The Distribution of Phosphatidylinositol in Microsomal Membranes from Rat Liver after Biosynthesis *de novo*

EVIDENCE FOR THE EXISTENCE OF DIFFERENT POOLS OF MICROSOMAL PHOSPHATIDYLINOSITOL BY THE USE OF PHOSPHATIDYLINOSITOL-EXCHANGE PROTEIN

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1. The phosphatidylinositol-exchange protein from bovine brain was used to determine to what extent phosphatidylinositol in rat liver microsomal membranes is available for transfer. 2. The microsomal membranes used in the transfer reaction contained either phosphatidyl[2-³H]inositol or ³²P-labelled phospholipid. The ³²P-labelled microsomal membranes were isolated from rat liver after an intraperitoneal injection of [³²P]P_i. The ³H-labelled microsomal membranes and rough- and smooth-endoplasmic-reticulum membranes were prepared *in vitro* by the incorporation of *myo*-[2-³H]inositol into phosphatidylinositol by either exchange in the presence of Mn²⁺ or biosynthesis *de novo* in the presence of CTP and Mg²⁺. 3. Tryptic or chymotryptic treatment of the microsomes impaired the biosynthesis *de novo* of phosphatidylinositol. It was therefore concluded that the biosynthesis of phosphatidylinositol and/or its immediate precursor CDP-diacylglycerol takes place on the cytoplasmic surface of the microsomal membrane. 4. Under the conditions of incubation 42% of the microsomal phosphatidyl[2-³H]inositol was transferred with an estimated half-life of 5 min; 38% was transferred with an estimated half-life of about 1 h; the remaining 20% was not transferable. Identical results were obtained irrespective of the method of *myo*-[2-³H]inositol incorporation. 5. Both measurement of phosphatidylinositol phosphorus in the microsomes after transfer and the transfer of microsomal [³²P]phosphatidylinositol indicate that phosphatidyl[2-³H]inositol formed by exchange or biosynthesis *de novo* was homogeneously distributed throughout the microsomal phosphatidylinositol. 6. We present evidence that the slowly transferable pool of phosphatidylinositol does not represent the luminal side of the microsomal membrane; hence we suggest that this phosphatidylinositol is bound to membrane proteins.

Although phosphatidylinositol is a relatively minor constituent of membranes, its metabolism appears to be intimately involved with the cellular response towards electrical and pharmacological stimuli (Hokin, 1969; Hawthorne, 1973; Michell, 1975). To appreciate more fully the role of phosphatidylinositol in membrane receptor function one should know its distribution over, and arrangement in, the two halves of the membrane bilayer. Rothman *et al.* (1976) reported that phosphatidylinositol is present in the outer leaflet of influenza virus. By inference these investigators presumed that phosphatidylinositol has a similar distribution in the plasma membrane of the host bovine kidney cells. On the other hand, most of the phosphatidylinositol in sheep erythrocyte membrane (Low & Finean, 1977; Billington *et al.*, 1977)

and the surface membrane of human blood platelets (Chap *et al.*, 1977) is present in the inner leaflet.

van Golde *et al.* (1974) have suggested that the biosynthesis of phosphatidylinositol occurs primarily in the endoplasmic reticulum of rat liver (van Golde *et al.*, 1974). Thus the plasma membrane, for example, depends on the endoplasmic reticulum for its supply of phosphatidylinositol. This raises the question where phosphatidylinositol is localized in the endoplasmic reticulum and on what side of the membrane the biosynthesis occurs. Studies by Nilsson & Dallner (1977*a,b*) on the phospholipid asymmetry in subcellular membranes of rat liver indicated that about 10% of the microsomal phosphatidylinositol is accessible to phospholipase A₂ from *Naja naja* venom. Similarly, a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* degraded up to a maximum of 15% (Low & Finean, 1976). Approx. 15% of the phosphatidylinositol in

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rat brain microsomes was detectable by anti-(phosphatidylinositol) antibody (Guarnieri, 1975).

The above studies suggest that the phosphatidylinositol pool on the cytoplasmic side of the endoplasmic reticulum is very limited. In spite of this apparently asymmetric distribution, the cytoplasm of a variety of bovine and rat tissues contain phospholipid-exchange proteins that catalyse the transfer of phosphatidylinositol between membranes (Helmkamp *et al.*, 1974, 1976; Lumb *et al.*, 1976; Bloj & Zilversmit, 1977). It is a property of phospholipid-exchange proteins to interact only with those phospholipids of the membrane bilayer that are in direct contact with the bulk solution, i.e. phospholipids in the outer half of the bilayer (Bloj & Zilversmit, 1976; Rothman *et al.*, 1976; Barsukov *et al.*, 1976). In addition, Demel *et al.* (1977) have demonstrated that the phosphatidylinositol-exchange protein from bovine brain stimulates the net transfer of phosphatidylinositol to membranes devoid of this phospholipid.

In the present study we have used the phosphatidylinositol-exchange protein from bovine brain to determine what fraction of the microsomal phosphatidylinositol is available for transfer after synthesis *in vitro* and *in vivo*. In addition, we have established whether synthesis of phosphatidylinositol can occur on the cytoplasmic side of the microsomal membrane.

Experimental

Materials

Egg-yolk phosphatidylcholine, phosphatidic acid and phosphatidylinositol from rat liver were prepared as previously described (Wirtz *et al.*, 1976a). Trypsin was obtained from Merck (Darmstadt, Germany), and chymotrypsin and CTP were from Boehringer (Mannheim, Germany). Carrier-free sodium ortho[^{32}P]phosphate was purchased from Philips Duphar (Amsterdam, Holland); *myo*-[2- ^3H]inositol (2.8 mCi/ μmol) and cholesteryl [1- ^{14}C]oleate (10.4 mCi/mmol) were from The Radiochemical Centre (Amersham, Bucks., U.K.). Phosphatidylinositol-exchange protein was purified from bovine brain as described by Demel *et al.* (1977), except that the final isoelectric-focusing step was omitted. Protein was stored at -20°C in 50% (v/v) glycerol.

Methods

Preparation of microsomal membranes containing radioactively labelled phosphatidylinositol. Rat liver was homogenized in 0.25 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, and the microsomal fraction isolated as described previously (Wirtz & Zilversmit, 1969). Microsomes containing [^{32}P]phospholipids

were prepared by an intraperitoneal injection of [^{32}P]P_i (1 mCi) into each rat 16 h before decapitation. The microsomal fraction was washed once by resuspension in the sucrose/EDTA/Tris buffer.

A microsomal fraction containing phosphatidyl-[2- ^3H]inositol was prepared by *myo*-[2- ^3H]inositol exchange as described previously (Helmkamp *et al.*, 1974). After the initial sedimentation the microsomal pellet was resuspended in 20 mM-Tris/HCl, pH 7.4, and incubated with 10 mM-MnCl₂ and *myo*-[2- ^3H]inositol (5 μM ; 1.5 μCi /mg of microsomal protein). After 90 min at 37°C , the incubation was stopped by sedimentation of the microsomal membranes at 37000g for 30 min. The microsomal membranes were washed twice by resuspension in the sucrose/EDTA/Tris buffer.

Microsomal membranes containing phosphatidyl-[2- ^3H]inositol were prepared by synthesis *de novo* as follows. A microsomal fraction was isolated from rat livers homogenized in 0.25 M-sucrose/16.6 mM-potassium phosphate, pH 7.4; rough- and smooth-endoplasmic-reticulum fractions were prepared from livers homogenized in 0.25 M-sucrose (Dallner, 1974). Each fraction was resuspended in the sucrose/phosphate buffer (2 mg of protein/ml) with optimum concentrations of *myo*-[2- ^3H]inositol (0.5 mM, 5 $\mu\text{Ci}/\mu\text{mol}$), CTP (1.5 mM) and MgCl₂ (10 mM). After incubation for periods up to 90 min at 37°C , the membranes were re-isolated and washed in sucrose/EDTA/Tris as described above for membranes labelled by inositol exchange. T.l.c. of a lipid extract from the microsomes labelled *in vitro* with *myo*-[2- ^3H]inositol indicated that the total ^3H radioactivity in microsomes is accounted for by phosphatidyl[^3H]inositol (methods described below).

All membrane preparations were resuspended in sucrose/EDTA/Tris (about 10 mg of protein/ml) and used the same day, stored at 4°C overnight or at -20°C for longer periods.

Proteolytic treatment of microsomal membranes. Microsomal membranes were prepared after homogenization of livers in 0.25 M-sucrose/16.6 mM-potassium phosphate, pH 7.4, and the microsomal fraction was washed once by sedimentation of the membranes at 105000g_{av.} for 60 min, followed by resuspension in the same buffer. Trypsin or chymotrypsin (0.1 mg of enzyme protein/mg of microsomal protein) were incubated with microsomal membranes (9.6 mg of microsomal protein) in a total volume of 3 ml of sucrose/potassium phosphate buffer, pH 7.4, at 30°C as described by Nilsson & Dallner (1977a). Microsomal suspensions, including controls that contained no proteinase, were incubated for different periods up to 90 min; the membranes were subsequently sedimented at 105000g_{av.} for 120 min. The protein content of the supernatants was measured as described below and the microsomal pellets were resuspended in 3 ml of 0.25 M-sucrose/16.6 mM-

potassium phosphate, pH 7.4. A portion of the microsomal suspension (0.94 ml) was incubated for 60 min at 37°C in a total volume of 1 ml to assay phosphatidylinositol synthesis *de novo* as described above.

Measurement of phosphatidylinositol transfer from microsomal membranes. The measurement of phosphatidylinositol transfer from microsomal membranes containing phosphatidyl[2-³H]inositol to sonicated liposomal acceptor membranes in the presence or absence of exchange protein has been described in detail elsewhere (Helmkamp *et al.*, 1974). Sonicated liposomes were prepared from egg phosphatidylcholine and egg phosphatidic acid (49:1 molar ratio) and included a trace of cholesteryl [1-¹⁴C]oleate as an internal marker for the recovery of liposomes after incubation and extraction (generally 80–90%). The purity of the phospholipids was checked before and after sonication to ensure that no lysophospholipids were formed during this process. Assays were conducted at 37°C and usually contained 1.5 mg of microsomal protein, 6 μmol of liposomal phospholipid and 81 μg of exchange protein in a total volume of 3.0 ml of 0.25 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4. Further addition of liposomes and exchange protein were made as indicated in the Results section. Liposomes were recovered from the incubation mixtures and their content of radioactively labelled phosphatidylinositol was measured as described by Helmkamp *et al.* (1974).

In those experiments in which the transfer of [³²P]phosphatidylinositol from ³²P-labelled microsomal membranes was studied, the assay conditions were similar to those described above. After extraction of the liposomal lipids half of the extract was used to determine the ³²P and ¹⁴C radioactivity. By comparison with the ¹⁴C label in the liposomes before incubation, the total transport of [³²P]phospholipid was calculated. After addition of carrier phosphatidylinositol (0.13 μmol) to the other half of the extract, [³²P]phosphatidylinositol was separated by t.l.c. and the radioactivity determined as described below. Transport to the liposomes was expressed as the fraction of [³²P]phosphatidylinositol present in the microsomes before incubation.

Lipid and protein determinations. Lipids from microsomal membranes and liposomes were extracted by the method of Bligh & Dyer (1959); the lipid extracts were washed as described previously (Helmkamp *et al.*, 1974). The phospholipids were separated by two-dimensional t.l.c. by the method of Broekhuysse (1969) and detected by iodine staining. Phosphatidylinositol is resolved from phosphatidylserine in this system. Ninhydrin was used to detect phosphatidylserine in the instances where the resolution was less distinct. Spots containing [³²P]-phosphatidylinositol were scraped off into counting

vials and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer as described by Helmkamp *et al.* (1974). For phosphatidyl[2-³H]inositol the lipid was eluted from the silica gel with chloroform/methanol (1:2, v/v) and the radioactivity determined. Phosphorus was determined directly on the silica-gel scrapings by the method of Rouser *et al.* (1970).

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Incorporation of myo[2-³H]inositol into microsomal phosphatidylinositol

The incorporation of *myo*-inositol into phosphatidylinositol of liver microsomal membranes *in vitro* can occur by synthesis *de novo* and by inositol exchange (Agranoff *et al.*, 1958; Paulus & Kennedy, 1960). Synthesis *de novo* is a two-step reaction that requires CTP for the formation of CDP-diacylglycerol by the CTP-phosphatidate cytidyltransferase (EC 2.7.7.41); CDP-diacylglycerol is the immediate precursor of phosphatidylinositol in the second step, which is catalysed by CDP-diacylglycerol-*myo*-inositol phosphatidyltransferase (EC 2.7.8.11). In agreement with these earlier results we found that incorporation of *myo*-[2-³H]inositol into phosphatidylinositol in the presence of Mg²⁺ will only occur if CTP is included in the incubation mixture. By contrast, the *myo*-inositol-exchange reaction can take place in the absence of CTP, yet requires Mn²⁺. After incorporation of *myo*-[2-³H]inositol by synthesis *de novo* or exchange as described in the Experimental section the specific radioactivity of microsomal phosphatidylinositol was 2500 ± 500 and 9600 ± 1900 d.p.m./nmol respectively (mean ± s.d.; three experiments). The phospholipid content of the rat liver microsomes after incorporation of *myo*-[2-³H]inositol was 604 ± 50 nmol/mg of protein, of which the major components were: phosphatidylcholine, 348 ± 16 nmol; phosphatidylethanolamine, 98 ± 15 nmol; and phosphatidylinositol, 60 ± 10 nmol (means ± s.d.; four different microsomal preparations).

Effect of proteolysis on synthesis of phosphatidylinositol de novo

Nilsson & Dallner (1977a) have shown that trypsin and chymotrypsin do not affect the permeability barrier of the microsomal membranes to macromolecules, so that only those proteins on the cytoplasmic side of the microsomal vesicles are subject to proteolysis. In this way they demonstrated that several microsomal enzymes are distributed asym-

Fig. 1. Effect of chymotrypsin and trypsin on protein content and phosphatidylinositol biosynthesis in microsomal membranes

Microsomal membranes were incubated with either chymotrypsin or trypsin (0.1 mg/mg of microsomal protein) for different times at 30°C as described in the Experimental section. Incubations were stopped by sedimentation of the microsomal membranes at 105 000 g_{av} for 120 min at 4°C. Protein was measured in the supernatants and phosphatidylinositol biosynthesis *de novo* was measured in the microsomal membranes after resuspension as described in the Experimental section. After proteolysis, microsomal protein content (○, chymotrypsin; △, trypsin) and phosphatidylinositol-biosynthetic activity (●, chymotrypsin; ▲, trypsin) are expressed as a percentage of control values for membranes incubated under the same conditions but in the absence of proteinases.

metrically in the transverse plane of the membranes. Fig. 1 shows the result of a similar study on the susceptibility to proteolysis of the enzymes responsible for phosphatidylinositol synthesis *de novo*. After incubation for different times with either trypsin or chymotrypsin (0.1 mg/mg of microsomal protein) at 30°C, microsomal membranes were re-isolated by centrifugation and their ability to catalyse phosphatidylinositol biosynthesis was measured as described in the Experimental section. Values are expressed as a percentage of those obtained for control incubations. In the absence of trypsin or chymotrypsin the decrease in the control values was less than 10%. Fig. 1 shows that both trypsin and chymotrypsin treatment impairs the ability of microsomal membranes to synthesize phosphatidylinositol *de novo*. Within 10 min tryptic proteolysis caused the loss of almost 100% of the original microsomal activity. A residual activity of 15% was preserved after a prolonged chymotryptic

treatment. Both proteinases eventually released 45% of the microsomal protein. Hence it is likely that one or both of the enzymes involved in the biosynthesis of phosphatidylinositol are localized on the cytoplasmic side of the microsomal membranes. It is of course possible that enzymic activity may also be present on the luminal side of the microsomal vesicles; nevertheless it is evident that such activity, if present, makes no contribution to phosphatidylinositol synthesis under our conditions. Next we wished to investigate how phosphatidylinositol formed by enzyme(s) localized on the cytoplasmic side of the microsomal membrane is distributed in this membrane. For this purpose we used the phosphatidylinositol-exchange protein from bovine brain.

Accessibility of microsomal phosphatidylinositol

Under the assay conditions used the phosphatidylinositol-exchange protein transfers phosphatidylinositol from microsomes to sonicated liposomes of phosphatidylcholine and phosphatidic acid (49:1 molar ratio). This implies that in the course of incubation the microsomes become depleted of phosphatidylinositol. To ensure the maximal transfer of microsomal phosphatidylinositol we first wished to know what amounts of exchange protein and liposomal acceptor membranes were required in the assay. The result of one such experiment is shown in Fig. 2(a), where microsomal membranes (1.5 mg of protein) in which *myo*-[2-³H]inositol has been incorporated by exchange, were incubated with 27 μg of exchange protein and 2 μmol of liposomal phospholipid. Further additions of the same amounts of exchange protein and liposomes were made after 60 min and 120 min. It appears that the additions at 120 min have little effect on the transfer. The results therefore suggest that a total of 81 μg of exchange protein and 6 μmol of liposomal phospholipid suffice to remove a maximal amount of phosphatidylinositol from the microsomes. Hence these amounts were used to investigate what fraction of the microsomal phosphatidyl[2-³H]inositol after biosynthesis *de novo* was accessible to the exchange protein (Fig. 2b). It is clear that when 80% of the microsomal phosphatidyl[2-³H]inositol had been removed, transfer became extremely slow; in six other experiments the upper value of 80% was not exceeded. Further addition of 27 μg of exchange protein and 2 μmol of liposomal phospholipid at the times indicated by the arrows ensured that the 80% value was determined by the microsomal membranes. It is noteworthy that identical results were obtained with microsomal fractions that contained phosphatidylinositol radioactively labelled by *myo*-[2-³H]inositol exchange. As indicated in Fig. 2(b), transfer of phosphatidyl[2-³H]inositol in the absence of exchange protein was 10% after 3 h of incubation. A further feature

Fig. 2. Effect of concentration of liposomal acceptor membrane and exchange protein on transfer of phosphatidyl[2-³H]inositol from microsomal membranes

Transfer was measured from membranes of the microsomal fraction to sonicated liposomes as described in the Experimental section. Each incubation mixture contained 1.5 mg of microsomal protein in which phosphatidylinositol had been radioactively labelled by either *myo*-inositol exchange (○) or by synthesis *de novo* (△) for 90 min at 37°C. Exchange protein (27 μg) and liposomal phospholipid (2 μmol) were added to start the transfer of phosphatidylinositol shown in Fig. 2(a). Three times this amount of exchange protein (81 μg) and liposomes (6 μmol) were added to start the transfer depicted in Fig. 2(b). Further additions of exchange protein (27 μg) together with liposomal phospholipid (2 μmol) are indicated by the arrows. The filled symbols represent values for phosphatidylinositol [labelled by exchange (●) or synthesis *de novo* (▲)] transfer in the absence of exchange protein.

with phosphatidyl[³H]inositol. This raises the possibility that the biphasic appearance of the phosphatidylinositol transfer reflects a different behaviour of the smooth- and rough-endoplasmic-reticulum membranes in the transfer assay. Therefore, rough- and smooth-endoplasmic-reticulum fractions were isolated and labelled independently with phosphatidyl[³H]inositol by synthesis *de novo* as described in the Experimental section. Phosphatidylinositol extracted from the rough- and smooth-membrane fractions (1 mg of protein) contained 26000 and 13000 d.p.m. respectively. The membrane fractions (1.5 mg of protein) were incubated with an excess of liposomal phospholipid (10 μmol) and pure exchange protein (41 μg) and the transfer of phosphatidyl[³H]inositol was measured with time as described in the Experimental section. Both fractions gave results that were essentially similar to those obtained with the total microsomal fraction. This supports the conclusion that the different phosphatidyl[³H]inositol pools are characteristic for the endoplasmic-reticulum membranes in the microsomal fraction.

Rothman & Lenard (1977) have pointed out that it is essential that all pools of a given phospholipid have the same specific radioactivity when studying the distribution of radioactive lipids in membranes. This was checked in the present system by isolation of phospholipids from microsomal membranes before and after phosphatidylinositol transfer. Measurements of the quantity of phosphatidylinositol remaining in the membranes after transfer were corrected for losses of lipids incurred during the analytical procedure. Losses were estimated by measuring the amount of phosphatidylethanolamine recovered, since this phospholipid is not transferred from microsomal membranes by the phosphatidylinositol-exchange proteins of bovine brain (Helmkamp *et al.*, 1974). After 78% depletion of microsomal phosphatidyl[2-³H]inositol from membranes exchange-labelled with *myo*-inositol and 72% depletion of membranes labelled by phosphatidylinositol biosynthesis *de novo*, the corresponding values for depletion of microsomal phosphatidylinositol phosphorus were 72% and 63% respectively. These results show that the phosphatidyl[2-³H]inositol transferred from the membranes does not represent a specific fraction or pool into which *myo*-[2-³H]inositol is particularly incorporated.

To confirm that the behaviour of phosphatidyl[2-³H]inositol is a true reflection of the total microsomal phosphatidylinositol, microsomal membranes containing [³²P]phospholipids as described in the Experimental section were prepared. Nilsson & Dallner (1977a,b) have shown that phospholipids in such membranes are uniformly labelled with ³²P. Incubation of these microsomes (1.9 mg of protein) with sonicated liposomes (2 μmol of phospholipid phosphorus) and 27 μg of exchange protein resulted

of great interest is the biphasic appearance of phosphatidylinositol transfer, namely a fast phase and a slow phase independent of the exchange protein and liposomal acceptor membranes that are present in excess. By extrapolation of the slow transfer to zero time, it is estimated that approx. 42% of the microsomal phosphatidyl[³H]inositol is transferred with a half-life of 5 min. The remaining 38% becomes available for transfer with an estimated half-life of about 1 h (Fig. 2b). Three different experiments gave essentially the same results. In each experiment no difference was found for microsomal fractions labelled by either base exchange or synthesis *de novo*.

Under our conditions of synthesis *de novo*, both the rough- and smooth-endoplasmic-reticulum membranes in the microsomal fraction become labelled

in a transfer of 30–32% of the [^{32}P]phosphatidylinositol after 60 min (two experiments). This compares very well with the transfer of 34% shown in Fig. 2(a) for the membranes exchange-labelled with *myo*-inositol and incubated under identical conditions. From these results, it appears to be justified to assume that *myo*-[2- ^3H]inositol incorporated by either *myo*-inositol exchange or by biosynthesis *de novo* is homogeneously distributed throughout the phosphatidylinositol of the endoplasmic reticulum.

Nature of the slow phase of phosphatidylinositol transfer

Although 80% of the microsomal phosphatidylinositol is available for protein-mediated transfer, the results suggest that 42% of the lipid is present in an easily accessible pool; transfer of the remaining 38% is a much slower event. In view of the current membrane model one could assume that 38% of the phosphatidylinositol is present in the inner leaflet of the bilayer. The slow phase of transfer would then represent the rate of movement ('flip-flop') of phosphatidylinositol from the inside to the outside of the microsomal membrane. To test this hypothesis, microsomes containing phosphatidyl[2- ^3H]inositol by synthesis *de novo* were incubated with 6 μmol of liposomal phospholipid and 81 μg of exchange protein for 40 min to remove the easily accessible phosphatidylinositol pool (Fig. 3). After this incubation the microsomes were isolated by centrifugation as described in the Experimental section and re-suspended in 0.25 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4. For phosphatidylinositol of the inner leaflet to equilibrate with the outer leaflet, the depleted microsomes were preincubated at 37°C for 60 min, whereupon 6 μmol of liposomal phospholipid and 81 μg of exchange protein were added and the incubation was continued. As a control, microsomes were incubated under identical conditions without the preincubation. The results in Fig. 3 show that preincubation has no effect on the transfer of phosphatidylinositol. Identical results were obtained from the same experiment carried out with microsomes that were labelled by base-exchange. The absence of an induced pool of easily accessible phosphatidylinositol argues against the hypothesis that the slow phase of transfer reflects the movement of phosphatidylinositol from the inner- to the outer-membrane leaflet. In one series of experiments incubation was continued up to 90 min without interruption, except that an additional amount of liposomes and exchange protein was added at 40 min. Fig. 3 shows that transfer is virtually identical, independent of the incubation conditions.

When 40% of the microsomal phosphatidylinositol was removed, the microsomes were re-isolated, suspended in sucrose/EDTA/Tris and sonicated for

2.5 min at 70 W with a Branson tip sonifier. It was observed that sonication had no effect on the transfer of the residual phosphatidylinositol. This is further evidence that the slowly available phosphatidylinositol is not present in the inner-membrane leaflet, since one would expect sonication to randomize the residual phosphatidylinositol in a bilayer. In addition, the distribution of phosphatidylinositol between the two pools discussed so far was not affected by storage of radioactively labelled microsomes at -20°C .

Fig. 3. Effect of preincubation on the degree of phosphatidylinositol transfer from microsomal membranes *myo*-[2- ^3H]inositol (5 $\mu\text{Ci}/\mu\text{mol}$) was incorporated into microsomal phosphatidylinositol by biosynthesis *de novo* for 15 min at 37°C as described in the Experimental section. Microsomal membranes (1.5 mg of protein) were incubated at 37°C with exchange protein (81 μg) and liposomal phospholipid (6 μmol) and phosphatidylinositol transfer was measured (○); after 40 min, a further 6 μmol of liposomal phospholipid and 81 μg of exchange protein were added and the incubation was continued (●). Another portion of the microsomal fraction (24 mg of protein) was incubated under the same conditions with the same proportions of liposomes and exchange protein. After 40 min, the microsomes were re-isolated by sedimentation at 105 000 g_{av} for 45 min at 4°C and the pellet was resuspended in 8 ml of 0.25 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4. The resuspended microsomes (1.5 mg of protein) were incubated in 2 ml of sucrose/EDTA/Tris at 37°C for 60 min, after which time the usual amounts of liposomes and exchange protein were added and the transfer was measured (▲). In a parallel experiment the transfer of phosphatidylinositol was measured without preincubation of the microsomes (△).

Discussion

The endoplasmic reticulum in animal cells is the principal site of phospholipid biosynthesis (Dawson, 1973; McMurray, 1973; van den Bosch, 1974). In view of recent evidence (Nilsson & Dallner, 1977a) that phospholipids in the endoplasmic reticulum are asymmetrically distributed, it is important to identify the site of phospholipid biosynthesis in the membrane; such knowledge may help to clarify how this asymmetric distribution comes about. The present study is an attempt to determine with the phosphatidylinositol-exchange protein from bovine brain the localization of phosphatidylinositol in the microsomal fraction from rat liver after synthesis *de novo*.

Studies with phospholipases (Nilsson & Dallner, 1977a,b; Low & Finean, 1976) and anti-(phosphatidylinositol) antibody (Guarnieri, 1975) have suggested that about 85% of the microsomal phosphatidylinositol was localized on the luminal side. From the results of the trypsin and chymotrypsin treatment, however, it is evident that the biosynthesis of phosphatidylinositol can occur on the cytoplasmic side of the endoplasmic reticulum. Previous studies have demonstrated that CDP-diacylglycerol is the putative intermediate in the synthesis *de novo* (Agranoff *et al.*, 1958; Paulus & Kennedy, 1960; Thompson *et al.*, 1963; Prottey & Hawthorne, 1967). Our data do not provide an answer to whether CTP-phosphatidatecytidyltransferase (EC 2.7.7.41) and/or CDP-diacylglycerol-*myo*-inositol phosphatidyltransferase (EC 2.7.8.11) are inactivated by the proteolytic treatment. The cytoplasmic localization of CTP-phosphatidatecytidyltransferase, however, would be expected from the fact that CTP is a cytoplasmic constituent for which the endoplasmic reticulum is probably impermeable (Nilsson *et al.*, 1973). While the present study was in progress, it was reported that the synthesis of phosphatidylcholine and phosphatidylethanolamine occurs on the cytoplasmic side of microsomal vesicles (Coleman & Bell, 1978; Vance *et al.*, 1977).

Zilversmit & Hughes (1977) demonstrated with a number of phospholipid-exchange proteins that after homogeneous labelling of rat liver microsomes *in vivo* with [³²P]P_i, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol behaved as a single pool and 85–95% of all these phospholipids were exchangeable after 1–2 h of incubation. It was indicated that the exchangeability of phosphatidylinositol varied between 60 and 100% and depended somewhat on the exchange protein. In the present study it is shown with the phosphatidylinositol-exchange protein from bovine brain that microsomal phosphatidylinositol formed *in vitro* by synthesis *de novo* was partitioned between three pools. One pool of about 40% consisted of phosphatidylinositol that was easily accessible to the exchange

protein; a second pool of another 40% was transferred at a much slower rate and a third pool was not transferable. In addition it appeared that the newly synthesized phosphatidylinositol was homogeneously distributed over the total microsomal pool.

At present we cannot make a definite statement on the localization of these various pools in the microsomal membrane. In view of the non-perturbing nature of phospholipid-exchange proteins it is presumed that the easily accessible pool represents phosphatidylinositol exposed to the exchange protein on the cytoplasmic side of the microsomal vesicles. Zilversmit & Hughes (1977) have interpreted their data to indicate that most of the microsomal phospholipid is subject to rapid translocation over the microsomal membrane. This would imply that phosphatidylinositol can be present on both sides of the microsomal membrane and yet be easily accessible to the exchange protein. For reasons not yet understood, phospholipases would limit their action to the phosphatidylinositol that is actually present on the cytoplasmic side (Low & Finean, 1976; Nilsson & Dallner, 1977a,b).

The phosphatidylinositol that is transferred at a much slower rate by the exchange protein does not appear to represent the pool on the luminal side of the microsomal vesicles. This suggests that the microsomal membrane contains a substantial amount of phosphatidylinositol with a limited ability to interact with the exchange protein. The slow rate of transfer may reflect the gradual release of phosphatidylinositol bound to membrane protein into the easily accessible pool. In a study on the localization of phosphatidylglycerol in *Acholeplasma laidlawii* it was evident that part of the phosphatidylglycerol was not accessible to phospholipases (Bevers *et al.*, 1977). It was suggested that interaction with membrane-bound proteins might protect against enzymic hydrolysis. Lipid domains surrounding proteins have been shown in several types of membranes (Jost *et al.*, 1973; Trauble & Overath, 1973; Stier & Sackmann, 1973; Warren *et al.*, 1974). Exchange of spin-labelled phosphatidylcholine between boundary lipid and bilayer domains in reconstituted membranes containing cytochrome oxidase has been demonstrated (Jost *et al.*, 1977). There is also evidence that phospholipids as components of membrane lipoprotein structures are less exchangeable (Bergelson & Barsukov, 1977).

It is of considerable interest that phosphatidylinositol formed *in vitro* by enzyme(s) present on the cytoplasmic side of the microsomal membrane is partitioned between different pools that reflect the distribution of the bulk of the microsomal phosphatidylinositol. This suggests that it is the membrane proteins that control the distribution of phosphatidylinositol, and not so much the physical constraints imposed by the membrane bilayer (Sheetz & Singer,

1974; Bretscher & Raff, 1975; Rothman & Lenard, 1977). Similarly it can be argued that the different amounts of phosphatidylinositol in the various subcellular membranes is determined to a large extent by the membrane proteins present (Colbeau *et al.*, 1971). The phosphatidylinositol-exchange proteins would function as the vehicles by which the various subcellular membranes are continuously supplied with phosphatidylinositol (Helmkamp *et al.*, 1976; Wirtz *et al.*, 1976b).

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References

- Agranoff, B. W., Bradley, R. M. & Brady, R. O. (1958) *J. Biol. Chem.* **233**, 1077–1083
- Barsukov, L. I., Kulikov, V. I. & Bergelson, L. D. (1976) *Biochim. Biophys. Res. Commun.* **71**, 704–711
- Bergelson, L. D. & Barsukov, L. I. (1977) *Science* **197**, 224–230
- Beyers, E. M., Singal, S. A., Op den Kamp, J. A. F. & van Deenen, L. L. M. (1977) *Biochemistry* **16**, 1290–1295
- Billington, D., Coleman, R. & Lusak, Y. A. (1977) *Biochim. Biophys. Acta* **466**, 526–530
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Bløj, B. & Zilversmit, D. B. (1976) *Biochemistry* **14**, 1277–1283
- Bløj, B. & Zilversmit, D. B. (1977) *J. Biol. Chem.* **252**, 1613–1619
- Bretscher, M. S. & Raff, M. C. (1975) *Nature (London)* **258**, 43–49
- Broekhuysse, R. M. (1969) *Clin. Chim. Acta* **23**, 457–461
- Chap, H. J., Zwaal, R. F. A. & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta* **467**, 146–164
- Colbeau, A., Nachbaur, J. & Vignais, P. M. (1971) *Biochim. Biophys. Acta* **249**, 462–492
- Coleman, R. & Bell, R. M. (1978) *J. Cell Biol.* **76**, 245–253
- Dallner, G. (1974) *Methods Enzymol.* **31**, 191–201
- Dawson, R. M. C. (1973) *Sub-Cell. Biochem.* **2**, 69–89
- Demel, R. A., Kalsbeek, R., Wirtz, K. W. A. & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta* **466**, 10–22
- Guarnieri, M. (1975) *Lipids* **10**, 294–298
- Hawthorne, J. N. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 423–440, Elsevier, Amsterdam
- Helmkamp, G. M., Harvey, M. S., Wirtz, K. W. A. & van Deenen, L. L. M. (1974) *J. Biol. Chem.* **249**, 6382–6389
- Helmkamp, G. M., Nelemans, S. A. & Wirtz, K. W. A. (1976) *Biochim. Biophys. Acta* **318**, 313–325
- Hokin, L. E. (1969) *Ann. N.Y. Acad. Sci.* **165**, 695–709
- Jost, P. C., Griffith, O. H., Capaldi, R. A. & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 480–484
- Jost, P. C., Nadakavukaren, K. K. & Griffith, O. H. (1977) *Biochemistry* **16**, 3110–3114
- Low, M. G. & Finean, J. B. (1976) *Biochem. J.* **154**, 203–208
- Low, M. G. & Finean, J. B. (1977) *Biochem. J.* **162**, 235–240
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lumb, R. H., Kloosterman, A. D., Wirtz, K. W. A. & van Deenen, L. L. M. (1976) *Eur. J. Biochem.* **69**, 15–22
- McMurray, W. C. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 205–251, Elsevier, Amsterdam
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Nilsson, O. S. & Dallner, G. (1977a) *J. Cell Biol.* **72**, 568–583
- Nilsson, O. S. & Dallner, G. (1977b) *Biochim. Biophys. Acta* **464**, 453–458
- Nilsson, R., Peterson, E. & Dallner, G. (1973) *J. Cell Biol.* **56**, 762–776
- Paulus, H. & Kennedy, E. P. (1960) *J. Biol. Chem.* **235**, 1303–1311
- Prottey, C. & Hawthorne, J. N. (1967) *Biochem. J.* **105**, 379–392
- Rothman, J. E. & Lenard, J. (1977) *Science* **195**, 743–753
- Rothman, J. E., Tsai, D. K., Dawidowicz, E. A. & Lenard, J. (1976) *Biochemistry* **15**, 2361–2370
- Rouser, G., Fleischer, S. & Yamamoto, A. (1970) *Lipids* **5**, 494–496
- Sheetz, M. P. & Singer, S. J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4457–4461
- Stier, A. & Sackmann, E. (1973) *Biochim. Biophys. Acta* **311**, 400–408
- Thompson, W., Strickland, K. P. & Rossiter, R. J. (1963) *Biochem. J.* **87**, 136–142
- Trauble, H. & Overath, P. (1973) *Biochim. Biophys. Acta* **307**, 491–512
- Vance, D. E., Choy, P. C., Farren, S. B., Lim, P. H. & Schneider, W. J. (1977) *Nature (London)* **270**, 268–269
- van den Bosch, H. (1974) *Annu. Rev. Biochem.* **43**, 243–277
- van Golde, L. M. G., Raben, J., Batenburg, J. J., Fleischer, B., Zambrano, F. & Fleischer, S. (1974) *Biochim. Biophys. Acta* **360**, 179–192
- Warren, G. B., Toon, P. A., Birdsall, W. J. H., Lee, A. G. & Metcalfe, J. C. (1974) *Biochemistry* **13**, 5501–5507
- Wirtz, K. W. A. & Zilversmit, D. B. (1969) *Biochim. Biophys. Acta* **193**, 105–116
- Wirtz, K. W. A., Geurts van Kessel, W. S. M., Kamp, H. H. & Demel, R. A. (1976a) *Eur. J. Biochem.* **61**, 515–523
- Wirtz, K. W. A., Jolles, J., Westerman, J. & Neys, F. (1976b) *Nature (London)* **260**, 354–355
- Zilversmit, D. B. & Hughes, M. E. (1977) *Biochim. Biophys. Acta* **469**, 99–110