Proc. Natl. Acad. Sci. USA
Vol. 74, No. 8, pp. 3350–3354, August 1977
Biochemistry

Evidence against phospholipid asymmetry in intracellular membranes from liver*

(phospholipases/isethionyl acetimidate/microsomes/Golgi vesicles/phospholipid vesicles)

ROGER SUNDLER, SHARON L. SARCIONE, ALFRED W. ALBERTS, AND P. ROY VAGELOS

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065

Contributed by P. Roy Vagelos, June 9, 1977

ABSTRACT **We have studied the distribution of phospholipids across the membrane of microsomal vesicles and Golgi-derived secretory vesicles from rat liver by the use of phospholipases. Model studies on single-bilayer phospholipid vesicles showed that phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) cleaved at least 80% of the lipids on the outer surface of such vesicles without significant attack on the inner surface. In microsomal vesicles approximately 40% of the outer surface phospholipids were cleaved before the enzyme gained access to the interior of the vesicles. The same conclusion was reached for Golgi vesicles. By following the degradation of the three major phospholipids in intact microsomes and in extracted lipids we found that the same fraction of each of these phospholipids was exposed on the outer surface of the microsomal vesicles. Corresponding experiments with Golgi vesicles showed that distinctly different fractions of phosphatidylcholine and phosphatidylethanolamine were present on the surface of these vesicles. However, the difference was accounted for by enrichment of phosphatidylcholine in intravesicular particles rather than by asymmetry across the vesicle membrane. The results from specific hydrolysis of phosphatidylinositol confirmed an essentially symmetric distribution of this phospholipid across the microsomal and the Golgi vesicle membranes.**

Over the last few years compelling evidence for an asymmetric distribution of phospholipids across the erythrocyte membrane has accumulated (1–7). Because such asymmetry could be relevant to many processes associated with cellular membranes, it is important to determine if it is generally occurring. A step in this direction was taken by Rothman and coworkers (8), who analyzed the membrane of influenza viruses grown in tissue culture cells. They found that while phospholipids as a group were displaced towards the inner half of the viral membrane, the degree of asymmetry among the major individual phospholipids was considerably smaller than in the erythrocyte membrane. To extend such studies to membranes of nucleated mammalian cells, we have chosen microsomal vesicles and Golgi-derived secretory vesicles from rat liver. We found that phospholipases of type A_2 degrade a significant part of the phospholipids on the outer surface of these vesicles before gaining access to the inner surface. Under these conditions, the fraction of each phospholipid exposed on the vesicle surface could be determined. The result of such analyses provided no evidence for significant asymmetry among the three major phospholipids in the microsomal or the Golgi vesicle membrane.

MATERIALS AND METHODS

Radioactively labeled compounds ([*methyl*-^{3}H]choline, 4.2 Ci/mmol; *myo*-[2-^{3}H]inositol, 17.4 Ci/mmol; [*U*-^{14}C]ethanolamine, 25 mCi/mmol; and carrier-free [^{32}P]phosphoric acid) were obtained from New England Nuclear. Phosphatidyl[^{3}H]choline was isolated from LM cells grown as described (9) in choline-free medium supplemented with 11 μM [^{3}H]-choline. Phosphatidyl[^{14}C]ethanolamine was isolated from rat liver after intraperitoneal injection of [^{14}C]ethanolamine (10). Unlabeled phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) were isolated from egg yolk (11). Isethionyl acetimidate (IAI) was from Pierce.

Phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) from *Naja naja* was purified from lyophilized venom (Sigma) by the method of Cremona and Kearney (12) as modified by Zwaal *et al.* (5). A purified phospholipase A_2 from *Crotalus atrox* venom was purchased from P-L Biochemicals. Phosphatidylinositol inositolphosphohydrolase (EC 3.1.4.10) (phospholipase C_{II}) was isolated from crude *Bacillus cereus* phospholipase C (Calbiochem or Sigma) by chromatography on DEAE-cellulose and Sephadex G-100 (unpublished). A different purification procedure was described recently (13). Our enzyme was 98% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. It lacked detectable activity against PtdCho, PtdEtn, phosphatidylserine, phosphatidylglycerol, sphingomyelin, and phosphatidic acid.

Preparation of Phospholipid Vesicles. PtdCho and PtdEtn, labeled with ^{3}H and ^{14}C, respectively, were mixed in chloroform solution to give a molar ratio of 6:4. After solvent removal, the lipids were hydrated in 50 mM NaCl (to give 1–5 μmol of lipid per ml) and then subjected to ultrasonic treatment in pulses of 0.4 sec/sec from the microprobe of a Branson Sonifier (W-350) at a power output of 50–70 W. The sample was constantly flushed with nitrogen and kept below 15°. After sonication for 30–45 min and following centrifugation (200,000 × *g* for 90 min) at least 90% of the supernatant phospholipids were in single-bilayer vesicles, as determined by column chromatography on Sepharose 4B (14).

Amidination of vesicle surface PtdEtn with IAI (at pH 8.6–9.0) and subsequent isolation of the vesicles on Sephadex G-50 followed the procedure of Roseman *et al.* (15). The chromatographic behavior of the vesicles on Sepharose 4B was not changed by this treatment.

Preparation of Microsomes. Male Sprague–Dawley rats (180–220 g) were used. Liver phospholipids were prelabeled by intraperitoneal injection of one of the following isotope mixtures: [^{3}H]choline + [^{32}P]phosphate; [^{3}H]choline + [^{14}C]-ethanolamine; or [^{3}H]inositol + [^{32}P]phosphate. After 2 hr the

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; IAI, isethionyl acetimidate; phospholipase C_{II}, phosphatidylinositol inositolphosphohydrolase.

* Part of this work was presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, IL, April 1977.

liver was excised and homogenized in 4 volumes of ice-cold 0.25 M sucrose/1 mM Na_2EDTA and centrifuged (12,000 × g_{max} for 10 min). Microsomes were then sedimented from the supernatant by centrifugation at 100,000 × g_{av} for 1 hr and washed once in 0.25 M sucrose. The procedure was carried out at 0–5°. In some experiments microsomes (from 5 to 10 g of liver) were treated with IAI. The reagent (0.2 g) was dissolved in 2 ml of 0.25 M sucrose, adjusted to pH 8.0, and immediately added to the microsomal suspension (15 ml). The reaction was allowed to proceed for 40 min at 0° with pH maintained at 8.0 ± 0.1. The reaction mixture was then layered over 0.5 M sucrose/10 mM TrisCl (ph 7.5) and the microsomes were pelleted by centrifugation (100,000 × g_{av} for 1 hr). Assays for mannose-6-phosphatase contained in 0.5 ml of 0.25 M sucrose: 0.1–0.5 mg of microsomal protein, sodium mannose 6-phosphate (18 mM), and Na_2EDTA (1 mM) with a final pH of 6.5. Incubation was at 37° for 5 or 10 min. Total (latent + nonlatent) activity was determined in the presence of 0.02% sodium deoxycholate (16, 17).

Preparation of Golgi Vesicles. Golgi-derived secretory vesicles were prepared from rat liver as described by Ehrenreich *et al.* (18) and Redman *et al.* (19). Five rats fasted for 16–20 hr were pretreated with (*a*) intraperitoneal injection of 1 mCi of [^{32}P]phosphate (at time 0) and (*b*) intraperitoneal injection of 10 mg of colchicine and feeding by gastric tube of 2.5 ml of 50% ethanol (at 2.5 hr) and were then sacrificed 1.5 hr later (at 4 hr). Only GF_1, the lightest, most homogeneous fraction (18) was used. Its identity and purity were assessed by electron microscopy and assay of marker enzymes. The morphology and the specific activities of galactosyltransferase and acid phosphatase were almost identical to those reported (19, 20); glucose-6-phosphatase assay indicated 2–8% contamination with microsomes.

In order to determine ^{32}P-labeled phospholipids associated with triglyceride-rich particles in the Golgi vesicle content, vesicles were prepared from rats injected with [^{32}P]phosphate intraperitoneally and albumin-bound [^{3}H]oleic acid intravenously. Isolated vesicles were disrupted by dilution with 5 volumes of 10 mM TrisCl/1 mM Na_2EDTA (pH 8.5) followed by forceful passage four times through a 25-gauge syringe needle. Sucrose (to a final concentration of 0.25 M) and lipoproteins of density $d < 1.006$ g/cm^3 from rat plasma were then added, pH was adjusted to 7.5, and the sample (8 ml) was loaded in a centrifuge tube and overlayered with 3 ml of 5 mM Na_2EDTA (pH 7.5). After centrifugation at 10° in a Beckman SW 40 rotor at 40,000 rpm (200,000 × g_{av}) for 3 hr, the top 1.5 ml containing 65% of the ^{3}H-labeled triglyceride was collected with a tube slicer.

Phospholipid Analysis. Conditions for phospholipase treatments are given in figure legends. Incubations containing phospholipid vesicles or detergent/phospholipid mixtures were stopped with 3 volumes of chloroform/methanol/12 M HCl (2:1:0.02 by volume) while incubations containing microsomes or Golgi vesicles were extracted consecutively with 6 volumes and then 3 volumes of chloroform/methanol (1:1) after addition of Na_2EDTA to a concentration of 10 mM (phospholipases A_2) or HCl to a concentration of 0.1 M (phospholipase C_{II}).

Phospholipids were separated by thin-layer chromatography on silica gel G (solvent: chloroform/methanol/4 M ammonia, 65:35:5). In this system *N*-acetimidoyl-PtdEtn, PtdEtn, and PtdCho are well separated from each other and from their corresponding lysophospholipid. For isolation of phosphatidylinositol (PtdIns), two-dimensional thin-layer chromatography (solvent I: chloroform/methanol/8 M ammonia, 65:35:8; solvent II: acetone/chloroform/methanol/acetic acid/water, 40:30:10:10:5) on Redi-coat plates (Supelco) was used. Radioactivity in individual phospholipids was determined by liquid scintillation spectrometry. To samples scraped from thin-layer plates, two drops of acetic acid and 0.5 ml of water were added before addition of 10 ml of Aquasol-2 (New England Nuclear).

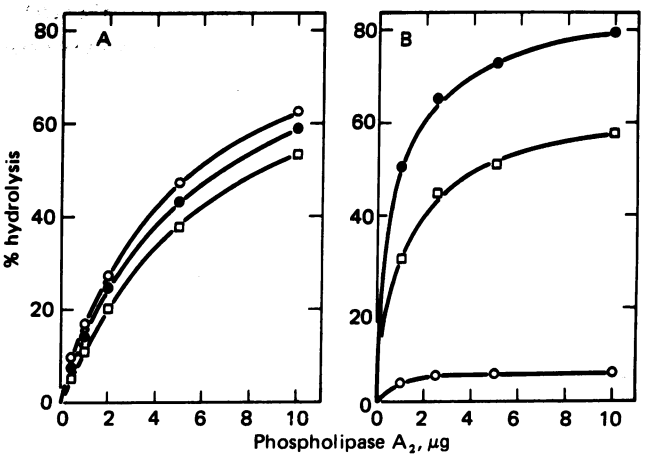

FIG. 1. Hydrolysis by phospholipase A_2 (*C. atrox*) of PtdCho/PtdEtn/*N*-acetimidoyl-PtdEtn (6:2:2). In *A* the three lipids were mixed with a 2-fold molar excess of Triton X-100 and taken up in buffer by sonication. In *B* single-bilayer vesicles were prepared from PtdCho/PtdEtn (6:4) and were then amidinated with IAI, with conversion of 51% of the PtdEtn into *N*-acetimidoyl-PtdEtn. Incubations were for 10 min at 25° in 50 mM Tris/10 mM $CaCl_2$ (pH 8.0). ○, PtdEtn; ●, *N*-acetimidoyl-PtdEtn; □, PtdCho.

RESULTS

Asymmetric Phospholipid Vesicles. To verify that phospholipases could be used to selectively probe one side of a bilayer, experiments were conducted using single-bilayer phospholipid vesicles. Such vesicles, made up from a mixture of PtdCho and PtdEtn, were made asymmetric by amidination of approximately 90% of the PtdEtn exposed on the vesicle surface with the nonpermeating reagent isethionyl acetimidate (4, 15). These vesicles would be expected to contain in the outer monolayer all of the amidinated PtdEtn, approximately 70% of the PtdCho, and very little unreacted PtdEtn (15). As shown in Fig. 1*B*, treatment of the vesicles with phospholipase A_2 from *C. atrox* resulted in exclusive hydrolysis of lipids expected to constitute the outer monolayer. This pattern of hydrolysis could not be ascribed to the substrate specificity of the enzyme (Fig. 1*A*). Phospholipases A_2 from several other sources gave corresponding results. By varying the extent of amidination, the degree of asymmetry induced across the vesicle bilayer could be changed. In each case the hydrolysis pattern with phospholipase A_2 was consistent with exclusive attack on the outer vesicle monolayer. A more extensive account of this work will be presented elsewhere.

Microsomal Vesicles Made Asymmetric by Amidination. A similar approach was used to determine to what extent hydrolysis by phospholipase A_2 was restricted to the outer half of the microsomal membrane. In order to minimize damage to the microsomal membrane, amidination was carried out at pH 8.0 and 0° with a relatively low level of IAI. Mannose-6-phosphatase, an enzyme localized to the inner (cisternal) side of the microsomal membrane (21), is highly latent in intact microsomes, probably due to lack of penetration of mannose 6-phosphate to the site of hydrolysis (22). The fact that the latency of mannose-6-phosphatase was well preserved after amidination (80–85% of the activity was latent after amidination compared

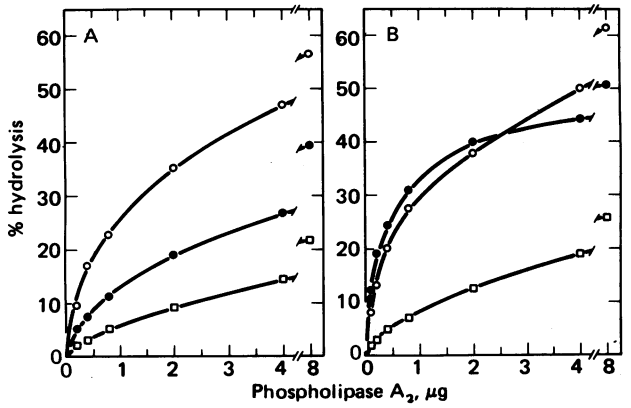

FIG. 2. Hydrolysis by phospholipase A_2 (*N. naja*) of partially amidinated microsomal lipids, dispersed with Triton X-100 as in Fig. 1*A* (*A*) or amidinated intact microsomes (*B*), in which 15% of the PtdEtn was converted into *N*-acetimidoyl-PtdEtn. Phospholipids were prelabeled *in vivo* with [^{3}H]choline *plus* [^{14}C]ethanolamine. Phospholipase treatment was for 10 min at 0° in 10 mM Tris/1 mM $CaCl_2$ (pH 7.5). Symbols as in Fig. 1.

to 86–90% before this treatment) suggests that the vesicles remained intact. As expected, the degree of amidination was low under the conditions employed and only 15% of the microsomal PtdEtn became derivatized. The distribution of PtdEtn across the microsomal membrane would therefore be much less affected than in the experiments with phospholipid vesicles, but the amidinated PtdEtn would again provide a marker for phospholipids exposed on the vesicle surface.

The amidinated microsomes were treated with phospholipase A_2 from *N. naja* (Fig. 2*B*) and the extracted lipids, solubilized with detergent, were used to assess the substrate specificity of this enzyme (Fig. 2*A*). In the case of microsomes (Fig. 2*B*) the degradation of *N*-acetimidoyl-PtdEtn was enhanced 2-fold

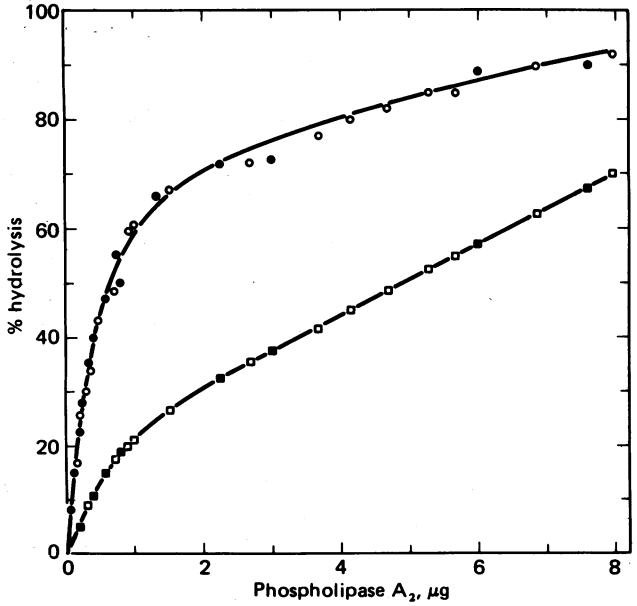

FIG. 3. Treatment of detergent-dispersed rat liver phospholipids (filled symbols) and intact microsomes (open symbols) with *N. naja* phospholipase A_2. Conditions were as in Fig. 2, except that prelabeling was with [^{3}H]choline plus [^{32}P]phosphate and treatment with IAI was omitted. Only data on detergent-dispersed lipids were used to construct the curves. To enable an accurate comparison, microsomal data were then plotted *not* on the basis of the amount of phospholipase added but on the basis of percentage hydrolysis of PtdCho. ○ And ●, PtdEtn; □ and ■, PtdCho.

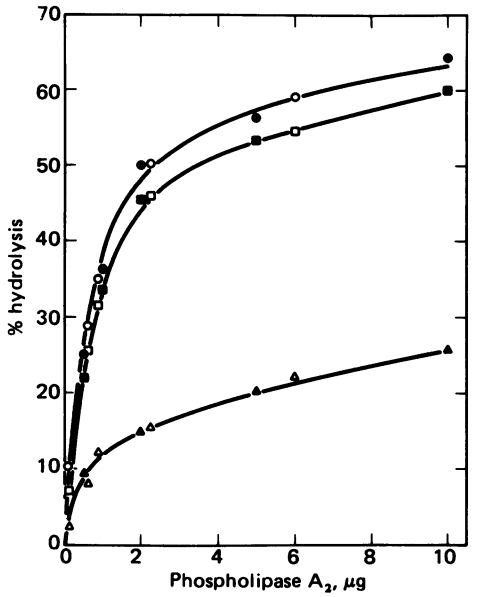

FIG. 4. Hydrolysis of detergent-dispersed rat liver phospholipids (filled symbols) and intact microsomes (open symbols) by phospholipase A_2 from *C. atrox*. Phospholipids were prelabeled *in vivo* with [^{3}H]inositol *plus* [^{32}P]phosphate. Incubation conditions were as in Fig. 2 except that $CaCl_2$ was 5 mM and the temperature was 20°. As in Fig. 3, the data for microsomes were plotted on the basis of percentage hydrolysis of PtdCho. ○ And ●, PtdEtn; □ and ■, PtdCho; △ and ▲, PtdIns.

relative to PtdEtn and PtdCho at low levels (<2 μg) of enzyme (compare Fig. 2*A*). With more extensive hydrolysis (>2 μg of enzyme), the pattern approached that seen with extracted lipids. These results indicate that at low levels of enzyme, hydrolysis was restricted to a pool of microsomal phospholipids containing approximately half of the PtdCho and PtdEtn but essentially all of the *N*-acetimidoyl-PtdEtn. Most likely this pool represents the outer half of the microsomal membrane. The change in hydrolysis pattern for microsomes with more extensive hydrolysis (Fig. 2*B*) indicates that the inner (cisternal) half of the microsomal membrane then became available to the enzyme.

Localization of Phospholipids in Microsomal Vesicles. Because phospholipid asymmetry induced by amidination was clearly detected by subsequent treatment with phospholipase A_2 (Figs. 1 and 2), any asymmetry inherent to the microsomal membrane should also be detectable. The relative degradation of PtdEtn and PtdCho in microsomes was therefore followed using phospholipase A_2 from either *N. naja* or *C. atrox*. As already shown (compare Figs. 1*A* and 2*A*), these enzymes have clearly different substrate specificities. However, when hydrolysis data for microsomes were superimposed on corresponding hydrolysis data for extracted lipids, no deviation from the pattern dictated by the substrate specificity of the phospholipase was detected, whether the *N. naja* enzyme (Fig. 3) or that from *C. atrox* (Fig. 4) was used. In the latter case also the hydrolysis of PtdIns was followed; again with no significant difference between microsomes and extracted lipids. A third phospholipase A_2 (from the venom of *Bungarus multicinctus*), which again has a different substrate specificity and preferentially hydrolyzes PtdCho, also gave corresponding results. These results show that during phospholipase treatment of microsomes a virtually identical fraction of each of the three phospholipids PtdCho, PtdEtn, and PtdIns is exposed to the enzyme at all stages of hydrolysis. Previous results (Fig. 2) indicated that phospholipase attack is initially restricted to the

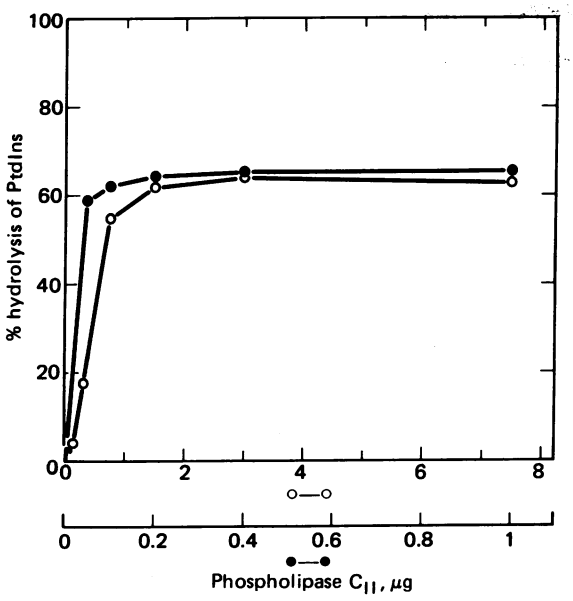

FIG. 5. Hydrolysis of PtdIns in microsomes (○) and Golgi vesicles (●) by a phospholipase C specific for PtdIns. Microsomes and Golgi vesicles were treated with phospholipase C_{II} at 0° for 20 min in 0.25 M and 0.4 M sucrose, respectively, and the hydrolysis of PtdIns prelabeled with [^{3}H]inositol *plus* [^{32}P]phosphate (microsomes) or [^{32}P]phosphate (Golgi vesicles) was determined.

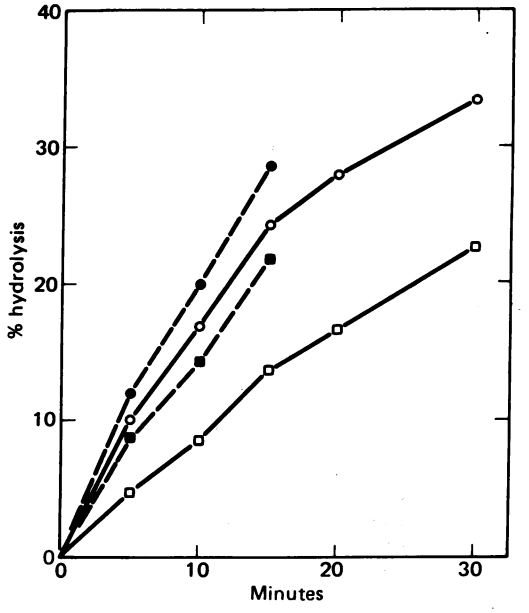

FIG. 6. The degradation of phospholipids in Golgi vesicles by phospholipase A_2 from *C. atrox*. Golgi vesicles (GF_1, ref. 18) prelabeled *in vivo* with [^{32}P]phosphate were treated with phospholipase A_2 (4 μg) at 20° in 0.4 M sucrose/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/5 mM $CaCl_2$ (pH 7.0). Open symbols represent total vesicles while closed symbols represent the vesicle membrane. The latter data were computed from data on total vesicles by subtracting phospholipids recovered in intravesicular particles (see *text*) under the assumption that these were not exposed to the enzyme in the initial phase. ○ And ●, PtdEtn; □ and ■, PtdCho.

outer half of the microsomal membrane. An identical exposure of the three phospholipids must therefore mean that they are distributed in an identical manner between the two halves of the microsomal membrane.

Localization of PtdIns as Determined with Phospholipase C_{II}. As an independent means to analyze the distribution of PtdIns across the microsomal membrane and the membrane of Golgi vesicles, we used isolated phospholipase C_{II} from *B. cereus*. This enzyme hydrolyzes 98–100% of the PtdIns in unsealed erythrocyte ghosts or in detergent/phospholipid micelles, but only PtdIns exposed on the outer surface of closed vesicles in which PtdIns is a relatively minor component (unpublished data). When microsomes were treated with phospholipase C_{II} at 0°, a maximum of approximately 60% of the microsomal PtdIns was cleaved (Fig. 5) and almost identical results were obtained for Golgi-derived secretory vesicles. Because PtdIns constitutes less than 15% of the phospholipids in these membranes, cleavage should be restricted to the outer surface of the vesicles.

Phospholipase Analysis of Golgi Vesicles. When the Golgi-derived vesicles were treated with phospholipase A_2 from *C. atrox* for increasing periods of time (Fig. 6) or with increasing amounts of enzyme (not shown), the rate of degradation of PtdEtn was twice that for PtdCho until approximately 30% of the vesicle phospholipids were hydrolyzed. Further hydrolysis affected both phospholipids similarly. The rapid hydrolysis of PtdEtn compared to PtdCho in the initial phase cannot be explained by the substrate specificity of this phospholipase (Fig. 4); but it is consistent with a difference in the distribution of PtdEtn and PtdCho either across the vesicle membrane or between the membrane and the content of the vesicles. By subjecting Golgi vesicles to disruptive treatment followed by gradient centrifugation, we isolated a large part of the triglyceride-rich particles known to be present within these vesicles (18) (see *Materials and Methods*). Analysis of the particle fraction showed that it contained a higher proportion PtdCho and a lower proportion PtdEtn than did the phospholipids of either total vesicles or the membranes sedimented after vesicle disruption.

Hydrolysis data for the vesicle membrane were then computed from the results on total vesicles by correcting for phospholipids associated with the triglyceride-rich particles (Fig. 6). A large part of the difference between PtdEtn and PtdCho must be attributed to the enrichment of PtdCho in intravesicular particles. The remaining difference is somewhat larger than that expected with identical exposure of the two phospholipids (Fig. 4). It is therefore possible that a slight asymmetry exists across the vesicle membrane (with a relative ratio of PtdEtn/PtdCho on the vesicle surface of 1.2–1.3). However, it is equally possible that additional particles within the vesicles, such as precursors of plasma high-density lipoprotein (23), account for the difference. These particles would not be isolated by our flotation procedure.

DISCUSSION

We have used phospholipases to analyze how phospholipids are distributed across the membrane of microsomal and Golgi-derived vesicles from rat liver. Our results, limited to PtdCho, PtdEtn, and PtdIns, are most consistent with a random (symmetric) distribution of phospholipids across the membranes.

The experimental support for our conclusion that a unilateral analysis of the membranes was achieved is summarized below.

(*i*) In microsomes, amidination with IAI as well as the initial phase of phospholipase A_2-catalyzed hydrolysis was restricted to a phospholipid pool containing approximately half of the major microsomal phospholipids. Further hydrolysis made most or all of these phospholipids available to the enzyme (Fig. 2). These results also suggest that little exchange of phospholipids

across the membrane (flip-flop) occurred between amidination and phospholipase treatment (approximately 2 hr at 0–4°).

(*ii*) In Golgi-derived secretory vesicles, treatment with phospholipase A_2 showed a clear change in the hydrolysis pattern at a certain degree of phospholipid hydrolysis (Fig. 6). Analysis of triglyceride-rich particles known to be present within the vesicles indicated that phospholipids associated with these particles were not available to the phospholipase initially.

(*iii*) Experiments with sonicated (single-bilayer) phospholipid vesicles have shown that even a low degree of asymmetry across a bilayer, such as the documented spontaneous asymmetry in mixed vesicles of PtdCho and PtdEtn (24, 25), can be determined with phospholipase A_2 (unpublished data).

The relevance of following hydrolysis of biosynthetically labeled phospholipids in this study has been verified in the following way. Prelabeling with [^{3}H]choline + [^{14}C]ethanolamine results in incorporation of both isotopes into PtdCho, but by different metabolic pathways and into phosphatidylcholines that differ greatly in fatty acid composition (26, 27). The degradation of ^{3}H- and ^{14}C-labeled microsomal PtdCho was, however, not significantly different. Labeling with [^{32}P]phosphate for 2 or 16 hr resulted in hydrolysis data that could not be differentiated.

It is well known that microsomal vesicles form from the tubular structures of the endoplasmic reticulum during homogenization. Although there is ample evidence that vesicles formed have the same orientation (28), it cannot be *a priori* excluded that a randomization of lipids does not occur during this process. However, the facts that erythrocytes can be lysed and resealed with full maintenance of phospholipid asymmetry and that inside-out vesicles can be prepared with only minor changes in phospholipid distribution (6) suggest that randomization of lipids does not necessarily accompany membrane rupture and resealing. In this respect, the Golgi-derived secretory vesicles are ideal because they are isolated in the native state and in addition in highly purified form. They do, however, contain lipoprotein particles that are selectively enriched in certain lipids and to obtain information on the vesicle membrane this must be taken into account.

While extensive phospholipid asymmetry has been documented for the human (1–5) and the rat (6, 7) erythrocyte membrane, no conclusive evidence has been presented regarding the membranes of nucleated mammalian cells. In erythrocytes and also influenza viruses (8), sidedness analysis has been facilitated by the fact that complete degradation of phospholipids in the outer half of these membranes has been achieved without membrane rupture. High levels of cholesterol and other membrane lipids not attacked by phospholipases may account for the stability of these membranes. It was recently proposed, on the assumption that the same procedure could be used for intracellular membranes, that various liver membranes are highly asymmetric with respect to individual phospholipids (28–30). However, it is clear from the present study that complete hydrolysis of the outer half of the microsomal membrane with phospholipase A_2, without attack on the inner half, is not obtained. The fact that virtually identical results were reported for several subcellular fractions, including intact nuclei (29), which are surrounded by two concentric separable membranes with identical phospholipid compositions (31), supports this conclusion. It appears that the substrate specificity of the phospholipase A_2 from *N. naja* (Fig. 3), which was used in these studies (28–30), may account for the results.

The results presented in this study provide strong support for the idea recently put forward (32) that the pattern of phospholipid asymmetry observed in the erythrocyte membrane is not a general feature of biological membranes. Our results further suggest that lack of phospholipid asymmetry might be a valid working hypothesis for future studies on the organization and biosynthesis of mammalian cell membranes.

We thank Dr. R. L. Vandlen for a sample of phospholipase A_2 from *B. multicinctus* and Ms. J. Rakowski for secretarial assistance. During part of this work, R.S. received financial support from the Swedish Medical Research Council (no. 4689).

1. Bretscher, M. S. (1972) *J. Mol. Biol.* **71**, 523–528.
2. Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurious, P., Kastelijn, D. & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta* **323**, 178–193.
3. Gordesky, S. E. & Marinetti, G. V. (1973) *Biochem. Biophys. Res. Commun.* **50**, 1027–1031.
4. Whiteley, N. M. & Berg, H. C. (1974) *J. Mol. Biol.* **87**, 541–561.
5. Zwaal, R. F. A., Roelofsen, B., Comfurius, P. & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta* **406**, 83–96.
6. Bloj, B. & Zilversmit, D. B. (1976) *Biochemistry* **15**, 1277–1283.
7. Renooij, W., van Golde, L. M. G., Zwaal, R. F. A. & van Deenen, L. L. M. (1976) *Eur. J. Biochem.* **61**, 53–58.
8. Rothman, J. E., Tsai, D. K., Dawidowicz, E. A. & Lenard, J. (1976) *Biochemistry* **15**, 2361–2370.
9. Schroeder, F., Perlmutter, J. F., Glaser, M. & Vagelos, P. R. (1976) *J. Biol. Chem.* **251**, 5015–5026.
10. Sundler, R. (1973) *Biochim. Biophys. Acta* **306**, 218–226.
11. Litman, B. J. (1973) *Biochemistry* **12**, 2545–2554.
12. Cremona, T. & Kearney, E. B. (1964) *J. Biol. Chem.* **239**, 2328–2334.
13. Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. & Ohyabu, T. (1976) *Biochim. Biophys. Acta* **450**, 154–164.
14. Huang, C. & Thompson, T. E. (1974) in *Methods in Enzymology*, eds. Fleischer, S. & Packer, L. (Academic Press, New York), Vol. 32, pp. 485–489.
15. Roseman, M., Litman, B. J. & Thompson, T. E. (1975) *Biochemistry* **14**, 4826–2830.
16. Gold, G. & Widnell, C. C. (1976) *J. Biol. Chem.* **251**, 1035–1041.
17. Cater, B. R., Trivedi, P. & Hallinan, T. (1975) *Biochem. J.* **148**, 279–294.
18. Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 45–72.
19. Redman, C. M., Banerjee, D., Howell, K. & Palade, G. E. (1975) *J. Cell Biol.* **66**, 42–59.
20. Bergeron, J. J. M., Ehrenreich, J. H., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 73–88.
21. Leskes, A., Siekevitz, P. & Palade, G. E. (1971) *J. Cell Biol.* **49**, 288–302.
22. Arion, W. J., Wallin, B. K., Carlson, P. W. & Lange, A. J. (1972) *J. Biol. Chem.* **247**, 2558–2565.
23. Hamilton, R. L., Williams, M. C., Fielding, C. J. & Havel, R. J. (1976) *J. Clin. Invest.* **58**, 667–680.
24. Litman, B. J. (1974) *Biochemistry* **13**, 2844–2848.
25. Berden, J. A., Barker, R. W. & Radda, G. K. (1975) *Biochim. Biophys. Acta* **375**, 186–208.
26. Arvidson, G. (1968) *Eur. J. Biochem.* **4**, 478–486.
27. Sundler, R., Arvidson, G. & Åkesson, B. (1972) *Biochim. Biophys. Acta* **28**, 559–568.
28. Depierre, J. W. & Dallner, G. (1975) *Biochim. Biophys. Acta* **415**, 411–472.
29. Nilsson, O. S. & Dallner, G. (1977) *Biochim. Biophys. Acta* **464**, 453–458.
30. Nilsson, O. S. & Dallner, G. (1977) *J. Cell Biol.* **72**, 568–583.
31. Virtanen, I., Brotherus, J., Renkonen, O. & Wartiovaara, J. (1977) *Biochem. Biophys. Res. Commun.* **76**, 142–149.
32. Rothman, J. E. & Lenard, J. (1977) *Science* **195**, 743–753.