Evidence for a compartmentation of brain microsomal diacylglycerol

Luciano Binaglia, Rita Roberti, Alba Vecchini, and Giuseppe Porcellati

Institute of Biological Chemistry, The Medical School, University of Perugia, 06100 Perugia, Italy

Abstract Phosphatidylcholine synthesis from CDP-[methyl-^{14}C]choline and membrane-bound diacyl-[U-^{14}C]-*sn*-glycerol, formed through the glycerol phosphate pathway, has been examined in vitro in rat brain microsomes. When labeled diacylglycerol was incubated in the presence of unlabeled CDP-choline, the rate of phospholipid labeling looked very different from that measured in incubations of unlabeled diacylglycerol with CDP-[methyl-^{14}C]choline. Evidence is given that diacylglycerol formed through the glycerol phosphate pathway belongs to a metabolic pool separate from the bulk membrane diacylglycerol.—**Binaglia, L., R. Roberti, A. Vecchini, and G. Porcellati.** Evidence for a compartmentation of brain microsomal diacylglycerol. *J. Lipid Res.* 1982. **23:** 955–961.

Supplementary key words cholinephosphotransferase • diacylglycerol • glycerol phosphate pathway • molecular species • phospholipase C

The synthesis of phosphatidylcholine and phosphatidylethanolamine in animal tissue has been extensively studied in vitro from labeled CDP-choline or CDP-ethanolamine in the presence of exogenously-added lipid precursors (1–5). In addition, the same steps have been examined by Kanoh and Ohno (6), Fallon and co-workers (7), and Roberti, Binaglia, and Porcellati (8) by incubating cold CDP-bases with membranes containing endogenously labeled diacylglycerol.

Some discrepancies between the results of these two experimental approaches led us to examine in more detail the final step of membrane phospholipid synthesis. The time-course of the microsomal cholinephosphotransferase reaction (EC 2.7.8.2), studied in an incubation system containing exogenous diacylglycerol and labeled CDP-choline, is very different from that obtained with the experiments carried out with cold CDP-choline and membrane-bound labeled diacylglycerol (8). This aspect of the reaction, therefore, has been re-examined by carrying out experiments in which lipid synthesis was studied from labeled CDP-choline or labeled membrane diacylglycerol, under the same experimental conditions. An evaluation of these results leads to interesting conclusions which regard the fate of lipid intermediates implicated in the glycerol phosphate pathway.

METHODS

Preparation of membrane-bound labeled diacylglycerol

Rat brain microsomes were prepared and assayed for contamination as previously described (9). The microsomal pellet was suspended in 0.32 M sucrose, 2 mM 2-mercaptoethanol, and the suspension was divided into two parts. The first (microsomes A) was incubated for 30 min at 37°C, in the presence of 19.1 μM [U-^{14}C]-*sn*-glycerol-3-phosphate (sp act 170 Ci/mol), 0.1 M potassium phosphate buffer (pH 7.0), 4 mM $MgCl_2$, 0.2 mM CoA-SH, 70 mM NaF, 6 mM ATP, and about 1.8 mg of protein/ml microsomal suspension. The second half of the same microsomal suspension (microsomes B) was incubated with 19.1 μM unlabeled *sn*-glycerol-3-phosphate, at the same experimental conditions. The incubation mixtures (final volume of 10 ml) were cooled to 3–4°C and the membranes were pelleted by centrifugation. The pellets were resuspended in 0.32 M sucrose, 2 mM 2-mercaptoethanol solution, again precipitated by centrifugation, and resuspended in the sucrose, mercaptoethanol solution (pH 7.0). The suspensions were incubated for 30 min at 37°C. The dephosphorylation of the membrane-bound phosphatidic acid, which takes place during incubation, was stopped by cooling the mixtures to 3–4°C (8). Aliquots of the suspensions were used for protein determination and analysis of the labeled lipids. The remaining suspensions were incubated with CDP-choline, as described below.

Synthesis of phosphatidylcholine

Diacylglycerol-labeled microsomes (A) were incubated at 37°C for different time intervals, using 1 mg protein/ml, in 50 mM Tris–HCl buffer (pH 8.0), 10 mM $MnCl_2$, and 1 mM CDP-choline. The final volume was 0.3 ml. The incubation was stopped by adding 8 vol of $CHCl_3$–CH_3OH 1:1 (v/v).

Abbreviation: TLC, thin-layer chromatography.

TABLE 1. Degradation of CDP-choline during incubation for phosphatidylcholine synthesis

Incubation Time	Molar Concentration		
	CDP-choline	CMP	Cytidine + Cytosine
min			
30	9.6×10^{-4}	0	3.9×10^{-5}
60	9.3×10^{-4}	2.0×10^{-5}	4.7×10^{-5}
120	9.0×10^{-4}	2.0×10^{-5}	8.1×10^{-5}
180	8.0×10^{-4}	1.7×10^{-5}	1.8×10^{-4}

Rat brain microsomes (about 1 mg of protein) were incubated at 30°C for given times with 1 mM CDP-choline, 10 mM $MnCl_2$, 50 mM Tris–HCl buffer (pH 8.0), in 0.3 ml final volume. Incubation was stopped by adding 1 ml of 0.5 M $HClO_4$; after neutralization and centrifugation, the supernate was lyophilized and the residue was suspended in 1 ml of 0.1 M 2-methyl-2-amino-1-propanol, 0.02 M NaCl buffer (pH 10.3). An aliquot of the buffered solution was chromatographed on Aminex A-14 ion exchange resin (chloride form, particle size 20 μm), using a Pye-Unicam LC-20 instrument, equipped with a UV-20 detection unit (254 nm). Cytidine plus cytosine and CDP-choline were eluted with 0.1 M 2-methyl-2-amino-1-propanol, 0.02 M NaCl buffer (pH 10.3). CMP was eluted with 0.1 M 2-methyl-2-amino-1-propanol, 0.17 M NaCl buffer (pH 11.0). Each value is the average of three determinations. Deviation from mean values was less than 5%.

Parallel incubations of microsomes B were carried out, but 1 mM CDP-[methyl-^{14}C]choline (sp act 1.07 Ci/mol) was now used under the same experimental conditions.

The specific activity of the newly synthesized glycerol-labeled phosphatidylcholine was calculated as:

$$1.07 \times \frac{\text{nCi phosphatidylcholine (microsomes A)}}{\text{nCi phosphatidylcholine (microsomes B)}}.$$

In some experiments, diacylglycerol-labeled microsomes (A) were incubated with 1 mM CDP-[methyl-^{14}C]choline (sp act 1.07 Ci/mol). Labeled phosphatidylcholine, isolated by TLC, was then digested with phospholipase C, as described (10). Labeled diacylglycerol and phosphorylcholine were then separated by partition according to Folch, Lees, and Sloane Stanley (11) and isolated by TLC (12, 13). Recoveries of more than 95% were constantly obtained. The specific activity of the glycerol moiety in the doubly-labeled newly synthesized phosphatidylcholine was calculated as follows:

$$1.07 \text{ Ci/mol} \times \frac{\text{nCi diacylglycerol}}{\text{nCi phosphorylcholine}}.$$

Kinetics of the synthesis of phosphatidylcholine molecular species

Microsomes were prepared from adult rat brain as described above (9). Each set of incubations contained 1 mM CDP-choline, 50 mM Tris–HCl buffer (pH 8.0), and 10 mM $MnCl_2$ in a final volume of 0.3 ml. The mixtures were equilibrated at 30°C and the reaction was started at the same time for each tube by adding microsomes (4 mg of protein/ml), previously equilibrated at 30°C. A supplement of CDP-[methyl-^{14}C]choline was immediately added to the first tube and the reaction was stopped after 5 min of incubation. The second tube was supplemented with labeled CDP-choline after 5 min of incubation and the reaction stopped at 10 min. In a similar way, the other incubations were arranged so that the time range 0–3 hr was examined. The reaction was carried out at 30°C. The specific activity of the CDP-[methyl-^{14}C]choline, after supplementing, was 2.14 Ci/mol, during the first 5 min of incubation. Due to little degradation of CDP-choline during incubation (**Table 1**), the specific activity of the nucleotide did not change appreciably during the next incubations.

Phospholipase C treatments of microsomal vesicles

Rat brain microsomes were incubated with phospholipase C from *Clostridium welchii* (5 I.U./mg protein, Sigma Chemical Company), according to Rousseau and Gatt (14). Enzyme to microsomal protein ratios of 0.003 to 0.06 were used. Incubations of 5 min at 25°C were carried out. The reaction was stopped by adding 30 ml of 1 mM EDTA in 0.32 M sucrose, 2 mM 2-mercaptoethanol solution. The final volume was 10 ml. Control microsomes were treated in the same way, but phospholipase was omitted.

Incubations of microsomes with phospholipase C from *Bacillus cereus*, according to Wood and Harlow (15), were also carried out by incubating microsomes (15 mg of protein) with 0.004–0.04 units of the enzyme in a medium containing 0.2 M phosphate buffer (pH 7.0), 1 mM 2-mercaptoethanol, and 0.4 mM $ZnCl_2$ in 10 ml final volume. Incubations of 5 min were carried out at 25°C and stopped with 30 ml 1 mM EDTA in 0.32 M sucrose, 2 mM 2-mercaptoethanol. After the phospholipase treatments, microsomes were incubated with labeled CDP-choline at 30°C, as described before.

Analytical methods

Lipid extraction and analysis were performed as previously reported (10). Lipid phosphorus and membrane protein were quantitated according to Bartlett (16) and Lowry and co-workers (17), respectively. Molecular species of intact phospholipids were separated on $AgNO_3$-impregnated silica plates, as described by Arvidson (18). Identification of the unsaturation level of the lipid spots was accomplished by gas–liquid chromatography of the fatty acid methyl esters, as previously described (10).

Membrane diacylglycerols were quantitated by fluorimetric measurement of their naphtoyl derivatives. For this purpose, diacylglycerols were isolated by thin-layer chromatography (12) and eluted from the silica layer with chloroform–methanol 2:1 (v/v). The eluates were evaporated to dryness and 0.5 ml of anhydrous

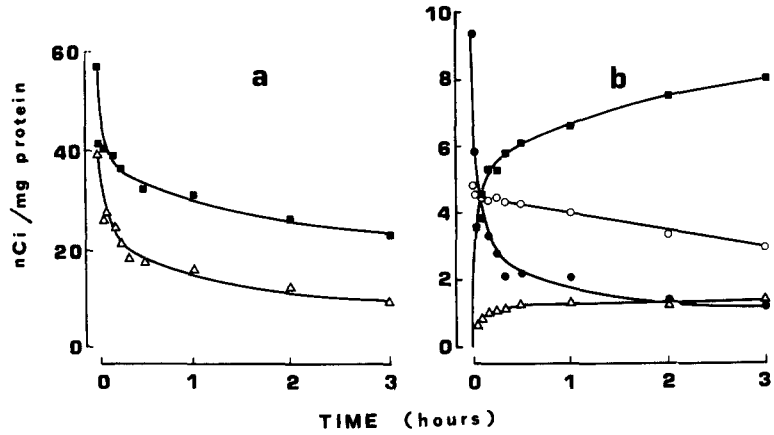

Fig. 1. Time-course of microsomal lipid labeling during incubation with CDP-choline. Rat brain microsomes were incubated with [U-^{14}C]-*sn*-glycerol-3-phosphate, as described in the text. The phosphatidate-labeled microsomes were then incubated under the conditions of the phosphatidate phosphohydrolase reaction. After this last incubation, microsomal lipid labeling was 57.1 nCi/mg protein in the present experiment (phosphatidic acid: 40.4 nCi; triacylglycerol: 4.8 nCi; diacylglycerol: 9.3 nCi; lysophosphatidic acid: 2.6 nCi). No labeled phosphatidylcholine was present at zero incubation time. Labeled microsomes were then incubated at 37°C in the presence of 10 mM Mn^{2+}, 50 mM Tris–HCl buffer (pH 8.0), and 1 mM CDP-choline. Lipid labeling was examined at different time intervals. Experiments repeated three times gave the same results. Only one experiment is reported, due to the variable extent of labeling of incubated phosphatidate and diacylglycerol. a: Change in radioactivity content of total lipid (■ — ■ — ■) and phosphatidic acid (△ — △ — △). b: Change in radioactivity content of diacylglycerol (● — ● — ●), triacylglycerol (○ — ○ — ○), phosphatidylcholine (■ — ■ — ■) and phosphatidylethanolamine (△ — △ — △).

pyridine containing 40 g/l of 2-naphtoyl chloride were added to each tube. The tubes were stoppered and kept for 1 hr at 60°C. The excess of 2-naphtoyl chloride was hydrolyzed by adding two or three drops of water and heating at 60°C for 5 additional minutes. Pyridine was evaporated under vacuum and the residue was dissolved into 4 ml of chloroform. The chloroform solution was washed twice with 1 vol of 0.1 N HCl and four times with 1 vol of 0.1 N NaOH. The chloroform phase was washed with 1 vol of water and the solvent was evaporated. Derivatized lipid was chromatographed on silica plates with petroleum ether (bp 30–60°C)–ethyl ether 85:15 (v/v) as developing system. Naphtoyl-diacylglycerol was identified under a short wavelength U.V. lamp and by automatic radiochromatoscanning (R_f = 0.45). The zones of the layers containing the diacylglycerol derivative were scraped into test tubes and 5 ml of chloroform–methanol 1:1 (v/v) was added. Tubes were agitated and centrifuged. The fluorescence of the clear supernatant was measured at 285 nm and 364 nm, excitation and emission wavelength, respectively, with appropriate standards and blanks. After fluorescence measurement, the samples were dried and assayed for radioactivity.

RESULTS

The distribution of the label among isolated lipid classes after incubation of rat brain microsomes with [U-^{14}C]-*sn*-glycerol-3-phosphate and partial dephosphorylation of phosphatidic acid is shown in **Fig. 1.** These incubations did not change the size of phosphatidic acid and diacylglycerol pools, which were, respectively, 14.5 ± 2.1 and 7.8 ± 0.5 nmol/mg protein (nine determinations). These values were comparable to those previously reported (12).

Incubation of labeled microsomes (microsomes A, see Methods) with 1 mM CDP-choline resulted in a rapid disappearance of the label in the total lipid fraction. As shown in Fig. 1a, the decrease of the total lipid radioactivity closely paralleled the decrease of phosphatidic acid labeling; this compound is supposed to be partially degraded via lysophosphatidic acid to glycerol phosphate. During the same incubation, a marked incorporation of the label into phospholipid was measured (Fig. 1b). There was a good correlation between the decrease of radioactivity of the neutral lipid fraction (diacylglycerol + triacylglycerol) and the increase in the phospholipid labeling (phosphatidylcholine + phosphatidylethanolamine). Although the incubation mixture did not contain added CDP-ethanolamine, reproducible labeling of phosphatidylethanolamine was found after incubation (Fig. 1b), as previously observed (8, 19).

The phosphatidylcholine labeling increased very rapidly in the first 10 min of incubation, but much less so from 10 min to 3 hr. The same observation was noted for phosphatidylethanolamine labeling.

The results obtained with parallel incubations of cold membrane diacylglycerol (microsomes B) and labeled CDP-choline are reported in **Table 2.** The figures of column B give a measure of the amount of phosphati-

TABLE 2. Time-course of phosphatidylcholine synthesis in brain microsomes

Incubation Time	A	B	C
min			
2	3.57	0.53	6.67
5	4.54	0.81	5.67
10	5.33	1.20	4.45
15	5.25	1.34	3.91
20	5.79	1.80	3.21
30	6.02	2.22	2.77
60	6.55	3.77	1.56
120	7.05	6.18	1.21
180	8.06	7.86	1.02

The synthesis of phosphatidylcholine was examined under the same experimental conditions from labeled CDP-choline or from membrane-bound labeled diacylglycerol. A: phosphatidylcholine synthesis from endogenously labeled diacylglycerol (nCi/mg protein); B: phosphatidylcholine synthesis from CDP-[methyl-^{14}C]choline (nmol/mg protein); C: specific activity of the newly synthesized glycerol-labeled phosphatidylcholine, calculated as the ratio of the figures A and B, at each incubation time. Experiments repeated three times gave the same results, but only one experiment is reported, due to the variable extent of the labeling of the incubated diacylglycerol.

dylcholine synthesized during incubation of membrane-bound cold diacylglycerol with labeled CDP-choline, while the figures of the column A represent the labeling of phosphatidylcholine from glycerol-labeled endogenous diacylglycerol prepared under the same conditions. The specific activity of the newly formed glycerol-labeled phosphatidylcholine (C) was calculated as the ratio A/B, for each incubation time. A constant decrease of this value with incubation time is evident, as a result of the different time-course of values A and B. In additional experiments, the specific activity of the newly formed phosphatidylcholine was compared with that of the precursor diacylglycerol, after different incubation times with CDP-choline. It is evident from **Table 3** that the specific activity of the glycerol-labeled phosphatidylcholine synthesized during the incubation is higher than that of its precursor diacylglycerol.

The data reported in Tables 2 and 3 indicate that the kinetics of phosphatidylcholine synthesis from labeled membrane diacylglycerol and cold CDP-choline are very different from that measured in the experiments carried out with labeled CDP-choline and unlabeled diacylglycerol (**Fig. 2**). The kinetics of labeled diacylglycerol consumption and that of glycerol-labeled phosphatidylcholine synthesis are almost superimposable, and seem to be compatible with a pseudo first order reaction. On the other hand, as expected from the incorporation data (Table 2, B), the kinetics of phosphatidylcholine synthesis from labeled CDP-choline follows a quite different behavior, and pseudo first order kinetics cannot be considered.

Preincubation of diacylglycerol-labeled microsomes at 37°C for different times does not produce any change in the specific activity of the newly formed phosphatidylcholine (**Table 4**).

The distribution of label among the molecular species of phosphatidylcholine is shown in **Table 5.** The percent labeling of oligoenoic and tetraenoic species decreased up to 10 min of incubation and then increased to about 30% and 46% of the whole class, respectively. As a consequence, the percent labeling of the polyenoic species increased initially until 40% and then decreased to 25% during the last hour of incubation. The label distribution among molecular species of phosphatidylcholine was very similar to the mass distribution of the molecular species of membrane diacylglycerol (8). This suggests that cholinephosphotransferase utilizes the lipid substrates already available in the membrane.

An increase of diacylglycerol availability in the membrane, produced through the glycerol phosphate pathway, increased the reaction rate only in the first minutes of incubation with labeled CDP-choline (**Table 6**). On the contrary, the pretreatment of microsomes with phospholipase C affected the reaction rate considerably in the time interval of 20 min–3 hr, but was less effective during the first 20 min of the reaction (**Fig. 3**).

DISCUSSION

In the present work phosphatidylcholine has been synthesized in rat brain microsomes both from labeled CDP-choline and from labeled endogenous diacylglycerol. The time-course of synthesis strongly depended on the choice of the isotopically labeled precursor. Membrane diacylglycerol labeled from [U-^{14}C]-*sn*-glyc-

TABLE 3. Specific activity of diacylglycerol and of glycerol-labeled phosphatidylcholine during incubation of microsomes with CDP-choline

Incubation Time	Specific Activity		
		Phosphatidylcholine	
	Diacylglycerol	A	B
min	*nCi/nmol*	*Ci/mol*	
0	0.85 ± 0.11 (6)		
5	0.43 ± 0.04 (3)	7.9	7.2
15	0.31 ± 0.02 (3)	3.4	n.d.
30	0.25 ± 0.02 (3)	3.0	2.6
60	0.21 ± 0.02 (3)	1.9	1.9
120	0.22 ± 0.01 (2)	1.5	1.5

Microsomal glycerol-labeled diacylglycerol was incubated with 1 mM CDP-[methyl-^{14}C]choline (sp act 1.07 Ci/mol). The specific activity of membrane diacylglycerol is expressed as nCi/nmol; the concentration was determined fluorimetrically (19). The number of determinations is in brackets. The concentration of diacylglycerol remained nearly constant throughout the whole incubation time, ranging from 8.1 nmol/mg protein at zero time to 7.2 nmol/mg protein (2 hr). A and B refer to two separate determinations.

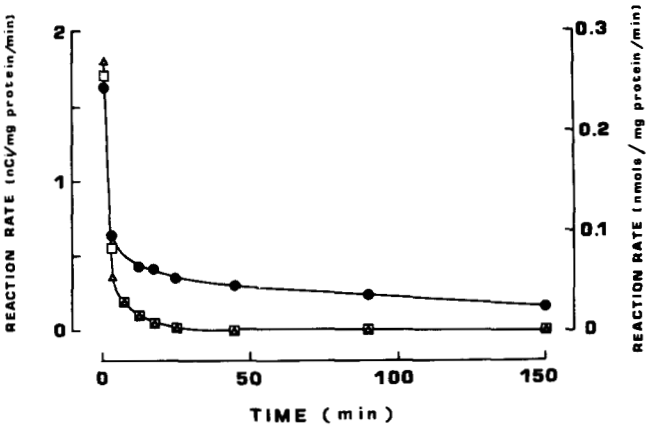

Fig. 2. Kinetics of phosphatidylcholine synthesis and diacylglycerol consumption. (● — ● — ●), Rate of synthesis of phosphatidylcholine from 1 mM CDP-[methyl-^{14}C]choline and cold membrane-diacylglycerol, expressed as nmol/mg protein per min. (□ — □ — □), Rate of synthesis of phosphatidylcholine from 1 mM CDP-choline and membrane-bound labeled diacylglycerol, expressed as nCi/mg protein per min. (Δ — Δ — Δ), Rate of diacylglycerol disappearance during incubation of labeled membrane-bound diacylglycerol with 1 mM CDP-choline, expressed as nCi/mg protein per min. Experiments repeated three times gave the same results. Only one experiment is reported, due to the variable extent of labeling of the incubated microsomes. Reaction rates have been calculated as the average rates between points in the time-course curves.

erol-3-phosphate was almost completely converted into phosphatidylcholine in a few minutes of incubation with unlabeled CDP-choline, although the phospholipid synthesis from labeled CDP-choline and unlabeled membrane diacylglycerol ran for more than 2 hr. As a consequence, the kinetics of phosphatidylcholine synthesis looks very different when the incubations are carried out according to different experimental protocols.

It might be supposed that labeled membrane diacylglycerols are rapidly converted into phospholipid during incubation and that new cold diacylglycerol molecules are produced by degradation of membrane phospholipids. However, this possibility does not seem feasible, since the "slow kinetics" begins when the diacylglycerol concentration in the membrane is still high (Table 3). Moreover, the label distribution among the molecular species of phosphatidylcholine, after incubation of microsomes with CDP-[methyl-^{14}C]choline, is not very different from the mass distribution of membrane diacylglycerol (Table 5 and reference 8).

The specific activities of both labeled diacylglycerol and glycerol-labeled phosphatidylcholine were measured after incubation of microsomes with CDP-choline. The specific activity of the newly formed phosphatidylcholine decreased by increasing the incubation time, and the specific activity of ^{14}C-labeled diacylglycerol was lower than that of the newly formed phosphatidylcholine, but also decreased during incubation (Table 3). This means that ^{14}C-labeled diacylglycerol is converted

TABLE 4. Dependence of the specific activity of the newly formed phosphatidylcholine on the preincubation time

Preincubation Time	A	B	C
min	*nCi*	*nmol*	*nCi/nmol*
0	6.6	1.2	5.5
30	7.8	1.4	5.6
60	7.4	1.3	5.7

Membrane-bound labeled diacylglycerol was synthesized through the glycerol phosphate pathway, as described under Methods (Microsomes A, see Experimental). Membranes were pelleted and resuspended in 80 mM Tris-HCl buffer (pH 8.0). The suspension was preincubated for different lengths of time at 37°C, then supplemented with 1 mM CDP-choline and 10 mM Mn^{2+} and incubated for an additional 20 min. Membrane-bound cold diacylglycerol was synthesized from cold glycerol phosphate in the same membrane preparation (Microsomes B, see Experimental). After preincubation, 10 mM Mn^{2+} and 1 mM CDP-[methyl-^{14}C]choline were supplemented and incubations for 20 min were carried out. A: nCi of glycerol-labeled phosphatidylcholine after incubation of microsomes A with cold CDP-choline. B: nmol of newly-formed phosphatidylcholine measured from incubations of cold microsomes B with labeled CDP-choline. C: specific activity of the newly formed glycerol-labeled phosphatidylcholine (A/B).

into phospholipid at a rate higher than the bulk membrane diacylglycerol, suggesting a sort of compartmentation among labeled and unlabeled pools.

A mixing of these hypothetic pools does not seem to take place very easily. In fact, the preincubation of diacylglycerol-labeled microsomes did not lower the specific activity of the newly formed phospholipid, as shown clearly in Table 4. Such a strong compartmentation can be explained only by a physical separation of the two pools, different from lateral or transverse membrane separation (20, 21).

The changes produced in the kinetics of phosphatidylcholine synthesis from labeled CDP-choline, following different pretreatments of the microsomes, strongly

TABLE 5. The distribution of label among phosphatidylcholine molecular species after incubation of brain microsomes with labeled CDP-choline

Incubation Time	Molecular Species of Phosphatidylcholine[a]		
	Oligoenoic	Tetraenoic	Polyenoic
0–5 min	27.8 ± 1.7	44.6 ± 3.2	26.5 ± 2.3
5–10 min	22.9 ± 1.2	37.4 ± 0.2	39.6 ± 1.4
10–20 min	23.2 ± 0.9	37.2 ± 0.4	39.6 ± 0.5
20–30 min	25.1 ± 1.0	34.3 ± 1.9	40.6 ± 0.9
30–60 min	25.0 ± 0.9	39.1 ± 0.4	35.8 ± 1.4
60–120 min	26.0 ± 1.1	40.5 ± 2.0	33.5 ± 1.0
120–180 min	28.9 ± 1.6	46.1 ± 1.4	25.0 ± 2.0

[a] Data are expressed as percent distribution of the label among the phosphatidylcholine species ± standard deviation (three experiments). Recovery was higher than 95%.

The tubes, containing the microsomal suspension, 1 mM CDP-choline, 50 mM Tris–HCl (pH 8.0), and 10 mM $MnCl_2$, were incubated at 30°C for different times. Each tube was supplemented with CDP-[methyl-^{14}C]choline during the time intervals reported in the table.

TABLE 6. Dependence of the rate of the cholinephosphotransferase reaction upon the membrane diacylglycerol availability (production of diacylglycerol through the glycerol phosphate pathway)

Incubation Time	Experiment 1		Experiment 2	
	A	B	A	B
	nmol PC synthesized · mg^{-1} · min^{-1}			
0–2 min	0.54	0.36	0.58	0.37
2–5 min	0.20	0.19	0.23	0.20
5–10 min	0.13	0.13	0.22	0.14
10–20 min	0.11	0.11	0.13	0.12
20–30 min	0.09	0.10	0.12	0.12
30–60 min	0.09	0.07	0.11	0.09
60–120 min	0.07	0.07	0.09	0.08
120–180 min	0.04	0.07	0.07	0.06

A: Membrane-bound cold diacylglycerol was synthesized through the glycerol phosphate pathway, as described under Experimental, but 1.4 mM glycerol-3-phosphate was incubated. Membranes were pelleted and then incubated with labeled CDP-choline, as described in the text. B: Control microsomes were incubated as in A, but glycerol-3-phosphate was omitted. PC, phosphatidylcholine.

support the compartmentation hypothesis. After digestion of microsomes with phospholipase C, the initial rate of phosphatidylcholine synthesis from labeled CDP-choline increased, as a consequence of the increased diacylglycerol availability. It is evident, however, that the phospholipase treatment affected mostly the "slow kinetics" part of the time curve (Fig. 3). On the other hand, when the diacylglycerol content was increased by incubating microsomes with 1.4 mM glycerol phosphate followed by dephosphorylation of phosphatidic acid, only the fast kinetics of phospholipid synthesis was affected (Table 6).

Two tentative explanations of these experimental evidences can be given. *a*) A sort of lipoprotein complex, containing the complete enzymic capacity for phospholipid synthesis, is segregated from the bilayer structure of the microsomal vesicles, so that a channeling of labeled lipid intermediates along these enzymes can be achieved (22); *b*) microsomal cholinephosphotransferase is contained in only a few microsomal vesicles, which contain also the complete enzymic store for diacylglycerol production from glycerol phosphate. Labeled diacylglycerol is then formed only in these vesicles and is not exchanged with the cold diacylglycerol contained in the others. During incubation of microsomes with cold CDP-choline, the labeled molecules, readily available for the enzyme, are rapidly converted into phospholipid, while the unlabeled diacylglycerol will reach the enzyme only by transfer from other vesicles to those containing the cholinephosphotransferase. This intervesicle transfer could be the rate-limiting step for the overall process.

The second model (*b*) requires that both cholinephosphotransferase and the enzymes that produce diacylglycerol from glycerol phosphate lie in restricted areas of the endoplasmic reticulum. Their closeness in the membrane, in fact, should allow their segregation in the same microsomal vesicle, during the homogenization process.

Both proposed models fit clearly with previously published data (8, 12). In fact, when microsomal phosphatidic acid is formed by acylation of labeled glycerol phosphate and then dephosphorylated to diacylglycerol, the distribution of label among the molecular species of diacylglycerol is very similar to that of phosphatidic acid.

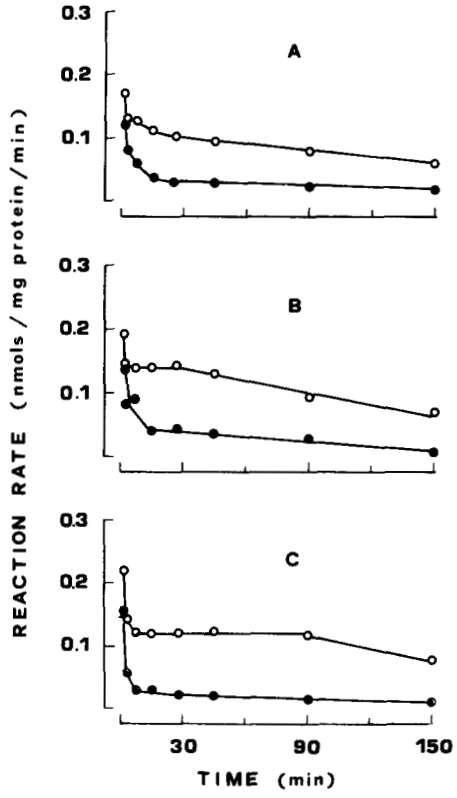

Fig. 3. Dependence of the rate of the cholinephosphotransferase reaction on the membrane diacylglycerol availability: production of diacylglycerol by incubation of microsomes with phospholipase C. A, Microsomes (17 mg of protein) were preincubated for 5 min at 25°C in a medium containing 0.003 I.U. of phospholipase C from *Clostridium welchii*. Incubation was stopped with EDTA. Control microsomes were preincubated in the same medium, but phospholipase C was omitted. After pelleting, the microsomes were resuspended and incubated with labeled CDP-choline, as described in the text. B, Microsomes (17 mg protein) were incubated as in A, but 0.06 I.U. of phospholipase C from *Clostridium welchii* was used. C, Microsomes (15 mg protein) were incubated at 25°C for 5 min in a medium containing 0.4 I.U. of phospholipase C from *Bacillus cereus* in a final volume of 10 ml. Incubations with labeled CDP-choline were carried out as indicated above on preincubated microsomes and in control microsomes. Reaction rates have been calculated as the average rates between points in the time-course curves. The results from three different experiments have not been mediated, due to different levels of diacylglycerol and free fatty acids produced during preincubations. (● — ● — ●), Control microsomes; (○ — ○ — ○), phospholipase-treated microsomes.

However, no evident relationship seems to occur between the specific activity of individual molecular species of phosphatidic acid and the label distribution among the homologous diacylglycerol species (12). Further, after incubation with CDP-choline of microsomal diacylglycerol, labeled through the glycerol phosphate pathway, a similar lack of connection between the specific activity of the diacylglycerol molecular species and the label distribution among the species of synthesized phosphatidylcholine is evident (8).

This investigation was supported in part by grants from the "Consiglio Nazionale delle Richerche, Rome" (Contracts N. 80.00543.04 and 81.0031804.115). Thanks are given to Mr. P. Caligiana for the skillful technical assistance and to Dr. C. A. Palmerini for help and advice in the high pressure liquid chromatography of nucleotides.

Manuscript received 4 June 1981, in revised form 4 March 1982, and in re-revised form 10 May 1982.

REFERENCES

1. Kennedy, E. P., and S. B. Weiss. 1956. The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.* **222:** 193–216.
2. Strickland, K. P., D. Subrahmanyam, E. T. Pritchard, W. Thompson, and R. J. Rossiter. 1963. Biosynthesis of lecithin in brain. Participation of cytidine diphosphate choline and phosphatidic acid. *Biochem. J.* **87:** 128–136.
3. McMurray, W. C. 1964. Metabolism of phosphatides in developing rat brain. I: Incorporation of radioactive precursors. *J. Neurochem.* **11:** 287–299.
4. McCaman, R. E., and K. Cook. 1966. Intermediary metabolism of phospholipids in brain tissue. III: phosphocholine-glyceride transferase. *J. Biol. Chem.* **241:** 3390–3394.
5. Porcellati, G., M. G. Biasion, and N. Pirotta. 1970. The labeling of brain ethanolamine phosphoglycerides from cytidine diphosphate ethanolamine in vitro. *Lipids.* **5:** 734–742.
6. Kanoh, H., and K. Ohno. 1975. Substrate selectivity of rat liver microsomal 1,2-diacylglycerol: CDP-choline (ethanolamine) choline (ethanolamine) phosphotransferase in utilizing endogenous substrates. *Biochim. Biophys. Acta.* **380:** 199–207.
7. Fallon, H. J., J. Barwick, R. G. Lamb, and H. van den Bosch. 1976. Studies on the specificity of the reactions of glycerol lipid biosynthesis in rat liver using membrane-bound substrates. *In* Lipids. Vol. 1. R. Paoletti, G. Porcellati, and G. Jacini, editors. Raven Press, New York. 67–74.
8. Roberti, R., L. Binaglia, and G. Porcellati. 1980. Synthesis of molecular species of glycerolphospholipids from diglyceride-labeled brain microsomes. *J. Lipid Res.* **21:** 449–454.
9. Porcellati, G., G. Arienti, M. G. Pirotta, and D. Giorgini. 1971. Base-exchange reactions for the synthesis of phospholipids in nervous tissue: the incorporation of serine and ethanolamine into the phospholipids of isolated brain microsomes. *J. Neurochem.* **18:** 1395–1417.
10. Porcellati, G., and L. Binaglia. 1976. Metabolism of phosphoglycerides and their molecular species in brain. *In* Lipids. Vol. 1. R. Paoletti, G. Porcellati, and G. Jacini editors. Raven Press, New York. 75–88.
11. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
12. Binaglia, L., R. Roberti, and G. Porcellati. 1978. A study on the turnover of rat brain phosphatidic acid through the glycerol phosphate pathway. *In* Enzymes of Lipid Metabolism. S. Gatt, L. Freysz, and P. Mandel, editors. Plenum Publishing Corporation, New York. 353–366.
13. Binaglia, L., R. Roberti, G. Michal, and G. Porcellati. 1973. The metabolism of phosphoric esters and of cytidine-diphosphate esters of choline and ethanolamine in the liver. *Int. J. Biochem.* **4:** 597–611.
14. Rousseau, A., and S. Gatt. 1979. Interaction of membranous enzymes with membranous lipid substrates. Hydrolysis of diacylglycerol by lipase in rat brain microsomes. *J. Biol. Chem.* **254:** 7741–7745.
15. Wood, R., and R. D. Harlow. 1969. Structural analyses of rat liver phosphoglycerides. *Arch. Biochem. Biophys.* **135:** 272–281.
16. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Arvidson, G. A. E. 1965. Fractionation of naturally occurring lecithins according to degree of unsaturation by thin-layer chromatography. *J. Lipid Res.* **6:** 574–577.
19. Binaglia, L., R. Roberti, and G. Porcellati. 1977. Conversion of individual phosphatidates into rat brain lipid in vitro. *Biochem. Soc. Trans.* **5:** 175–178.
20. Azzi, A., and C. Montecucco. 1977. Spin labels and biological membranes. *In* Horizons in Biochemistry and Biophysics. Vol. 4. Addison-Wesley Publishing Company, Reading, MA. 266–294.
21. Allan, D., P. Thomas, and H. R. Michell. 1978. Rapid transbilayer diffusion of 1,2-diacylglycerol and its relevance to control of membrane curvature. *Nature.* **276:** 289–290.
22. Coleman, R. 1973. Membrane-bound enzymes and membrane ultrastructure. *Biochim. Biophys. Acta.* **300:** 1–30.