Materials. [*Me*- ^{14}C]Choline (51.8 mc/m-mole), [2- ^{14}C]ethanolamine (4.8 mc/m-mole), [^{14}C]myoinositol (0.01 mc/0.05 mg.) and inorganic [^{32}P]phosphate ('carrier-free')

Fig. 1. Specific radioactivities of liver microsomal and mitochondrial phospholipids after the intravenous injection of [32 P]phosphate. [32 P]Phosphate (400 μ C) was injected into the femoral vein of 200 g. rats. (a) $\circ$, Mitochondrial phosphatidic acid; $\bullet$, microsomal phosphatidic acid; $\blacktriangle$, liver total acid-soluble phosphate. (b) $\circ$, Mitochondrial phosphatidylethanolamine; $\bullet$, microsomal phosphatidylethanolamine. (c) $\circ$, Mitochondrial phosphatidylinositol; $\bullet$, microsomal phosphatidylinositol. (d) $\circ$, Mitochondrial phosphatidylcholine; $\bullet$, microsomal phosphatidylcholine; $\square$, mitochondrial cardiolipin.

were obtained from The Radiochemical Centre, Amersham, Bucks. CDP-[1,2- 14 C]₂choline (0.14 mc/m-mole) was synthesized as described by McMurray (1964). Other chemicals were obtained as follows: cytochrome c from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; NADPH and ATP from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; CTP, CMP and CoA from Sigma Chemical Co., St Louis, Mo., U.S.A. 1,2-Diglyceride was prepared from o-oleicthoin by using *Clostridium perfringens* phospholipase C and separating the product by gradient elution from a silicic acid column with diethyl ether in light petroleum.

RESULTS

Turnover of the individual phospholipids of rat liver organelles in vivo

Turnover 0–1 hr. after intravenous injection. When [32 P]phosphate was injected into the femoral vein of rats, the specific radioactivity of the acid-soluble phosphate pool of the liver reached a maximum within 10 min. and then rapidly declined (Fig. 1a). This decrease presumably reflects a fall of the specific radioactivity of the plasma inorganic phosphate due to tissue exchange, and the exchange of the [32 P]phosphate pulse already in the liver pool with acid-insoluble compounds in the organ. The phosphatidic acid rapidly became labelled, that present in the mitochondria reaching a maximal specific radioactivity in approx. 15 min. The curve of specific radioactivity against time for mitochondrial phosphatidic acid showed a classical

product-precursor relationship (Zilversmit, Entenman & Fishler, 1943) with the acid-soluble phosphate pool (Fig. 1a). Although the incorporation of 32 P into the microsomal phosphatidic acid was also rapid, it appeared to reach a maximum somewhat more slowly than the mitochondrial phosphatidic acid. This is in contrast with the other phosphoglycerides, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol, whose specific radioactivity in the microsomal component always increased more rapidly than that in the mitochondria (Figs. 1b, 1c and 1d). The 32 P-labelling of the cardiolipin in the mitochondria was negligible compared with that of the other phosphoglycerides (Fig. 1d).

Turnover 0–4 hr. after intraperitoneal injection. The labelling of the phospholipids of liver organelles was much greater after the intraperitoneal injection of inorganic [32 P]phosphate. A comparison of the 1 hr. specific-radioactivity values with the corresponding values obtained after the intravenous injection show that the former were 5–10 times greater, even though the dose of [32 P]phosphate injected/100 g. body wt. was the same (Figs. 1 and 2). This is most probably a reflection of the specific radioactivity of the precursor acid-soluble phosphate pool, which was 3730 counts/min./μg. of P after the intraperitoneal injection compared with only 470 counts/min./μg. of P after the intravenous injection.

The rate of increase of the specific radioactivities of the individual phospholipids showed the same

Fig. 2. Specific radioactivities of rat liver microsomal phospholipids and inner- and outer-mitochondrial-membrane phospholipids after the intraperitoneal injection of inorganic [^{32}P]phosphate (0–4 hr.). [^{32}P]Phosphate (500 μC) was injected into 250 g. rats. ●, Microsomes; ○, whole mitochondria; □, inner mitochondrial membrane; ▲, outer mitochondrial membrane (corrected for microsomal contamination). (a) Top four curves, phosphatidylethanolamine; bottom curve, phosphatidylserine. (b) Phosphatidylcholine. (c) Top four curves, phosphatidylinositol; bottom curve, cardiolipin.

sequence as after intravenous injection (Figs. 2a, 2b and 2c). As after intravenous injection, the rate of labelling of phosphatidylinositol initially exceeded that of the phosphatidylcholine in the mitochondria and declined after 1 hr. so that the specific radioactivity of phosphatidylcholine was greater than that of phosphatidylinositol at longer times. One explanation of the time-course of labelling of phosphatidylinositol after intravenous (Fig. 1c) and intraperitoneal (Fig. 2c) injection could be that the organelles contain a small phosphatidylinositol pool, which is rapidly labelled with ^{32}P . Most of the phosphatidylinositol would then be labelled at a rate less than the rate of labelling of phosphatidylcholine (Figs. 2b and 2c). The slower increase in labelling of phosphatidylinositol observed in both series of experiments after the initial fast labelling is not due to the whole of this phospholipid pool achieving isotopic equilibrium with the acid-soluble phosphate pool: in fact, in Fig. 2 the specific radioactivity of the pool fell after 1 hr. (1 hr., 3730 counts/min./ $\mu\text{g. of P}$; 2 hr., 2590; 4 hr., 2524). Phosphatidylserine, which was present in appreciable concentrations only in the microsomes (Table 2), was labelled with ^{32}P only very slowly (Fig. 2a) but more rapidly than mitochondrial cardiolipin, which, as after intravenous injection, again showed a very slow incorporation of ^{32}P .

The increased extent of incorporation after the intraperitoneal injection of [^{32}P]phosphate enabled the labelling of the individual phospholipids in the

inner and outer membranes of mitochondria to be examined. The specific radioactivities of the outer membranes were corrected for microsomal contamination by measuring the NADPH-cytochrome c reductase activities in both the microsomes and the outer membranes. With all the phospholipids examined, i.e. phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, the specific radioactivities were in the order: microsomes > outer mitochondrial membranes > inner mitochondrial membranes (Fig. 2). The low concentrations of cardiolipin in outer mitochondrial membranes and microsomes (Table 2) precluded an accurate determination of its specific radioactivity; the inner-membrane cardiolipin had practically the same specific radioactivity as that in the whole mitochondria plotted in Fig. 2(c). Again the low concentration of phosphatidylserine in mitochondria prevented any measurement of its specific radioactivity.

Table 2 shows the distribution of the individual phosphoglycerides in the microsomes and inner and outer mitochondrial membranes. In confirmation of the results of Parsons *et al.* (1967) the inner membranes could be distinguished from the outer membranes by their much higher content of cardiolipin and lower content of phosphatidylinositol. The phospholipid composition of the outer membrane appeared to be much closer to that of the microsomes.

Turnover 4 hr.–22 days after intraperitoneal

Table 2. *Distribution of phosphoglycerides in rat liver microsomes, whole mitochondria and mitochondrial fragments*

Values are expressed as percentages of alkali-labile phospholipid phosphate; the ranges for six determinations are given in parentheses, followed by the means.

Phosphoglyceride	Content of each phosphoglyceride in fraction (% of total phospholipid in the fraction)			
	Fraction ...	Microsomes	Whole mitochondria	Inner mitochondrial membrane Outer mitochondrial membrane
Phosphatidylethanolamine	(16.4–20.6)	18.1	(25.2–32.6) 29.6	(20.4–31.7) 26.0 (18.1–29.7) 24.1
Phosphatidylcholine	(58.0–62.7)	59.9	(34.9–45.8) 42.0	(43.8–51.7) 46.6 (49.7–54.2) 51.6
Phosphatidylserine	(3.3–4.4)	3.9	(0.4–1.4) 0.8	(0.3–2.0) 0.9 (1.8–3.0) 2.3
Phosphatidylglycerol	(3.4–8.5)	6.0	(3.0–5.0) 4.0	(1.2–4.3) 2.1 (1.6–3.2) 2.6
Phosphatidic acid	(0.8–1.6)	1.2	(0.5–1.2) 0.9	(0.2–1.2) 0.7 (1.2–2.1) 1.3
Cardiolipin	(0.5–1.4)	0.8	(16.1–24.8) 19.0	(16.3–20.1) 17.9 (3.4–4.3) 3.6
Phosphatidylinositol	(8.7–10.8)	10.1	(5.3–6.4) 6.0	(5.1–8.0) 6.0 (12.1–15.5) 13.1

Fig. 3. Specific radioactivities of the phospholipids of microsomes and mitochondrial fractions of rat liver after intraperitoneal injection of inorganic [³²P]phosphate (4 hr.–22 days). [³²P]Phosphate (500 μc) was injected intraperitoneally into female 250 g. rats. ○, Whole mitochondria; □, mitochondrial inner membrane; △, mitochondrial outer membrane; ●, microsomes. (a) —, Phosphatidylcholine ——— cardiolipin. (b) Phosphatidylethanolamine; ▲, acid-soluble phosphate. (c) Phosphatidylinositol. Where superimposition of values occurs at a given time value, the points are inserted at the side for clarity.

injection. Fig. 3 shows the decline of the specific radioactivities of individual phospholipids in the various membrane fractions at longer periods after the intraperitoneal injection of [³²P]phosphate. The specific radioactivities of phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol showed an almost identical rate of decrease (Figs. 3a, 3b and 3c) with no evidence of a persistence of labelling in outer or inner mitochondrial membranes, whole mitochondria or microsomes. In fact the rate of decline was almost a replicate of the fall in the specific radioactivity of the liver acid-soluble phosphate pool (Fig. 3b). The phospholipids reached 'isotopic equilibrium' with this pool in 1 day and then their specific radio-

activities fell according to the general 'biological' turnover of the precursor acid-soluble phosphate pool. The exception was mitochondrial cardiolipin, the specific radioactivity of which, as could have been predicted from the short-term experiments, took several days to reach a maximum and then declined rather less rapidly than those of the other phospholipids (Fig. 3a).

Labelling of the phospholipids of isolated liver organelles

Labelling of the phospholipids of isolated mitochondria with ¹⁴C-labelled inositol, ethanolamine and choline. When liver mitochondria were incubated

Table 3. *Incorporation of labelled inositol, ethanolamine and choline into phospholipids by rat liver mitochondria*

The mitochondria (equivalent to 0.5 g. of liver) were incubated for 1 hr. at 37° in phosphate buffer with the labelled phospholipid precursors indicated. Results are expressed as specific radioactivities (counts/min./μg. of lipid P), with total radioactivities incorporated (counts/min.) in parentheses.

Incubation conditions	Precursor added ...	Specific and total radioactivity		
		[U- ¹⁴ C]Inositol (0.25 μC, 2.5 μmoles)	[2- ¹⁴ C]Ethanolamine (0.5 μC, 5 μmoles)	[Me- ¹⁴ C]Choline (0.5 μC, 5 μmoles)
Complete system		5.0 (345)	10.4 (625)	42.0 (2570)
No CTP		5.5 (360)	9.2 (550)	54.3 (3300)
No CMP		1.6 (150)	8.2 (490)	37.3 (2280)
No CTP or CMP		0.2 (70)	6.2 (370)	15.5 (970)
No CoA		4.5 (310)	12.2 (730)	18.8 (1170)
No ATP, malate or pyruvate; + 0.3 mM-KCN		5.5 (380)	5.2 (310)	19.3 (1200)
Complete system + microsomes (≅ 0.5 g. of liver)		35.2 (4780)	12.4 (1860)	48.5 (7280)
Complete system + supernatant (≅ 30 mg. of liver)		19.3 (1130)	30.0 (1800)	48.2 (2890)
Complete system + microsomes (≅ 0.5 g. of liver) + supernatant (≅ 30 mg. of liver)		51.2 (7710)	53.7 (9660)	72.0 (12960)

with radioactive inositol, ethanolamine or choline, the phospholipids subsequently isolated from the organelles were substantially labelled (Table 3). On alkaline degradation of the phospholipid fraction (Dawson *et al.* 1962) all the radioactivity recovered in the water-soluble phosphate esters was present in glycerylphosphorylinositol, glycerylphosphoryl-ethanolamine or glycerylphosphorylcholine, depending on the radioactive precursor used, and cross-labelling was not apparent. However, all the labelling did not appear to be through energy-dependent pathways, since in the absence of ATP and oxidizable substrate and in the presence of cyanide about 50% of the original labelling still occurred. Cytidine nucleotides (CTP or CMP) were only essential for about half the incorporation from labelled choline and ethanolamine; CMP but not CTP was required for optimum inositol incorporation. The results indicate that part of the incorporation under these conditions was due to exchange reactions rather than synthesis of the phospholipid molecule from its individual molecular moieties. In addition, with [¹⁴C]choline labelling, about half of the incorporation appeared to be into a substance that migrated on thin-layer chromatography to a position equivalent to that of palmitoylcholine rather than phosphatidylcholine. The synthesis of this compound (Kennedy, 1956; Berry & Whittaker, 1959) could account for the partial requirement for CoA for the choline incorporation that was not observed with ethanolamine or inositol. Addition of the microsomal and supernatant fractions greatly stimulated the incorporation, particularly with inositol and ethanolamine.

Labelling of the phospholipids of isolated liver

organelles with [³²P]phosphate. When inorganic [³²P]phosphate was incubated with isolated mitochondria there was a substantial incorporation of label into the phospholipid fraction, this being due to synthesis mainly of phosphatidic acid and to a smaller extent of phosphatidylinositol (Table 4). The remainder of the phosphoglycerides, including the nitrogen-containing phospholipids that comprise the bulk of the fraction, showed negligible labelling. On the addition of either supernatant or microsomes to the mitochondria, there was a markedly increased incorporation of ³²P into the phospholipid fraction. Phosphatidic acid was again the most heavily labelled, but significant labelling of the other phosphoglycerides also occurred. When both microsomes and supernatant were incubated with the mitochondria, the phosphatidic acid isolated from the system still had the maximum specific radioactivity, but more radioactivity was incorporated into phosphatidylethanolamine and there was also substantial labelling of phosphatidylcholine and phosphatidylinositol. As in the studies *in vivo*, cardiolipin was not labelled to any extent.

In a further experiment isolated mitochondria together with supernatant and various amounts of microsomes were incubated with [³²P]phosphate. The labelling of the individual phospholipids in terms of total radioactivity incorporated is plotted against the microsomal component in Fig. 4. The first point plotted is that for mitochondria with no added microsomes and represents the intrinsic microsomal contamination of the mitochondria, calculated by NADPH-cytochrome *c* reductase assays on the mitochondria and microsomes. For phosphatidylethanolamine and phosphatidyl-

Table 4. Incorporation of [32 P]phosphate in vitro into the individual phospholipids of rat liver mitochondria and microsomes

Mitochondria (equivalent to 0.5 g. of liver), alone or with microsomes (equivalent to 0.5 g. of liver) or supernatant (equivalent to 60 mg. of liver), were incubated for 60 min. at 37° in phosphate buffer (standard medium) supplemented with choline hydrochloride (1.7 mM), ethanolamine hydrochloride (1.7 mM), inositol (1.7 mM), NaF (2 mM), EDTA (2 mM) and 50 μ C of [32 P]phosphate; CMP inhibited and was omitted. Results are expressed as specific radioactivities (counts/min./ μ g. of P) with total radioactivities incorporated (counts/min.) in parentheses.

Phospholipid	Specific and total radioactivity			
	Fractions ... Mitochondria	Mitochondria + supernatant	Mitochondria + microsomes	Mitochondria + microsomes + supernatant
Total phospholipid	14.9 (1664)	31.7 (3490)	31.3 (5400)	89.3 (17600)
Phosphatidylcholine	0.1 (2)	6.7 (205)	1.7 (102)	28.4 (1580)
Phosphatidylethanolamine	0.2 (5)	10.0 (252)	0.5 (16)	199 (6690)
Phosphatidylinositol	13.8 (90)	49.3 (346)	54 (830)	120 (1800)
Phosphatidylglycerol	24.5 (17)	113 (84)	30 (27)	52 (85)
Phosphatidylserine	— (4)	— (26)	— (4)	10 (14)
Cardiolipin	1.1 (17)	3 (45)	5 (61)	2 (32)
Phosphatidic acid	1000 (1115)	3070 (1810)	3390 (3070)	2030 (3430)

Fig. 4. Incorporation of 32 P into the phospholipids of a liver mitochondrial preparation containing various amounts of added microsomes. Each tube contained mitochondria (equivalent to 1.0 g. of tissue) and supernatant (equivalent to 240 mg. of tissue). The microsomal suspension was equivalent to 2.5 g. of tissue/ml. The first point plotted on each curve represents no added microsomes, the microsomal contamination of the mitochondria being assessed by NADPH-cytochrome c reductase assay. The incubation medium (phosphate buffer) was as described in the Methods section; tubes were incubated at 37° for 60 min. with inorganic [32 P]phosphate (100 μ C). O, Phosphatidylinositol; ●, phosphatidylethanolamine; ▲, phosphatidic acid; □, phosphatidylcholine.

inositol there was a linear relationship between the incorporation and the quantity of microsomal component, whereas the incorporation into phosphatidylcholine was initially linear but then fell at higher microsomal concentrations. Each curve cut the origin or the abscissa near the origin when extrapolated back to 'zero' microsomal concentration (Fig. 4). This result indicates that the small labelling of these three phospholipids by isolated liver mitochondria can be attributed entirely to the residual microsomal enzyme systems contaminating the mitochondrial preparation. In contrast, there was evidence that mitochondria can synthesize phosphatidic acid from [32 P]phosphate in the complete absence of microsomes; in fact the incorporation initially fell on the addition of microsomes to the system (Fig. 4).

Liver microsomes, mitochondria and supernatant fraction were incubated with [32 P]phosphate for various times, and the fractions were reisolated from the incubation medium. Fig. 5 shows that under these conditions, although the microsomal phospholipids were the most heavily labelled, there was a substantial incorporation of 32 P into the mitochondrial phospholipids. Since the results shown in Fig. 4 indicated that mitochondria were incapable of synthesizing phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol from added [32 P]phosphate *in vitro*, this suggested that a transfer of phospholipids between microsomes and mitochondria had occurred. However, the time-sequence of the labelling for each individual phospholipid fraction in microsomes and mitochondria (Fig. 5) gave no evidence for this,

Fig. 5. Labelling of the phospholipids of liver mitochondria and microsomes on incubation with [^{32}P]phosphate and a liver supernatant fraction. Mitochondria (equivalent to 1.25 g. of liver), microsomes (equivalent to 1.25 g. of liver) and supernatant (equivalent to 120 mg. of liver) were incubated in supplemented phosphate buffer (see Table 4) containing [^{32}P]phosphate (200 μC). After the incubation the mitochondria and microsomes were separated and the phospholipids extracted as described in the Methods section. (a) $\circ$, Mitochondrial phosphatidylcholine; $\bullet$, microsomal phosphatidylcholine. (b) $\circ$, Mitochondrial phosphatidylethanolamine; $\bullet$, microsomal phosphatidylethanolamine. (c) $\circ$, Mitochondrial phosphatidylinositol; $\bullet$, microsomal phosphatidylinositol.

unless the rate of transfer was falling off rapidly with time in the system *in vitro*.

Phospholipid labelling in rat liver organelles from CDP-[1,2- $^{14}\text{C}_2$]choline. When mitochondria were incubated with radioactive CDP-choline, the phospholipid labelling (shown to be entirely in the phosphatidylcholine fraction) was not enhanced by the presence of supernatant (Table 5). As expected, the addition of microsomes to the system produced a greatly increased synthesis of phosphatidylcholine. When the mitochondria were fragmented by ultrasonic treatment, incorporation was decreased in all experiments, even though it is to be expected that any permeability barrier against CDP-choline entering the mitochondria would have been diminished. A number of experiments were carried out in which mitochondria alone or with various amounts of added microsomes were incubated with CDP-[^{14}C]choline. The total incorporation of ^{14}C into the phospholipids plotted against microsomal complement (Fig. 6a) was linear, and on extrapolation to 'zero' microsomal concentration indicated that mitochondria uncontaminated with

microsomes would not synthesize phosphatidylcholine.

Since the incorporation of labelled CDP-choline into the phospholipids of liver particles is greatly enhanced by the addition of an appropriate diglyceride acceptor (Weiss, Smith & Kennedy, 1958), the dependence of mitochondrial phosphatidylcholine labelling on the presence of microsomes in the present experiments might be attributed to a limited ability of the mitochondria to generate diglycerides. Preliminary experiments showed that the addition of synthetic 1,2-diolein or mixed diglycerides prepared by the action of phospholipase C on egg lecithin markedly stimulated (20–30-fold) the incorporation of CDP-[^{14}C]choline in both the mitochondrial fraction and the microsomal fraction. The effect of additions of various amounts of microsomes on the incorporation of CDP-[^{14}C]choline in the presence of constant amounts of diglyceride and mitochondria is shown in Fig. 6(b). As in the experiments in Fig. 6(a) the incorporation was proportional to the microsome concentration, and extrapolation to zero microsome

Table 5. Incorporation of radioactivity from CDP-[1,2-¹⁴C₂]choline into rat liver mitochondrial and microsomal phospholipids

Whole mitochondria (equivalent to 0.25 g. of liver), mitochondrial fragments (treated ultrasonically at 0° for 3 min. in a Mullard-MSE 60 w instrument) or microsomes were incubated alone or with various other cell fractions in tris buffer containing CDP-[1,2-¹⁴C₂]choline (28 μμc; 0.2 μmole) at 37° for 60 min.

	Incorporation into mitochondria or microsomes		Incorporation into mitochondria or microsomes	
	Specific radioactivity (counts/min./μg. of P)	Total radioactivity incorporated (counts/min.)	Specific radioactivity (counts/min./μg. of P)	Total radioactivity incorporated (counts/min.)
Mitochondria	80	1730	32	745
Mitochondria + supernatant (≡ 50 mg. of liver)	73	1640	27	520
Mitochondria + microsomes (≡ 12.5 mg. of liver)	123	2910	138	3110
Microsomes (≡ 125 mg. of liver)	632	11 100	450	6290

Fig. 6. Incorporation of radioactivity from CDP-[1,2-¹⁴C₂]choline into a liver mitochondrial preparation containing various amounts of added microsomes. (a) Each tube contained mitochondria equivalent to 0.25 g. of tissue. The microsomal suspension was equivalent to 2.5 g. of tissue/ml. Each line of points represents a separate experiment. The first point plotted in each series represents no added microsomes, the microsomal contamination of the mitochondria being assessed by NADPH-cytochrome c reductase assay. The incubation medium (tris buffer) was as described in the Methods section; tubes were incubated at 37° for 120 min. in medium containing CDP-choline (28 μμc, 0.2 μmole). (b) Each tube contained mitochondria equivalent to 0.1 g. of tissue. The microsomal suspension was equivalent to 1.0 g. of tissue/ml. Each point is the average of duplicate determinations. Diglyceride (1.25 μmoles) prepared from egg lecithin and emulsified by ultrasonic treatment was added to each tube in 0.05 ml. of 1% Tween 20; the tubes were incubated at 37° for 30 min. Other conditions of incubation were as in (a).

contamination indicated that there would be no incorporation in uncontaminated mitochondria. Thus the dependence of mitochondrial incorporation of CDP-[¹⁴C]choline on the presence of microsomes is not simply attributable to provision of diglycerides for mitochondrial phosphatidylcholine synthesis.

Phospholipid exchange processes between isolated mitochondria and microsomes. The unambiguous labelling of phosphatidylcholine with CDP-

[1,2-¹⁴C₂]choline was used to examine the possibility of exchange reactions between the phosphatidylcholine of microsomes and that of mitochondria. In initial experiments microsomes together with supernatant were incubated with labelled CDP-choline, and mitochondria were added either at the start or at the end of the incubation. The mitochondria and microsomes were again separated and the labelling in their phospholipids was examined. When mitochondria were added at

Table 6. *Incorporation of radioactivity into rat liver microsomal and mitochondrial phospholipids on incubation with CDP-[1,2-¹⁴C]choline*

Microsomes (equivalent to 0.25 g. of liver) + supernatant (equivalent to 0.1 g. of liver) were incubated in a phosphate buffer containing CDP-[1,2-¹⁴C]choline (Expt. 1: 56 μ C; 0.4 μ mole; Expts. 2 and 3: 28 μ C, 0.2 μ mole), and mitochondria (equivalent to 0.5 g. of liver) and unlabelled CDP-choline (2 μ moles) were added at the times indicated. After completion of the incubation the mitochondria and microsomes were separated and the phospholipids extracted. Microsomal contamination of mitochondria was assessed by NADPH-cytochrome *c* reductase assays.

Expt. no.	Time of changes in incubation conditions after start of incubation (min.)				Microsomal phospholipid			Mitochondrial phospholipid				Total radioactivity recovered in mitochondrial and microsomal phospholipids (counts/min.)
	Microsomes added	Mitochondria added	Unlabelled CDP-choline added	Incubation stopped	Total radioactivity incorporated (counts/min.)	Specific radioactivity (counts/min./ μg. of P)	Total radioactivity incorporated (counts/min.)	Specific radioactivity (counts/min./ μg. of P)	Maximum radioactivity that could be due to microsomal contamination (counts/min.)			
1	0	60	—	60	23 160	795	780	20	1350	23 940		
	0	0	—	60	16 040	663	8220	224	1210	24 260		
2	0	60	—	60	13 750	583	1050	45.5	1450	14 800		
	0	0	—	60	10 330	423	4100	127	1740	14 430		
3	0	60	60	60	15 850	370	330	11	500	16 180		
	0	60	60	120	12 100	381	5060	129	1100	17 160		

the end of the incubation, their phospholipid labelling could entirely be accounted for by microsomal contamination as determined by NADPH-cytochrome *c* reductase assay (Table 6). When mitochondria were added from the beginning of the incubation, their phospholipid fraction showed substantial labelling that could not be ascribed to microsomal contamination (Table 6, Expts. 1 and 2). Since the results reported in Figs. 4 and 6 had indicated that mitochondria are not capable of synthesizing phosphatidylcholine, this result suggested that phosphatidylcholine molecules were being transferred from the microsomal fraction to the mitochondria during the incubation. The increase in labelling of the mitochondrial fraction was in each case accompanied by a corresponding decrease in the total radioactivity of the microsomal fraction. In a variation of this type of experiment (Table 6, Expt. 3) microsomal phospholipids were labelled by incubation with radioactive CDP-choline and then subsequent labelling was blocked by the addition of an excess of unlabelled CDP-choline. Mitochondria were added to the system and then recovered by centrifuging either immediately or after 60 min. incubation with the labelled microsomes. Again on incubation the mitochondrial phospholipids became labelled to an extent that could not be accounted for by any direct microsomal contamination (Table 6), in spite of the fact that further synthesis of phosphatidyl-[¹⁴C]choline in the period 60–120 min. was effectively blocked by the dilution of the labelled CDP-choline, as indicated by the total radioactivity incorporated into mitochondrial and microsomal phospholipids.

In further experiments the time-sequence and the reversibility of the exchange were examined. Isolated mitochondria and microsomes were mixed and incubated with labelled CDP-choline. The labelled mitochondria and microsomes were isolated and incubated separately for various times with the opposite unlabelled membrane fraction together with unlabelled supernatant. The fractions were subsequently reisolated and their phospholipids assayed for radioactivity. When the labelled microsomes were incubated with unlabelled mitochondria, there was a rapid loss of radioactivity from the microsomal fraction and a corresponding increase in the labelling of the mitochondrial phospholipids (Fig. 7a). After 2 hr. about 40% of the radioactivity had been incorporated into the mitochondria, but there was little evidence of any equivalent transfer of the microsomal marker enzyme, NADPH-cytochrome *c* reductase, to the mitochondrial fraction (Fig. 7a). The same phenomenon was observed in the opposite direction when labelled mitochondria were incubated with unlabelled microsomes. The microsomal fraction acquired some of the labelled phospholipid from the

Fig. 7. Incubation of phosphatidyl[¹⁴C]choline-labelled microsomes with unlabelled mitochondria and vice versa. The mitochondrial (equivalent to 0.5 g. of liver) and microsomal fractions (equivalent to 0.25 g. of liver) were isolated from rat liver, mixed and incubated with CDP-[1,2-¹⁴C₂]choline (28 mμC, 0.2 μmole) as described in the Methods section (phosphate buffer medium). Each of the two labelled fractions was then isolated and incubated independently with the unlabelled supernatant and opposite membrane fraction. (a) Labelled microsomes + unlabelled mitochondria + unlabelled supernatant. (b) Labelled mitochondria + unlabelled microsomes + unlabelled supernatant. After incubation the microsomes and mitochondria were reisolated and assayed for radioactivity and enzymic activity. ○, Mitochondrial ¹⁴C; ●, microsomal ¹⁴C; ▲, microsomal NADPH-cytochrome c reductase; △, mitochondrial NADPH-cytochrome c reductase; □, mitochondrial cytochrome c oxidase; ■, microsomal cytochrome c oxidase.

mitochondria without any corresponding transfer of cytochrome c oxidase activity (Fig. 7b). Nor was there any increase in the monoamine oxidase activity of the reisolated microsomes, indicating that there had been no release of mitochondrial outer membranes into this fraction. The time-course of the curves indicates that after 2 hr. isotopic equilibrium between the phosphatidylcholine pools had almost been reached. During this reversible exchange of phospholipid there was no evidence of any significant change of concentration of the phosphatidylcholine contained in the recovered microsomes or mitochondria.

In further experiments the conditions necessary for the transfer of phosphatidylcholine from microsomes to mitochondria were investigated. Microsomes were labelled by incubation with CDP-[¹⁴C]choline and after being washed were incubated with unlabelled mitochondria. The mitochondria were reisolated and, after a washing, the radioactivity in their phospholipids was

measured. The transfer of phosphatidylcholine did not occur on incubation of the system at 0°, and omission of the supernatant or substitution of boiled supernatant decreased it dramatically (Table 7). On the other hand, there was no evidence of the need for added substrate, ATP or Mg²⁺.

The exchange of other phospholipids between microsomes and mitochondria was studied in the experiment shown in Table 8. Microsomal phospholipids were labelled by preincubation with [³²P]-phosphate in the presence of mitochondria, supernatant and the cofactors required for ³²P incorporation. On mixing of the labelled microsomes with unlabelled mitochondria and immediate reisolatation of the two fractions (0 min. values), the mitochondrial phospholipids had specific radioactivities 2–3% of those for the microsome fraction, or of the same order as the microsomal NADPH-cytochrome c reductase contamination of the mitochondria. After a 60 min. incubation of labelled microsomes with unlabelled mitochondria in the

Table 7. *Conditions necessary for the transfer of labelled phosphatidylcholine from microsomes to mitochondria*

Microsomes (equivalent to 0.25 g. of liver) were incubated with CDP-[1,2- ^{14}C]choline (28 m μC , 0.2 μmole) for 2 hr. at 37° in phosphate buffer. The microsomes were recovered by centrifuging, washed once in unlabelled medium and incubated with unlabelled mitochondria (equivalent to 0.8 g. of liver) and supernatant (equivalent to 0.1 g. of liver). The mitochondria were reisolated and washed as described in the Methods section.

Incubation conditions	Radioactivity incorporated into mitochondrial phospholipids (counts/min.)
No incubation	315
60 min. at 0°	300
60 min. at 37°	2135
60 min. at 37°; no pyruvate, malate or ATP	2535
60 min. at 37°; no Mg^{2+}	2365
60 min. at 37°; no liver supernatant	975
60 min. at 37°; liver supernatant boiled for 5 min.	1000

Table 8. *Exchange of ^{32}P -labelled microsomal phospholipids with mitochondrial phospholipids*

Microsomes (equivalent to 0.5 g. of liver), mitochondria (equivalent to 0.5 g. of liver) and supernatant (equivalent to 60 mg. of liver) were incubated together with 100 μC of [^{32}P]phosphate for 90 min. at 37° in the supplemented phosphate buffer described in Table 4. The labelled microsomes were recovered by centrifuging, washed once in unlabelled medium and incubated with unlabelled mitochondria (equivalent to 1.5 g. of liver) and supernatant (equivalent to 0.2 g. of liver) in 10 mM- KH_2PO_4 - K_2HPO_4 buffer, pH 7.4, at 37°. The mitochondria and microsomes were reisolated and washed as described in the Methods section. Values for phospholipids are expressed as specific radioactivities (counts/min./ μg . of P). Values for NADPH-cytochrome *c* reductase are expressed as $10^3 \times$ total activities (μmoles of cytochrome *c* reduced/min./fraction).

Incubation time	Specific radioactivity			
	0 min.		60 min.	
	Microsomes	Mitochondria	Microsomes	Mitochondria
Total phospholipid	545	10.3	397	78.3
Phosphatidylcholine	76.2	2.0	46.7	33.5
Phosphatidylethanolamine	1560	22.0	973	152
Phosphatidylinositol	213	5.3	122	116
Phosphatidic acid	733	12.5	392	48
Total NADPH-cytochrome <i>c</i> reductase activity	243	12.1	310	9.7

absence of the cofactors required for ^{32}P incorporation, the mitochondrial phospholipids showed considerable additional labelling, although the microsomal contamination of the reisolated mitochondria was not increased. The specific radioactivity of the microsomal phospholipids decreased during the incubation. Phosphatidylinositol and phosphatidylcholine of the two fractions approached isotopic equilibrium, whereas the exchange of phosphatidylethanolamine and phosphatidic acid appeared to be less rapid. In further experiments in which microsomal phospholipids were labelled by preincubation with [^{14}C]inositol or [^{14}C]ethanolamine the different rates of exchange of labelled phosphatidylinositol and phosphatidylethanolamine with mitochondrial phospholipids were confirmed. On incubation with

unlabelled mitochondria for various times the mitochondrial phosphatidylinositol acquired maximal radioactivity within 30 min., approaching isotopic equilibrium with the microsomes. The transfer of labelled phosphatidylethanolamine proceeded more slowly, so that at the end of 90 min. only approx. 25% of the total radioactivity had exchanged.

DISCUSSION

The present results strongly suggest that the limited capacity of rat liver mitochondria to synthesize nitrogen- and inositol-containing phosphoglycerides from [^{32}P]phosphate or phosphatidylcholine from CDP-[^{14}C]choline is dependent on the small microsomal component with which all mito-

chondrial fractions prepared by present methods are invariably contaminated. With both precursors the plots of total synthesis of individual phospholipids by mitochondria against 'added + intrinsic' microsome concentration are approximately linear and extrapolations back suggest that negligible phospholipid synthesis would occur in the complete absence of microsomes (Figs. 4 and 6). With phosphatidylcholine synthesis the component most likely to be missing from the mitochondria is cholinephosphotransferase, which Wilgram & Kennedy (1963) and Schneider (1963) showed to be predominantly microsomal. There is a possibility that the added CDP-[^{14}C]choline is unable to penetrate to 'sites' for phosphatidylcholine synthesis within the mitochondria, but this is rendered less likely by the lack of any stimulation produced by fragmenting of the mitochondria. Nor is the availability of diglyceride to the mitochondria likely to be a contributing factor, since the same results were observed in the presence of added diglyceride (Fig. 6b). Moreover, liver mitochondria are comparatively rich in neutral lipids including diglycerides (Getz, Bartley, Stirpe, Notton & Renshaw, 1962; Scherphof & van Deenen, 1966), and also contain a diglyceride-generating system, phosphatidate phosphohydrolase (Smith, Sedgwick, Brindley & Hübscher, 1967; Stoffel & Schiefer, 1968). The present results contrast with the experiments of Stoffel & Schiefer (1968), who found some CDP-choline-1,2-diglyceride cholinephosphotransferase in the outer mitochondrial membranes of rat liver. However, owing to the difficulty of solubilizing diglyceride the incorporation was rather low and the question of the contribution to synthesis by microsomal contamination was not specifically examined. Our results do not, of course, exclude the possibility of liver mitochondria being able to form phosphatidylcholine by processes that do not involve the synthesis of the whole phospholipid molecule. Such would include the methylation of phosphatidylethanolamine, the acylation of lysolecithin or the Ca^{2+} -activated incorporation of choline into the phospholipid fraction. Indeed, the present results show that isolated mitochondria can incorporate some labelled choline and ethanolamine into their lipid fractions by processes that do not appear to be energy-dependent, although this may again be a function of the contaminating microsomes. The results of Dils & Hübscher (1959) suggest that the Ca^{2+} -activated incorporation of choline into the phospholipids of liver homogenates is largely a function of the microsomal component, and our own results (Table 3) show a marked increase in the incorporation of choline when microsomes are added to a mitochondrial system. These results are in contrast with those of Kaiser & Bygrave (1968),

which suggest that the liver microsomes are somewhat inactive in incorporating labelled choline into phospholipids compared with liver mitochondria.

In contrast with the nitrogen-containing phosphoglycerides and phosphatidylinositol, the results of these same experiments (Fig. 4) clearly show that labelled phosphatidic acid can be formed by liver mitochondria incubated with [^{32}P]phosphate in the complete absence of microsomes. The synthetic pathway involved is not indicated, but both brain mitochondria (McMurray, Strickland, Berry & Rossiter, 1957) and liver mitochondria (Brandes, Olley & Shapiro, 1963; Wojtezak & Zborowski, 1967; Stoffel & Schiefer, 1968) contain a glycerol phosphate acyltransferase system. There is also a possibility that phosphatidic acid could be synthesized by diglyceride kinase, an enzyme known to occur in plant mitochondria (Bradbeer & Stumpf, 1960) and brain mitochondria (Strickland, 1962).

The present results showing that mitochondria can rapidly acquire labelled phospholipid from a microsomal preparation with which they are incubated, and vice versa, are in agreement with the concurrent experiments of Wirtz & Zilversmit (1968), who used [^{32}P]phosphate and [^{14}C]glycerol to follow the exchange. These workers also found, in agreement with our results, that the exchange was greatly stimulated by adding a supernatant fraction, and was almost abolished at 0° . The possibility that the mitochondria can synthesize labelled phosphatidylcholine from residual CDP-[^{14}C]choline in the microsomal fraction is rendered unlikely because of the known inability of mitochondria to use this substrate for phosphatidylcholine synthesis, discussed above. Moreover, Mg^{2+} , a known cofactor for choline phosphotransferase, is not required for the transfer, and transfer still occurs when the system is saturated with unlabelled CDP-choline. The exchange process is not limited to phosphatidylcholine; both phosphatidylinositol and phosphatidylethanolamine also appear to undergo exchange between the two fractions. The relative rates at which these exchanges took place (phosphatidylinositol > phosphatidylcholine > phosphatidylethanolamine) paralleled the rates at which the mitochondrial phospholipids approached isotopic equilibrium with those of the microsomes *in vivo* (Fig. 1) and *in vitro* (Fig. 5). Wirtz & Zilversmit (1968) also observed that phosphatidylcholine exchanged more rapidly between the two fractions than phosphatidylethanolamine.

As to the mechanism of the exchange it seems unlikely on physicochemical grounds that either membrane would liberate pure lipid micelles consisting partly or entirely of phospholipid, which would then diffuse to and react with the other type of membranes. Indeed, in general, phospholipid

micelles do not seem to react readily with mitochondria (Fleischer & Brierley, 1961) unless detergent is added to the system.

It has been suggested that, if intracellular transfers of phospholipid between membranes do occur, they could be carried out through the intermediary of a soluble lipoprotein or lipoprotein apoprotein, which carries the phospholipid through the cytoplasm (Dawson, 1966). It is indeed possible that the heat-labile supernatant factor that has been shown to enhance the phospholipid exchange reaction between microsomes and mitochondria could fulfil this role. Certainly exchanges between the phospholipids of blood-plasma lipoproteins and blood cells are well documented (e.g. Sakagami, Minari & Orii, 1965*a,b*), and evidence has been presented that there is an exchange of phospholipids between both the blood plasma and the cell sap and liver mitochondria; this exchange, like the present microsomal-mitochondrial exchange, is independent of the energy-generating metabolism of the mitochondria (Tarlov, 1968). There is evidence suggesting that labelled proteins from microsomes can also be transferred to rat liver mitochondria *in vitro* (Kadenbach, 1967), but unlike the phospholipid exchange this appears to require energy generation by the mitochondria.

Alternatively the supernatant factor could be fulfilling some other role and the mitochondria and microsomes could be forming a collision complex during the incubation in which the lowered energy barriers would allow phospholipids or whole lipoprotein moieties to exchange between the two membranes, a transfer that would be equivalent to the mechanism suggested for the phospholipid exchange reaction between 'soluble' plasma lipoproteins (Gurd, 1960). Whatever the mechanism of the exchange, it is clear that proteins and phospholipids cannot exchange indiscriminately and completely between the two membrane fractions. Thus the two membranes still maintain a different phospholipid composition even when the phosphatidylcholine exchange has practically achieved isotopic equilibrium. Further, there is no significant transfer of the marker enzymes, NADPH-cytochrome *c* reductase and cytochrome *c* oxidase, between the two membrane fractions (Fig. 7).

The question arises whether a similar intracellular exchange of phospholipids occurs in the intact animal. The distribution of the short-term (0-4 hr.) labelling of the individual liver phospholipids after intraperitoneal injection of [32 P]phosphate is consistent with the synthesis of phospholipids in the endoplasmic reticulum followed by an exchange between microsomes and mitochondria. Thus with all the phospholipids examined, apart from phosphatidic acid, the specific radioactivity of the microsomes is greater than that of the outer

membrane of the mitochondria (corrected for microsomal contamination) and this in turn is greater than that of the inner mitochondrial membrane. Gurr, Prottey & Hawthorne (1965) and Miller & Cornatzer (1966) also found that the specific radioactivity of rat liver phospholipids increased more rapidly in the microsomes than in the whole mitochondrial fraction. However, these experiments and also the labelling of phospholipids *in vivo* during the pulse-labelling of the liver acid-soluble phosphate pool by the intravenous injection give a time-sequence of incorporation that is not consistent with a simple precursor-product relationship between the phospholipid fractions of the two membranes. Thus one might expect the incorporation of radioactivity into mitochondrial phospholipids to show a lag phase relative to the labelling in the microsome phase. In fact the labelling of mitochondrial phosphatidylethanolamine with time is linear, and although that of the mitochondrial phosphatidylcholine shows a slight initial lag in incorporation this is approximately the same as that of the microsomal phosphatidylcholine (Fig. 1). However, the time-sequence of labelling of microsomal and mitochondrial phospholipids obtained when the two fractions are incubated together with [32 P]phosphate similarly gives little evidence for a precursor role of the whole microsomal phospholipid fraction (Fig. 5).

There are a number of possible ways in which this failure to demonstrate a simple precursor-product specific-radioactivity relationship between microsomal and mitochondrial phospholipids might be explained. First, the synthesis of these phospholipids could initially take place in a small part of the microsome pool that reaches a very rapid equilibrium with the acid-soluble [32 P]phosphate pool. This could, for example, be a labelled lipoprotein formed by the interaction of the newly synthesized phospholipid molecules on the surface of the synthesizing enzyme with an apoprotein or a specific lipoprotein of the microsome. It is possible that this actively labelled lipoprotein could then both leave the microsomes and exchange its phospholipid with the mitochondrial membranes and at the same time exchange with the unlabelled lipoproteins of the microsomes (including the lipoprotein unit where the marker enzyme is located). In such circumstances a simple precursor-product specific-radioactivity-time relationship between the total microsomal complement of an individual phospholipid and the same phospholipid in mitochondria would not occur. It is conceivable that such a rapidly labelled pool in the microsome might be formed by the more rapid transfer of a phosphorylated base to a diglyceride with a specific fatty acid composition. In this respect there is now a good deal of evidence accumulating that phospho-

lipids of differing fatty acid composition can turn over at widely different rates (Harris, Robinson & Getz, 1960; Collins, 1963; Arvidson, 1968).

Alternatively, it is also possible that in the intact cell there is no phospholipid exchange between the endoplasmic reticulum and the mitochondrial membranes. Certainly the conformation of the lipoproteins in isolated microsomes may be different from that in the endoplasmic reticulum, and moreover the spatial relationships between the endoplasmic reticulum and the mitochondrial membrane and their ionic environment would be expected to be different from that of the fractions incubated together *in vitro*. If this were so it would be necessary to assume either that our experiments *in vitro* failed to detect Kennedy's (1961) cytidine nucleotide pathway in isolated mitochondria, or that ^{32}P can be incorporated into the nitrogen-containing phospholipids of mitochondria by some alternative mechanism that again was not revealed by our experiments reported in Fig. 4.

In general our results confirm and extend previous reports on the rate of labelling by ^{32}P of the individual phospholipids of rat liver *in vivo* (Dawson, 1955; Marinetti, Erbland, Albrecht & Stotz, 1958), with the phosphatidylethanolamine pool showing a much more rapid turnover than the larger phosphatidylcholine fraction and with a slow incorporation into the small phosphatidylserine pool. The very low rate of synthesis of cardiolipin that we have demonstrated both *in vivo* and *in vitro* is in marked contrast with the report by Ulsamer & Glenn (1966), who claimed that 5 min. after the intravenous injection of [^{32}P]phosphate the cardiolipin fraction is the most heavily labelled. It is possible that the methods used by Ulsamer & Glenn (1966) do not adequately separate cardiolipin from the very rapidly labelled phosphatidic acid. Our results are in agreement with those of Gurr *et al.* (1965), who found that cardiolipin was synthesized very slowly by subcellular fractions of rat liver, and Taylor, Bailey & Bartley (1967), who showed that the ^{32}P labelling of cardiolipin in rat liver mitochondria *in vivo* was still increasing after 3 days whereas that of the ethanolamine- and choline-containing phosphoglycerides had already considerably decayed at this time (cf. Fig. 3a). Thus it appears that the cardiolipin, which the present analysis shows is localized almost exclusively in the inner mitochondrial membrane, can exchange only slowly with external [^{32}P]phosphate compared with the other mitochondrial phosphoglycerides. Wirtz & Zilversmit (1968) showed that the cardiolipin fraction of liver mitochondria did not exchange on incubation with microsomes and supernatant fraction, although this could be due to the microsomes containing negligible amounts of the phospholipid. Eichberg (1967) reported a proteolipid complex of heart mitochon-

dria whose lipid consists predominantly of tightly bound cardiolipin. The relative stability of cardiolipin in liver mitochondria contrasts sharply with the rapid turnover of this phospholipid in bacteria, which in addition also have a comparatively stable phosphatidylethanolamine fraction (Kanfer & Kennedy, 1963; Kanemasa, Akamatsu & Nojima, 1967).

W. C. McM. is grateful to the Canadian Medical Research Council for a Visiting Scientist Award. Miss N. Hemington is thanked for valuable assistance. Dr E. Munn kindly provided electron micrographs of the inner- and outer-mitochondrial-membrane preparations.

REFERENCES

- Aldridge, W. N., Emery, R. C. & Street, B. W. (1960). *Biochem. J.* **77**, 326.
 Arvidson, G. A. E. (1968). *Europ. J. Biochem.* **4**, 478.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
 Berry, J. F. & Whittaker, V. P. (1959). *Biochem. J.* **73**, 447.
 Bradbeer, C. & Stumpf, P. K. (1960). *J. Lipid Res.* **1**, 214.
 Brandes, R., Olley, J. & Shapiro, B. (1963). *Biochem. J.* **86**, 244.
 Bygrave, F. L. & Kaiser, W. (1968). *Europ. J. Biochem.* **4**, 578.
 Collins, F. D. (1963). *Biochem. J.* **88**, 319.
 Dawson, R. M. C. (1955). *Biochem. J.* **61**, 552.
 Dawson, R. M. C. (1966). In *Essays in Biochemistry*, vol. 2, p. 69. Ed. by Campbell, P. N. & Greville, G. D. London: Academic Press (Inc.) Ltd.
 Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
 Dils, R. R. & Hübscher, G. (1959). *Biochim. biophys. Acta*, **32**, 293.
 Eichberg, J. (1967). *Abstr. 7th int. Congr. Biochem., Tokyo*, p. 737.
 Fleischer, S. & Brierley, G. (1961). *Biochim. biophys. Acta*, **53**, 609.
 Galliard, T., Mitchell, R. H. & Hawthorne, J. N. (1965). *Biochem. biophys. Res. Commun.* **106**, 551.
 Garbus, J., DeLuca, H. F., Loomans, M. E. & Strong, F. M. (1963). *J. biol. Chem.* **238**, 59.
 Getz, G. S., Bartley, W., Stirpe, F., Notton, B. M. & Renshaw, A. (1962). *Biochem. J.* **83**, 181.
 Griffith, J. Q. & Jeffers, W. A. (1949). In *The Rat in Laboratory Investigation*, p. 289. Ed. by Farris, E. J. & Griffith, J. Q. Philadelphia: J. Lippincott, Co.
 Gurd, F. R. N. (1960). In *Lipide Chemistry*, p. 282. Ed. by Hanahan, D. J. New York: John Wiley and Sons Inc.
 Gurr, M. I., Prottey, C. & Hawthorne, J. N. (1965). *Biochim. biophys. Acta*, **106**, 357.
 Hajra, A. K. & Agranoff, B. W. (1967). *J. biol. Chem.* **242**, 1074.
 Harris, P. M., Robinson, D. S. & Getz, G. (1960). *Nature, Lond.*, **188**, 742.
 Kadenbach, B. (1967). *Biochim. biophys. Acta*, **134**, 430.
 Kaiser, W. & Bygrave, F. L. (1968). *Europ. J. Biochem.* **4**, 582.
 Kanemasa, Y., Akamatsu, Y. & Nojima, S. (1967). *Biochim. biophys. Acta*, **144**, 382.
 Kanfer, J. & Kennedy, E. P. (1963). *J. biol. Chem.* **238**, 2919.

- Kennedy, E. P. (1956). *Canad. J. Biochem. Physiol.* **34**, 334.
- Kennedy, E. P. (1961). *Fed. Proc.* **20**, 934.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McCaman, R. E. & Cook, K. (1966). *J. biol. Chem.* **241**, 3390.
- McMurray, W. C. (1964). *J. Neurochem.* **11**, 287.
- McMurray, W. C., Strickland, K. P., Berry, J. F. & Rossiter, R. J. (1957). *Biochem. J.* **66**, 634.
- Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1958). *Biochim. biophys. Acta*, **30**, 543.
- Miller, J. E. & Cornatzer, W. E. (1966). *Biochim. biophys. Acta*, **125**, 534.
- O'Brien, T. W. & Kalf, G. F. (1967). *J. biol. Chem.* **242**, 2172.
- Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. & Chance, B. (1967). In *Mitochondrial Structure and Compartmentation*, p. 29. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.
- Sakagami, T., Minari, O. & Orii, T. (1965a). *Biochim. biophys. Acta*, **98**, 111.
- Sakagami, T., Minari, O. & Orii, T. (1965b). *Biochim. biophys. Acta*, **98**, 356.
- Scherphof, G. L. & van Deenen, L. L. M. (1966). *Biochim. biophys. Acta*, **113**, 417.
- Schnaitman, C. & Greenawalt, J. W. (1968). *J. Cell Biol.* **38**, 158.
- Schnaitman, C., Erwin, V. G. & Greenawalt, J. W. (1967). *J. Cell Biol.* **32**, 719.
- Schneider, W. C. (1963). *J. biol. Chem.* **238**, 3572.
- Smith, M. E., Sedgwick, B., Brindley, D. N. & Hübscher, G. (1967). *Europ. J. Biochem.* **3**, 70.
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967). *J. Cell Biol.* **32**, 415.
- Stoffel, W. & Schiefer, H. G. (1968). *Hoppe-Seyl. Z.* **340**, 1017.
- Strickland, K. P. (1962). *Canad. J. med. Sci.* **30**, 484.
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954). *J. biol. Chem.* **208**, 645.
- Tarlov, A. (1968). *Fed. Proc.* **27**, 458.
- Taylor, C. B., Bailey, E. & Bartley, W. (1967). *Biochem. J.* **105**, 605.
- Ulsamer, A. G. & Glenn, J. L. (1966). *Biochim. biophys. Acta*, **125**, 525.
- Weiss, S. B., Smith, S. W. & Kennedy, E. P. (1958). *J. biol. Chem.* **231**, 53.
- Wilgram, G. F. & Kennedy, E. P. (1963). *J. biol. Chem.* **238**, 2615.
- Wirtz, K. W. A. & Zilversmit, D. B. (1968). *J. biol. Chem.* **243**, 3596.
- Wojtczak, L. & Zborowski, J. (1967). *Abstr. 4th Meet. Fed. Europ. biochem. Soc., Oslo*, p. 91.
- Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.