Phospholipid Synthesis and Exchange in Isolated Liver Cells

By F. B. JUNGALWALA AND R. M. C. DAWSON

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

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1. The [^{32}P]phosphate incorporated into the phospholipids of isolated rat hepatic cells is present in phosphatidic acid and to a smaller extent in phosphatidylinositol. 2. The ability to synthesize nitrogen-containing phospholipids is restored by adding a liver supernatant fraction, and it is suggested that the metabolic deficiency is caused by the leakage of cytoplasmic enzymes of the synthetase system from the cells. 3. Fortified cell preparations were pulse-labelled with [^{32}P]phosphate, [$\text{Me-}^{14}\text{C}$]choline, [$2\text{-}^{14}\text{C}$]ethanolamine and [$\text{U-}^{14}\text{C}$]inositol and the subsequent fate of the labelled microsomal and mitochondrial phospholipids followed. 4. A fall in the specific radioactivity of microsomal phospholipids and a rise in that of mitochondrial phospholipids is interpreted as providing evidence of a transfer of labelled phospholipid molecules from the synthetic site (endoplasmic reticulum) to the mitochondrial membranes in the intact cells. 5. The formation of the phospholipids of mitochondrial membranes is discussed.

Ever since the demonstration by Wilgram & Kennedy (1963) and Schneider (1963) that the main location of the phosphatidylcholine-synthesizing enzyme, CDP-choline-1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2.), in rat liver was in the endoplasmic reticulum, there has been speculation as to how the other membrane fractions of the cell acquire their complement of newly synthesized phospholipids during isotopic labelling (Dawson, 1966). There have been a number of reports that isolated mitochondria from rat tissues can synthesize phosphatidylcholine through the CTP-mediated pathway (McCaman & Cook, 1966; Bygrave & Kaiser, 1968; Stoffel & Schiefer, 1968; Bosmann & Case, 1969). However, recent work in our laboratory with [^{32}P]phosphate, [$\text{Me-}^{14}\text{C}$]choline and CDP-[$\text{Me-}^{14}\text{C}$]choline precursors has indicated that the limited synthesis of most phospholipids by isolated mitochondria can be largely, if not wholly, accounted for by microsomal contamination (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970*a,b*; E. K. Miller & R. M. C. Dawson, unpublished work). Phosphatidic acid seems to be an exception, and it is now generally accepted that this phospholipid can be synthesized by isolated mitochondria independently of microsomes (Wojtezak & Zborowski, 1967; Hajra & Agranoff, 1967; McMurray & Dawson, 1969; Wlodawer & Boguslawska, 1969; Shephard & Hübscher, 1969).

Since liver mitochondria *in vivo* show an appreci-

able capacity for incorporating labelled precursors into their phospholipids (see, e.g., McMurray & Dawson, 1969; Bygrave, 1969) the question arises as to whether this is by a transfer of newly synthesized material from the endoplasmic reticulum. It has recently been demonstrated independently in a number of laboratories that a reversible exchange of phospholipids can occur when isolated rat liver microsomes are incubated with mitochondria (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969; Akiyama & Sakagami, 1969). However, experiments in intact animals designed to demonstrate a precursor-product relationship between the phospholipids of the endoplasmic reticulum and mitochondria offer only limited evidence for such a transfer *in vivo* (McMurray & Dawson, 1969; Schiefer, 1969; Bygrave, 1969), although the specific radioactivities of the phospholipids in each membrane rose in the expected order, i.e. microsomal > mitochondrial outer membrane > mitochondrial inner membrane. The experiments reported below were designed to show whether a transfer of labelled phospholipids from the endoplasmic reticulum to the mitochondria occurred *in vitro* in a system still showing cellular integrity, namely isolated liver cells. The cells were pulse-labelled with various phospholipid precursors and the subsequent fate of the labelled phospholipids was followed after the incorporation of label had ceased.

EXPERIMENTAL

Preparation of liver cell suspensions. The preparation was based on that described by Jacob & Bhargava (1962). The rat (female Wistar albino, 200g) was decapitated and the liver immediately perfused through the portal vein with 16ml of ice-cold Ringer-Locke solution containing 27mM-sodium citrate, pH7.0. The perfusion was completed in about 10min. All subsequent operations were performed at 0–4°C in a cold-room unless stated otherwise. The liver was removed and washed twice with a 'cell-suspension medium' containing 0.25M-sucrose, 30mM-potassium phosphate buffer, pH7.4, 1% (w/v) bovine serum albumin (fraction V; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.), pH7.4, and 0.1mM-EDTA.

The liver (about 8.5g) was then chopped into pieces, suspended in 35ml of the cell-suspending medium and very lightly dispersed by using a homogenizer tube with a loose-fitting rubber plunger. The maximum number of intact cells was produced by six to nine up-and-down strokes. The suspension was filtered through a layer of cheesecloth and the filtrate was centrifuged in an MSE table-model centrifuge at 700rev./min for 3min. The supernatant, which contained particulate matter from broken cells and erythrocytes, was decanted and recentrifuged at 50000rev./min in a Spinco model L centrifuge (rotor no. 50) for 1h. The supernatant was collected and is designated 'cell supernatant'. The pellet of intact cells was gently resuspended in 35ml of the cell-suspending medium and recentrifuged as above to remove material other than whole cells. The washing procedure was repeated twice and the washed cells from one liver were resuspended in the medium to a final volume of 3ml. The cell suspension contained about 35–40mg of protein/ml. Recovery of the intact cells on a wet-weight basis was approx. 25–30%. The cell supernatant contained 8–10mg of protein/ml. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949), with bovine serum albumin as the standard.

Incubation conditions. The incorporation of radioactivity into the phospholipids of whole cells was studied in 1.5ml of incubation mixture containing (final concentrations) 20mM-sodium pyruvate, 1.3mM-sodium malate, 5mM-MgCl₂, 1.7mM-ATP, 0.25mM-CTP, 0.1mM-CoA, 1.7mM-choline, 1.7mM-ethanolamine, 1.7mM-inositol, 2mM-NaF, 2mM-EDTA and [³²P]P_i (50μCi) together with 1ml of 'cell supernatant' (which contained 8–10mg of protein, 0.25M-sucrose and 30mM-potassium phosphate buffer, pH7.4) and 0.2ml of cell suspension (7–8mg of protein) in a final volume of 1.5ml. The final pH of the incubation mixture was 7.4. In experiments with [*M*e-¹⁴C]choline (0.5μCi, 54mCi/mmol) or [2-¹⁴C]-ethanolamine (0.5μCi, 27.3mCi/mmol) or [U-¹⁴C]-inositol (0.2μCi, 0.1mCi/0.5mg), non-radioactive choline, ethanolamine and inositol, and NaF and EDTA were omitted and 1.7mM-CMP was added. When mitochondria and microsomes were isolated from the incubated cells, the amounts of cell suspension, supernatant and labelled precursor were increased fourfold and the volume of the incubation mixtures was increased to 6.0ml. Samples were incubated at 37°C with gentle shaking under O₂ + CO₂ (95+5).

Pulse-'chase' experiments. In pulse-'chase' experi-

ments, the cells were incubated with the labelled precursor and the latter was then quickly removed by centrifugation at 700rev./min (MSE bench centrifuge) for 3min at 0°C. The supernatant, containing most of the radioactivity as well as some broken cells, was discarded and the cells were washed three times with the cell-suspending medium as described above. The cells were then reincubated at 37°C together with the necessary cofactors and cell supernatant but without the addition of labelled precursor. One tube containing the washed cells was, however, reincubated with the same amount of fresh labelled precursor as added originally to check the amount of incorporation after the washing procedure. The cells reincubated for various periods were quickly cooled to 0°C and collected by centrifuging. They were then resuspended in 10ml of 0.25M-sucrose–0.1mM-EDTA, pH7.4, and homogenized in a motor-driven Tri-R-Teflon tissue homogenizer for 3min. The homogenate was centrifuged at 2500rev./min (800g) for 10min in a Servall RC-2 centrifuge (rotor SS-34), the supernatant was decanted and the pellet was resuspended in 10ml of 0.25M-sucrose–0.1mM-EDTA, pH7.4, rehomogenized for 1min and recentrifuged as described above. The pellet, containing nuclei, was discarded. Mitochondrial and microsomal fractions were isolated from the supernatant by the procedure described by Jungalwala & Dawson (1970b). The mitochondrial fraction after being washed five times still contained 5–9% contamination with microsomes, as estimated by NADPH-cytochrome *c* reductase activity (McMurray & Dawson, 1969). The microsomal fraction was essentially free of mitochondria, as judged by cytochrome *c* oxidase assays (McMurray & Dawson, 1969).

Phospholipid analyses. The cells or membrane fractions were extracted overnight at 0°C with 20 vol. of chloroform-methanol (2:1, v/v). Next day the samples were warmed at 40–45°C for 15min and filtered through a small pad of glass wool. The filtrates were washed to remove water-soluble radioactive compounds (McMurray & Dawson, 1969). A portion from the washed lipid extracts labelled with ¹⁴C-labelled precursors was evaporated to dryness in a scintillation vial, and 10ml of scintillator solution containing 4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/1 of toluene was added. Samples were counted in a Packard automatic liquid-scintillation spectrometer. Another portion from the same samples was evaporated to dryness and P_i was determined after digestion with 72% (w/v) HClO₄ (Bartlett, 1959). Determination of the specific radioactivity of individual phospholipids labelled with ¹⁴C-containing precursors was based on the observation that incorporation of the precursor only occurred into those phosphoglycerides containing the precursor molecule, e.g. [¹⁴C]choline into choline phosphoglycerides. The lipid extract was deacylated with ethanolic NaOH and a portion of the water-soluble portion of the ethanolsate was taken for measurement of radioactivity. The remainder of the water-soluble products was subjected to paper chromatography followed by ionophoresis to determine the amount of the individual phospholipid present and to calculate the specific radioactivity (Dawson, Hemington & Davenport, 1962).

The lipid extracts labelled with ³²P were evaporated to dryness and the phospholipids deacylated (Dawson *et al.* 1962). After separation the individual glycerylphosphoryl

derivative spots on the paper were digested with 72% (w/v) HClO_4 . The digest was made up to 10 ml with water and the radioactivity was measured in a scintillation counter without addition of scintillator (Čerenkov radiation giving an efficiency of about 30%; von Haberer, 1965), followed by determination of P_i on the same sample (Bartlett, 1959).

Materials. [$\text{Me-}^{14}\text{C}$]Choline (54 mCi/mmol), [$2\text{-}^{14}\text{C}$]ethanolamine (27.3 mCi/mmol), [$\text{U-}^{14}\text{C}$]myoinositol (0.1 mCi/0.5 mg) and [^{32}P] P_i (45 Ci/mg of P) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phosphoryl[$\text{Me-}^{14}\text{C}$]choline was prepared as described by Schneider, Fiscus & Lawler (1966). CDP-[$1,2\text{-}^{14}\text{C}$]choline (0.14 mCi/mmol) was a gift from McMurray (1964). Cytochrome *c* was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. NADPH, ATP, CTP, CMP and CoA were from Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents were of A. R. grade.

RESULTS

Synthesis of phospholipids by isolated liver cells. Various methods were tried for preparing suspensions of whole liver cells. These included the citrate perfusion method of Jacob & Bhargava (1962), the ethanedioxybis(ethylamine)tetra-acetic acid perfusion method of Ontko (1967) and the enzymic digestion method of Howard, Christensen, Gibbs & Pesch (1967). The cells looked acceptable by phase-contrast microscopy; however, on incubation with [^{32}P]phosphate and a number of cofactors they incorporated label only into phosphatidic acid and to a smaller extent into phosphatidylinositol, and little into the nitrogen-containing phospholipids. Electron-microscopic examination of cells prepared by perfusion showed damage to the endoplasmic reticulum, with the development of large vesicles. However, if microsomes and mitochondria were isolated from such cells they were able to incorporate ^{32}P into all phospholipids provided they were incubated under conditions (McMurray &

Dawson, 1969) that included the presence of a supernatant fraction. Eventually a method of isolation and incubation of whole cells was developed that allowed adequate synthesis of all the phospholipids. To do this it was found essential to carry out the isolation of the cells in a medium containing 0.25 M-sucrose and 30 mM-potassium phosphate buffer, pH 7.4, rather than in one where iso-osmoticity had been achieved by the addition of salts (Jacob & Bhargava, 1962). Further, reasonable synthesis of the nitrogen-containing phospholipids from [^{32}P]phosphate by the whole cells was obtained only when a cell supernatant fraction was added to the cell suspension during the incubation (Table 1).

This supernatant fraction was incapable of synthesizing phospholipids on its own. It retained its activity on dialysis, but all was lost on keeping it for 5 min at 100°C. The supernatant factor could not be replaced by bovine serum albumin, which is known to activate many lipid-synthesizing systems *in vitro* (Shapiro, 1964). Marinetti, Erbland, Chatterjee & Fang-Chiang (1968) found that NAD^+ was essential for the adequate synthesis of phospholipids in whole liver homogenates, but on addition of this coenzyme to the hepatic cells it inhibited the already minimal ^{32}P incorporation into the nitrogen-containing phospholipids, although the synthesis of phosphatidic acid and phosphatidyl-inositol was not affected.

Examination of the phosphorylcholine in the cells (Dawson, 1956) after ^{32}P -labelling in the absence of supernatant revealed that little of this phosphate ester was labelled in the system. This suggested that some of the enzymes responsible for the incorporation of ^{32}P into phosphatidylcholine by the cytidine nucleotide pathway may have leaked from the cells, since there is no doubt that the phosphorylcholine and CDP-choline intermediates become rapidly labelled in the liver *in vivo*

Table 1. *Effect of supernatant fraction on the distribution of ^{32}P incorporated into the phospholipids of hepatic cells*

Details of the incubation mixture are given in the Experimental section. Samples were incubated for 60 min at 37°C.

Vol. of cell suspension (ml)	Vol. of supernatant (ml)	Total radioactivity (c.p.m.)	^{32}P incorporated into alkali-labile phosphoglycerides			
			% distribution of radioactivity			
			Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidic acid
0.3	0	12160	1	1	22	76
0.3	0.1	15130	2	2	28	68
0.3	0.2	11503	5	5	41	48
0.3	0.4	15865	7	7	40	35
0.3	0.8	31455	37	3	27	33
0.1	1.0	25648	52	7	22	19

Table 2. *Effect of supernatant fraction on the incorporation of choline from [Me-¹⁴C]choline, phosphoryl[Me-¹⁴C]choline and CDP-[Me-¹⁴C]choline into the phospholipids of liver cells*

Cell suspension (0.2ml) and supernatant (1 ml), prepared as described in the Experimental section, were incubated with the labelled precursor in cell-suspension medium (final vol. 1.5ml) for 1 h at 37°C.

Expt. no.	Substrate	Radioactivity added (c.p.m.)	Supernatant	Radioactivity in phospholipid (c.p.m.)	Ratio $\frac{\text{incorporation without supernatant}}{\text{incorporation with supernatant}}$ (%)
1	[Me- ¹⁴ C]choline	708 000	—	8 580	27
			+	32 986	
	Phosphoryl-[Me- ¹⁴ C]choline	46 500	—	1 264	28
			+	4 508	
2	CDP-[Me- ¹⁴ C]choline	37 600	—	17 878	69
			+	25 896

Fig. 1. Labelling of the phospholipids of the microsomes and mitochondria of isolated hepatic cells with [³²P]phosphate. ●, Microsomal phospholipids; ○, mitochondrial phospholipids.

(Dawson, 1956; Bjørnstad & Bremer, 1966). Support for this suggestion was obtained by comparing the effect of the cell supernatant on the incorporation of methyl-labelled choline from [Me-¹⁴C]choline, phosphoryl[Me-¹⁴C]choline and CDP-[Me-¹⁴C]choline precursors into the choline phosphoglycerides of cells. Whereas in the absence of supernatant the incorporation from choline and phosphorylcholine was less than 30% of that incorporated in its presence, the corresponding value for CDP-choline was 69% (Table 2).

Electron micrographs of the cell preparations showed no evidence of contamination with erythrocytes or free mitochondria. The endoplasmic reticulum appeared to be relatively intact, although the mitochondria had somewhat shrunken inner membranes. The general appearance was similar to that of cells in the intact liver. Disintegration of some cells occurred on incubation and this was not prevented by adding 1 mM or 5 mM-calcium chloride to the medium. Consequently during exchange experiments observations were always limited to

the whole cells recovered from the incubation medium by centrifuging.

The time-course of the incorporation of [³²P]-phosphate into individual phospholipids of mitochondria and microsomes of the hepatic cells is shown in Fig. 1. Each individual phospholipid showed an initial lag in the incorporation, presumably due to a delay in the entry of the phosphate and the build-up of labelled intermediates required for the incorporation. The lag phase for phosphatidylinositol synthesis was less than for the nitrogen-containing phospholipids, possibly because the immediate ³²P-labelled precursor, phosphatidic acid, becomes very rapidly labelled. Phosphatidyl-ethanolamine showed a more rapid turnover than phosphatidylcholine, and with each of these phospholipids the labelling was more rapid in the microsomes than in the mitochondria. Similar relationships between the turnovers of the nitrogen-containing phospholipids have been observed in the intact liver (McMurray & Dawson, 1969). On the other hand phosphatidylinositol showed a more

Fig. 2. Pulse-labelling of the phospholipids of the microsomes and mitochondria of isolated hepatic cells with $[^{32}\text{P}]$ phosphate. The cells were labelled for 1 h, washed to remove the radioactive precursor and then re-incubated in the absence of precursor: ●, microsomal phospholipids; ○, mitochondrial phospholipids. Some cells after washing were re-incubated with fresh radioactive precursor: ■, microsomal phospholipids; □, mitochondrial phospholipids.

Fig. 3. Pulse-labelling of the phospholipids of the microsomes and mitochondria of isolated hepatic cells with $[^{14}\text{C}]$ ethanolamine. The cells were labelled for 1 h, washed to remove the radioactive precursor and then re-incubated in the absence of precursor [in the experiment on the right a 'chase' of non-radioactive ethanolamine (1.7 mM) was added]: ●, microsomal phospholipids; ○, mitochondrial phospholipids. Some cells after washing were re-incubated with fresh radioactive precursor: ■, microsomal phospholipids; □, mitochondrial phospholipids.

rapid labelling compared with phosphatidylethanolamine in isolated cells than that found *in vivo*, although in both situations there was little difference

between the turnovers of the mitochondrial and microsomal fractions. The general form of all the curves suggests that, if a transfer of labelled

Fig. 4. Pulse-labelling of the phospholipids of the microsomes and mitochondria of isolated hepatic cells with [^{14}C]choline. The cells were labelled for 1 h, washed to remove the radioactive precursor and then reincubated in the absence of precursor: ●, microsomal phospholipids; ○, mitochondrial phospholipids. Some cells after washing were reincubated with fresh radioactive precursor: ■, microsomal phospholipids; □, mitochondrial phospholipids.

individual phospholipids from the endoplasmic reticulum to the mitochondria occurs, it must be a relatively rapid process.

Figs. 2–5 record the changes in the specific radioactivities of the whole phospholipid fractions or of individual phosphoglycerides in the two membrane fractions after the pulse-labelling of whole cells with [^{32}P]phosphate, [$2\text{-}^{14}\text{C}$]ethanolamine, [$\text{Me-}^{14}\text{C}$]choline and [$\text{U-}^{14}\text{C}$]inositol. Without exception, the removal of the medium containing the labelled precursor resulted in a rapid fall of the specific radioactivity of the microsomal phosphoglycerides on subsequent incubation of the cells. That this was not caused by any damage to the cells resulting from the centrifuging and washing procedure during the change of medium was showed by adding fresh medium containing labelled precursor to some cells: on subsequent incubation the specific radioactivity of their microsomes did not decrease but usually showed an appreciable increase. Microsomes isolated from hepatic cells, labelled with radioactive choline or ethanolamine, also showed no decrease in the specific radioactivity of phospholipids when incubated independently (Table 3).

After the pulse-labelling with [^{32}P]phosphate both the microsomal and mitochondrial phosphatidic acid showed a rapid fall in radioactivity within the first 20 min. This was probably because of the rapid turnover of the phosphatidic acid pool, and indicated that the labelled ATP from which the phospholipid was synthesized was rapidly being replaced by unlabelled nucleotide, presumably in both the cytoplasm and the mitochondria. The amount of lipid phosphorus in the phosphatidic acid fraction was too small to be measured and consequently specific radioactivities could not be calculated. The specific radioactivity of the mitochondrial ^{32}P -labelled phosphatidylinositol fell, in line with that of the microsomes (Fig. 2). This again is probably consistent with the close similarity between the labelling patterns of the two organelles mentioned above (Fig. 1).

The specific radioactivities of the nitrogen-containing phospholipids of the cell mitochondria showed a pattern different from those of the microsomes after pulse-labelling and subsequent reincubation (Figs. 2, 3 and 4). With all precursors there was invariably a rise in the specific radioactivities of the mitochondrial choline phospho-

Fig. 5. Pulse-labelling of the phospholipids of the microsomes and mitochondria of isolated hepatic cells with [^{14}C]myoinositol. The cells were labelled for 40 min, washed to remove the radioactive precursor and then reincubated in the absence of precursor: ●, microsomal phospholipids; ○, mitochondrial phospholipids. Some cells after washing were reincubated with fresh radioactive precursor: ■, microsomal phospholipids; □, mitochondrial phospholipids.

glycerides and ethanolamine phosphoglycerides for the first 20 min of reincubation after replacement of the medium containing labelled precursor. Sometimes this increase went on for longer, and sometimes there was a small fall in specific radioactivity. Nevertheless the curves for both membrane fractions indicate that the system was moving towards some type of isotopic equilibrium after the synthesis of labelled phospholipid had ceased. This was also clearly seen after pulse-labelling of the cells with radioactive inositol (Fig. 5). Microsomal phosphatidylinositol acquired a much higher specific radioactivity after [^{14}C]inositol labelling than did the mitochondrial phosphatidylinositol, which is in contrast with the equivalent labelling with ^{32}P . This could be due to the exchange of free inositol with lipid inositol, a reaction which is known to

take place in rat liver microsomes (Paulus & Kennedy, 1960).

DISCUSSION

It has been known for some years that isolated parenchymal cells of liver show certain metabolic deficiencies compared with the cells in the intact tissue or in liver slices (Iype & Bhargava, 1965). Consequently it was perhaps not surprising to find that cell suspensions prepared by a variety of methods were incapable of bringing about synthesis of phospholipids *de novo*, apart from phosphatidic acid and phosphatidylinositol, from [^{32}P]phosphate. Although it has been shown that liver cell suspensions are capable of incorporating labelled palmitic acid into phospholipids (Ontko, 1967; Wright & Green, 1969), this could be through fatty acid exchange reactions (Lands, 1965) rather than by the cytidine nucleotide pathway responsible for the assembly of whole phospholipid molecules.

It seems likely that the deficiency of the synthetase system was due to leakage of the mainly cytoplasmic enzymes, ATP-choline phosphotransferase (EC 2.7.1.32), CTP-choline phosphate cytidyltransferase (EC 2.7.7.15) and CTP-ethanolamine phosphate cytidyltransferase (EC 2.7.7.14), from the cells during their preparation. Isolated liver cells appear to be permeable to chloramphenicol (Jacob & Bhargava, 1965) and to macromolecules such as RNA (Shanmugam & Bhargava, 1966), and are known to lose appreciable quantities of certain cytoplasmic enzymes, such as L-lactate-NAD oxidoreductase (EC 1.1.1.27), during their preparation (Henley, Sorensen & Pollard, 1959). This would explain why the dialysed supernatant fraction greatly enhanced the ability of the liver cells to synthesize labelled nitrogen-containing phospholipids from [^{32}P]phosphate, [$\text{Me-}^{14}\text{C}$]choline, and [$2\text{-}^{14}\text{C}$]ethanolamine precursors but to a much smaller extent from CDP-[$\text{Me-}^{14}\text{C}$]choline.

Up to the present the evidence for the exchange of whole phospholipid molecules between endoplasmic reticulum and mitochondria has been largely circumstantial. The inability of isolated mitochondria to synthesize any of the phospholipids that comprise the bulk of those present in their membranes could be ascribed to damage suffered during the isolation or to the absence of essential cofactors, and similarly in experiments showing that isolated mitochondria incorporate ^{32}P into their phospholipids at only 10% of the rate of tissue slices in the same incubation medium (Kadenbach, 1968). Also the greater rate of labelling of the phospholipids of microsomal membranes compared with those of mitochondria does not constitute a proof of transfer.

There is now good evidence that an exchange of

Table 3. *Specific radioactivities of phospholipids of labelled microsomes and mitochondria on incubation in non-radioactive media*

The subcellular particles were isolated from hepatic cells that had been labelled with [2-¹⁴C]ethanolamine or [Me-¹⁴C]choline during a 60 min incubation. They were then incubated in the cell suspension medium with added supernatant (total vol. 1 ml).

		Sp. radioactivity (c.p.m./μg of total lipid P)					
Time of incubation (min)		Choline labelling		Ethanolamine labelling			
		Expt. 1	Expt. 2	Expt. 3†	Expt. 4	Expt. 5	
Microsomes	0	212	183	173	1878	1029	1114*
	20	220	157	177	1800	1062	
	40	185	167	187	1835	1111	
	60	195	—	228	1828	1068	
Mitochondria	0	151	55	74	832	465	1001*
	20	133	52	73	566	455	
	40	139	—	69	734	459	
	60	135	—	73	725	462	

* No supernatant added.

† Unlabelled ethanolamine (1.7 mM) added to incubation media.

phospholipids occurs between microsomal membranes and mitochondria when they are incubated together *in vitro*. Since the spatial relationship between these membrane fractions in free suspension is different from that existing between the endoplasmic reticulum and the mitochondria in the intact tissue, it can be argued that an equivalent exchange may not occur *in vivo*. However, it is difficult to explain the present results obtained with whole liver cells, where the membranes appear to be maintained in a condition similar to that in the intact tissue, without assuming the existence of an exchange process. The fall in specific radioactivity in the microsomal phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol after pulse-labelling could readily be explained by an exchange with lower-specific-radioactivity material from the non-endoplasmic-reticulum phospholipids (e.g. mitochondrial, nuclear, plasma membranes). The fall is too large to be explained by assuming that freshly synthesized phospholipid of low specific radioactivity is diluting the whole microsomal pool, since calculations show that with the nitrogen-containing phospholipids this contribution would amount to, at the most, only a few per cent of the total mass in the time-period considered. Further, the breakdown of the microsomal pool as a whole by microsomal phospholipases would lead to a decrease in total radioactivity but not in specific radioactivity. However, this argument would not apply if the phospholipase hydrolysis were confined to a small rapidly turning-over portion of the whole microsomal phospholipid pool, and experiments in

which microsomes, obtained from whole cells after labelling with [2-¹⁴C]ethanolamine or [Me-¹⁴C]choline, were incubated in isolation gave little evidence of any such selective hydrolysis. The specific radioactivity of the microsomal phosphatidylethanolamine did not decrease on incubation (Table 3). That of the phosphatidylcholine showed a slight fall, but not nearly sufficient to account for that observed in the intact cells (Fig. 4), neither was there any consistent change in the specific radioactivities of the phospholipids of labelled mitochondria incubated alone (cf. Waite, 1969). Fractionation of rat liver microsomes has not revealed the presence of any morphological pool of phospholipid with a very rapid turnover (Glaumann & Dallner, 1968; Bygrave & Bücher, 1968), although the turnover of microsomal phosphoglycerides of different fatty acid composition can be quite variable (Rytter, Miller & Cornatzer, 1968).

The continuing increase in the specific radioactivity of mitochondrial phospholipids after pulse-labelling can most readily be explained by assuming that there is a transfer of phospholipid from the more rapidly labelled endoplasmic reticulum. It is not to be expected that the rise in specific radioactivity of the mitochondrial phospholipids would be as marked as the fall of those in the endoplasmic reticulum, since the mitochondria and other non-microsomal phospholipid-containing membranes represent a far larger pool of phospholipids. An alternative explanation for the phenomenon would be that phospholipid synthesis was still occurring from a pre-labelled mitochondrial pool of inter-

mediates that was not immediately affected by the change of incubation medium. However, there is no evidence that such a pool exists; mitochondria possess little ability to synthesize nitrogen-containing phosphoglycerides (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970a,b) and if they are capable of limited synthesis this would be confined to the outer membrane (Bygrave, 1969), presumably involving a cytoplasmic pool of precursors.

The present results, coupled with all the other evidence available, therefore strongly suggest that the formation of most, if not all, of the mitochondrial phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol occurs within the endoplasmic reticulum. Phosphatidylcholine and phosphatidylethanolamine comprise the bulk of the phospholipids present in the mitochondrial membranes. It does not follow that once deposited these phospholipids cannot be modified by subsequent exchange reactions of the mitochondria, but this would not amount to synthesis *de novo*. For example, Nachbaur, Colbeau & Vignais (1969) claim to have detected both phospholipase A and a lysophospholipid-acylation system that could carry out fatty acid exchange in both the inner and outer membranes of rat liver mitochondria, although Stoffel & Schiefer (1968) could detect acyl-CoA-lysocleithin acyltransferase only in the outer membranes, and Eibl, Hill & Lands (1969) in the mitochondria not at all.

It appears therefore that, like the mitochondrial proteins (Kadenbach, 1968; Work, 1968), the mitochondrial phospholipids can be synthesized both extra- and intra-mitochondrially. Thus the synthesis of phosphatidic acid, cardiolipin, acyl-dihydroxyacetone phosphate and sphingomyelin is probably mainly intramitochondrial (Hajra & Agranoff, 1967; Shephard & Hübscher, 1969; Sribney, 1968; Kiyasu, Pieringer, Paulus & Kennedy, 1963), whereas that of the choline and ethanolamine phosphoglycerides is largely extra-mitochondrial. How the latter are transported to the mitochondria is not yet clear. Wirtz & Zilversmit (1969) have isolated an active protein fraction from the cell supernatant that is essential for the exchange of phospholipids when mitochondria and microsomes are incubated together (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969). This may act as an exchange apoprotein for the newly synthesized phosphoglyceride molecules on the active centre of the microsomal synthetase and thus ferry the phospholipid as a rudimentary lipoprotein complex to the mitochondria (Dawson, 1966). Once a collision complex had been formed, phospholipid would pass by exchange diffusion first into the outer membrane and then into the inner cristae of the mitochondria.

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