# Membrane Interactions of Rat Intestinal Alkaline Phosphatase: Role of Polar Head Groups†

Bellur Seetharam,* Chinnaswamy Tiruppathi, and David H. Alpers
*Department of Gastroenterology, Washington University School of Medicine, St. Louis, Missouri 63110*
*Received January 21, 1985*

ABSTRACT: Lipid–protein interactions with purified membranous intestinal alkaline phosphatase have been studied by using rat intestine. The enzyme was incorporated equally well into neutral lecithin and anionic liposomes, including those made from phosphatidic acid alone. It could not be solubilized with chaotropic salts nor by phospholipases C and D from either native membranes or phospholipid vesicles. Detergents effected nearly complete release of enzyme from the vesicles. Phosphatase activity was lost upon treatment with phospholipase D alone. The activity was restored with free choline, or choline containing phospholipids, but not by the addition of other phospholipids or amines. The catalytic activity was also lower when the enzyme was bound to a phosphatidylcholine vesicle containing additional phosphatidic acid. Neither phosphatidylserine nor phosphatidylinositol addition altered enzyme activity. These results show that the enzyme binds to the membrane by a primary hydrophobic interaction with membrane phospholipids without requiring the polar head group and that the enzyme activity is affected via a secondary interaction with choline. We suggest that choline protects the active site of brush border alkaline phosphatase from inhibition by endogenous membrane phosphate groups.

Alkaline phosphatase is a membrane protein present in the cell wall of prokaryotic and plasma membranes of eukaryotic cells, where its primary function is to hydrolyze a variety of monophosphate esters (Stein & Koshland, 1952). Although extensive work has been carried out on the isolation and characterization of alkaline phosphatase from various sources (Fishman, 1974), studies directed toward understanding its immediate membrane surroundings are few. The enzyme in kidney and liver has been shown to be linked to the membrane via interactions involving phosphatidylinositol (Low & Finean, 1977a; Low & Zilversmit, 1980). On the other hand, our earlier studies (Yedlin et al., 1981) had shown that rat intestinal alkaline phosphatase preferentially bound to cationic liposomes, suggesting the possibility that membrane interactions involving alkaline phosphatase in the intestinal brush border might be different from those in kidney and liver membranes. In order to explore this possibility and to define further the membrane topography of alkaline phosphatase, we undertook the current studies.

Our results show that in the intestinal brush border the enzyme is not linked through phosphatidylinositol and that the primary interactions are with hydrophobic lipids. We further show that choline affects the active site of the enzyme in the membrane.

## Materials and Methods

*Materials.* All phospholipids (99.9% purity as assessed by thin-layer chromatography), phospholipase C (*Bacillus cereus* and *Clostridium welchii*), phospholipase D (peanut), and papain were purchased from Sigma Chemical Co., St. Louis, MO. Phospholipase C from *Staphylococcus aureus* was partially purified according to the method of Low & Finean (1977b). Bio-Gel A-0.5m (50–100 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of analytical grade.

Electrophoretically homogeneous membranous alkaline phosphatase from adult rat intestine was purified and assayed according to the method of Yedlin et al. (1981). Brush border membranes from rat intestinal mucosa were prepared according to the method of Kessler et al. (1978). The alkaline phosphatase activity was enriched 20-fold in the brush border membrane with a recovery of 50%.

*Preparation of Liposomes.* Liposomes were prepared by using single phospholipids (2 μmol) and alkaline phosphatase (2.5 g of protein) according to the cholate dialysis method (Low & Zilversmit, 1980) with cholate (40 mM) in a final volume of 1 mL. The above reaction mixture (1 mL) was dialyzed against 2 L of 10 mM Tris-HCl,[1] pH 7.4, containing 100 mM NaCl and 5 mM KCl (buffer A). Dialysis was carried out for 48 h with exchange of 2 L of buffer every 12 h. When more than phospholipid was included in the liposomes, they were prepared as follows: 1 mL of buffer A containing DMPC (0–2 μmol), anionic phospholipids (0–2 μmol), sodium cholate (40 mM), and alkaline phosphatase (2.5 μg of protein and 1 unit of enzyme activity) was incubated for 1 h at 37 °C and dialyzed as described above. Separation of vesicle-bound and free enzyme was carried out by centrifugation at 105000*g* for 4 h and, for confirmation of the results, by gel filtration or density centrifugation. When gel filtration was employed, the dialyzed liposome fraction (500 μL) was placed on a Bio-Gel A-0.5m column (1 × 50 cm) and the column was washed with buffer A. Fractions (500 μL) were collected every 7–8 min. Separation of the vesicle-bound enzyme in the liposome fraction obtained after dialysis against buffer A was also carried out by sucrose density (5–20%) centrifugation at 150000$g_{av}$ for 16 h. Fractions (250 μL) collected by gravity were assayed for enzyme activity and lipid

† This research was supported by National Institutes of Health Grants AM-26638 and AM-14038.
* Author to whom correspondence should be addressed.

[1] Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; DMPC, dimyristoylphosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; PE, phosphatidylethanolamine; $P_i$, inorganic phosphate.

0006-2960/85/0424-6603$01.50/0 

phosphate. The amount of enzymes bound to vesicle varied by only 5–10% depending upon the method employed.

*Enzymatic Digestion of Liposomes and Membranes.* (*1*) *Papain.* Brush border membrane (100 μg of protein; 600 milliunits of enzyme activity) and DMPC–alkaline phosphatase liposomes (1.3 μg of protein; 500 milliunits of enzyme activity) were incubated in buffer A and DTT (1 mM) in a final volume of 1 mL. The reaction was initiated by addition of 10 μg of papain for membranes and 1 μg for liposomes. At the end of 1 h the reaction was stopped by the addition of PMSF (1 mM). The total liposomal mixture or the supernatant fraction ($105000g_{av}$, 1 h) from the digested membranes was fractionated on a Bio-Gel A-0.5m column as described above.

(*2*) *Phospholipases.* Brush border membrane (10–12 mg of protein containing 70–80 units of activity) and DMPC–alkaline phosphatase liposomes (2.6 μg of protein and 1 unit of enzyme activity) were digested in a total volume of 3 mL with various phospholipases as follows: Incubation with nonspecific phospholipase C (15 units, *B. cereus* or *Cl. welchii*) and phospholipase D (15 units) was carried out according to the method of Sundler et al. (1978) and with phospholipase C (*S. aureus*) according to the method of Low & Finean (1977b). The reaction was initiated by addition of brush border membranes or liposomes after a 15-min preincubation of phospholipases in the presence of 0.1 mM phenylmethanesulfonyl fluoride and *N*-ethylmaleimide. The reaction mixture was rapidly chilled and centrifuged for 4 h at $105000g_{av}$, and the supernatant and particulate fractions were assayed for protein and enzyme activity. The supernatant fraction obtained after phospholipase D treatment was first neutralized to pH 7.4. All particulate fractions were suspended in 10 mM Tris-HCl, pH 7.4, containing 70 mM NaCl and 5 mM KCl.

Soluble fractions were obtained from membranes before and after phospholipase D treatment as follows: Brush border membranes (1 mg of protein/mL in buffer A) were treated with 1% Triton X-100, papain (100 μg/mL), or 30% 1-butanol for 1 h at 37 °C. The reaction mixture was centrifuged for 1 h at $105000g$. The supernatant fraction in the case of papain-treated and Triton X-100 treated membranes and the aqueous extract of the 1-butanol-treated membranes were carefully removed. The papain and butanol extracts were dialyzed against 4 L of buffer A for 18 h. Either the membranes or the solubilized extracts were incubated with or without 1.5 units of phospholipase D.

*Addition of Phospholipids.* Polar head groups, phospholipids, and other amines (5 mg/mL) were suspended in buffer A, and the suspension was sonicated for 5 min at 37 °C at 220-W power with a Braunsonic 1510 sonicator. The lipids were added to various fractions, the mixture was preincubated for 15 min at 37 °C, and the assay for alkaline phosphatase activity was initiated by the addition of the substrate. Assessment of phospholipid binding to control and phospholipase D treated membranes was carried out as follows: After treatment with phospholipase D, the membranes were incubated for 1 h at 22 °C with 0–2 μmol of diheptanoyl-PC in 1 mL of reaction mixture. The membranes containing >95% of the total enzyme activity in each incubation were collected by centrifugation for 1 h at $105000g_{av}$. A lipid extract was prepared from these membranes according to the method of Folch et al. (1957). The samples were dried under nitrogen and were hydrolyzed with 70% perchloric acid. Phosphate estimation was carried out according to the method of Bartlett (1959).

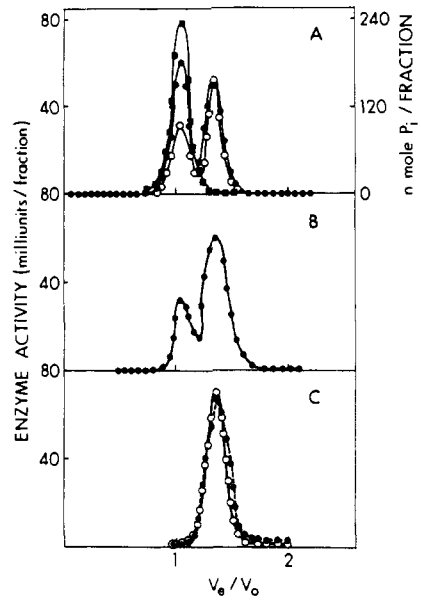


FIGURE 1: Gel filtration of bound and free intestinal alkaline phosphatase. Liposomes containing alkaline phosphatase and phospholipid were prepared and fractionated on a Bio-Gel A-0.5m column as described under Materials and Methods. (A) Phospholipid phosphate content (■) and enzyme activity in the presence of DMPC (●) and egg PA (○) liposomes; (B) enzyme activity after treatment of DMPC–alkaline phosphatase vesicles with papain; (C) enzyme activity in Triton X-100 (○) and papain (●) extracts of brush border membranes.

The phospholipid content of native and phospholipase D treated membranes was also estimated using two-dimensional thin-layer chromatography (Rouser et al., 1969) after an initial extraction with chloroform–methanol solvent (2:1). Protein content was estimated according to the method of Lowry et al. (1951).

## Results

*Primary Interaction between Alkaline Phosphatase and Phospholipid Vesicle.* The enzyme treated with DMPC or PA in the presence of cholate and later dialyzed was subjected to gel filtration on a Bio-Gel A-0.5m column (Figure 1A). Some of the enzyme activity eluted with a $V_e/V_0$ of 0.9–1.15 and an estimated molecular weight exceeding $500 \times 10^3$. This fraction contained greater than 95–98% of the lipid phosphorus and thus corresponded to the vesicle-bound enzyme. The remainder of the enzyme activity eluted with a $V_eV_0$ of 1.25–1.45, corresponding to an apparent molecular weight of $180–200 \times 10^3$. Similar separations were obtained with the enzyme treated with PI or PS (data not shown). The percent of total enzyme activity eluting in this region varied with the phospholipid used and was 50–55% with DMPC, PI, and PS and only 25–30% with egg PA liposomes due to inhibitory effect of PA on enzyme activity (see below). However, total binding of phosphatase was the same with PA vesicles as with other phospholipids. After treatment of the vesicles (enzyme activity eluting with a $V_e/V_0$ of 0.9–1.15 in Figure 1A) with papain, over two-thirds of the enzyme activity eluted at a $V_e/V_0$ between 1.25 and 1.45 (Figure 1B). This elution position was identical with that of the enzyme solubilized by Triton X-100 or papain from brush border membranes (Figure 1C) and probably represents non-vesicle-bound enzyme.

Further validation of the significance of the two peaks on Bio-Gel A-0.5m columns was obtained. When papain-treated liposomes or brush border membranes were centrifuged for 4 h at $105000g_{av}$, the supernatant fraction contained only membrane- or vesicle-free enzyme ($V_e/V_0$ of 1.25–1.45 on the

Table I: Effect of Phospholipase Digestion on Membrane- or Liposome-Bound Alkaline Phosphatase[a]

| | alkaline phosphatase activity | | | |
|---|---|---|---|---|
| | brush border membranes | | DMPC liposomes | |
| additions | total | soluble | total | soluble |
| none | 100 ± 2 | 0 | 100 ± 1 | 0 |
| phospholipase C | | | | |
| *S. aureus* | 96 ± 3 | 10 ± 2 | 95 ± 2 | 8 ± 4 |
| *B. cereus* | 100 ± 1 | 14 ± 2 | 103 ± 2 | 12 ± 2 |
| *Cl. welchii* | 98 ± 2 | 6 ± 2 | 98 ± 2 | 10 ± 3 |
| phospholipase D | 37 ± 2 | 3 ± 2 | 45 ± 3 | 0 |

[a] Membranes and liposomes were incubated as described under Materials and Methods. Total activity was determined before centrifugation. Soluble activity was determined after centrifugation for 4 h at $105000g_{av}$. All activity measurements after phospholipase D treatment were carried out after neutralizing the incubation mixture to pH 7.4 from pH 6.0. The results are expressed as percent of activity of control membranes incubated at pH 7.4 and pH 6.0 in the absence of phospholipases C and D, respectively. The values represent a mean ± SD of three separate experiments.

Bio-Gel A-0.5m column) whereas the pellet from the liposomes contained only vesicle-bound enzyme. The enzyme–phospholipid mixtures were also subjected to sucrose density centrifugation. The fraction containing phosphate as well as enzyme achieved a density between 1.025 and 1.03, while a fraction containing enzyme activity alone was found at a heavier density between 1.035 and 1.045 (data not shown). The percent of enzyme activity in each fraction was within 5–10% of the values obtained on the Bio-Gel column or obtained by centrifugation in the absence of sucrose. Moreover, vesicle-bound enzyme remained bound, even when subjected again to gel filtration or ultracentrifugation. Thus, we consider that, under the conditions of these incubations, the Bio-Gel column and ultracentrifugation accurately separated vesicle-bound and free enzyme.

*Solubilization of Alkaline Phosphatase by Detergents but Not by Chaotropic Salts.* Chaotropic salts, e.g., 1 M NaCl, KCl, KI, $KNO_3$, or KSCN, were unable to solubilize phosphatase bound either to native membranes or to DMPC vesicles. Incubation of bound phosphatase with acidic buffers (pH 5.0) or alkaline buffers (pH 11.0) did not result in any release of the enzyme but resulted in a 30–40% loss in total activity. However, neutral (Triton X-100) and anionic (sodium deoxycholate) detergents (1%) solubilized over 85% of the enzyme from both native membranes and DMPC liposomes (data not shown).

*Phospholipase C and D Effect on Membranes and Alkaline Phosphatase Activity.* Incubation of either native brush border membranes or DMPC liposomes with phosphatidylinositol-specific (*S. aureus*) or nonspecific phospholipase C did not result in any change in total enzyme activity or in any significant release of the enzyme (Table I), despite the fact that phospholipid hydrolysis occurred under the conditions used. When the unhydrolyzed membrane phospholipids were analyzed (Table II), the majority of PI and PC (65–90% and 60–90%, respectively) was found to have been released by phospholipase C digestion. These results demonstrate that enzyme release from membranes or liposomes was not the result of hydrolysis of phospholipid head groups. Treatment with phospholipase D resulted in the loss of nearly 60–65% of phosphatase activity, even though no enzyme was released (Table I). The polar head groups (choline and inositol) were extensively removed (75–80%) by phospholipase D treatment, and the membrane content of phosphatidic acid increased 980% from 5 to 49 nmol/mg of protein (Table II). These data

Table II: Phospholipid Composition of Brush Border Membranes after Treatment with Phospholipases C and D[a]

| | phospholipid content (% of control) | | |
|---|---|---|---|
| enzyme added | PC | PI | PA |
| phospholipase C | | | |
| *S. aureus* | 41 ± 1 | 11 ± 1 | 100 ± 1 |
| *B. cereus* | 10 ± 1 | 14 ± 1 | 95 ± 3 |
| *Cl. welchii* | 6 ± 1 | 35 ± 2 | 95 ± 2 |
| phospholipase D | 23 ± 1 | 22 ± 2 | 980 ± 50 |

[a] Membranes were incubated and analyzed as described under Materials and Methods. The values are expressed as the percent of individual phospholipids present in untreated membranes and represent the mean ± SD of three experiments.

Table III: Effect of Phospholipids on Restoration of Alkaline Phosphatase Activity in Phospholipase D Treated Membranes[a]

| | | | alkaline phosphatase activity (% control activity) | |
|---|---|---|---|---|
| chemical class | additions | amt added (μg) | brush border | DMPC liposomes |
| | none | | 38 ± 3 | 48 ± 3 |
| polar head group | choline | 500 | 80 ± 5 | 80 ± 2 |
| | inositol | 500 | 35 ± 3 | 52 ± 3 |
| | ethanolamine | 500 | 44 ± 2 | 50 ± 4 |
| | serine | 500 | 35 ± 4 | 45 ± 3 |
| phosphatidyl-cholines (PC) | egg PC | 50 | 95 ± 5 | 90 ± 3 |
| | egg lyso-PC | 50 | 85 ± 2 | 80 ± 2 |
| | diheptanoyl-PC | 50 | 100 ± 5 | 90 ± 4 |
| | dioctanoyl-PC | 50 | 103 ± 3 | 95 ± 4 |
| | distearoyl-PC | 50 | 65 ± 4 | ND |
| | diarachidoyl-PC | 50 | 56 ± 2 | ND |
| other phospholipids | sphingomyelin | 50 | 66 ± 4 | 70 ± 2 |
| | phosphatidyl-inositol | 50 | 38 ± 2 | 56 ± 4 |
| | phosphatidylserine | 50 | 21 ± 2 | 52 ± 2 |
| | phosphatidyl-ethanolamine | 50 | 35 ± 2 | 54 ± 3 |
| | stearylamine | 500 | 36 ± 2 | ND |
| | dodecylamine | 500 | 34 ± 4 | ND |
| | *N*-trimethyl-*n*-decylamine | 500 | 37 ± 4 | ND |

[a] Membranes and DMPC liposomes were prepared and treated with phospholipase D as described under Materials and Methods. Sonicated lipids were added to the incubation mixture and incubated for 30 min at pH 7.4 before the addition of the substrate. The amount of lipid added represents that which produced maximal effect. At least 100 μg (0.83 μmol) of choline or 10 μg (0.015 μmol) of lecithin was needed to produce a significant effect. The values represent the mean ± SD of three separate incubations and are expressed as percent of activity present in a control incubation carried out in the absence of phospholipase D.

again demonstrated the lack of correlation between phosphatase release and the membrane content of major phospholipid subclasses.

*Secondary Interaction between Choline Head Groups and Membrane-Bound Alkaline Phosphatase.* The loss of enzyme activity noted after treatment of membranes with phospholipase D was unique to alkaline phosphatase and was not seen with other brush border enzymes such as disaccharidases (data not shown). The factors responsible for the loss of enzyme activity were further investigated (Table III). When free polar head groups were added to an incubation mixture containing the phospholipase D treated membranes, only choline was able to reverse the activity lost by phospholipase D digestion. When phospholipids were added, only choline-containing phospholipids were effective in the restoration of the activity. Water-soluble cholines or phosphatidylcholines that are in the liquid phase at 37 °C were more effective in the restoration

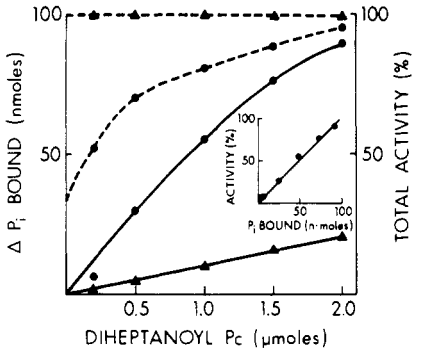


FIGURE 2: Binding of diheptanoyl-PC to control and phospholipase D treated membranes. Phospholipase D treatment and binding of PC to membranes were carried out as described under Materials and Methods. Enzyme activity in control (▲---▲) and phospholipase D treated (●---●) membranes and bound phosphate in control (▲—▲) and phospholipase D treated (●—●) membranes were measured. Enzyme activity was present in native membranes incubated in the absence of phospholipase D. The phosphate values reported were adjusted for the endogenous phosphate present in the membranes before addition of diheptanoyl-PC. The insert shows the relationship between activity restored (Δ activity) and the increment in bound phosphate (Δ $P_i$ bound).

of activity than were water-insoluble lecithins. The restoration of activity by lecithins was unique since other quaternary ammonium compounds or other hydrophobic amines were not as effective. Equal increments in activity were obtained at all concentrations of choline and diheptanoyl-PC as long as the choline was added in approximately 40 times molar excess. Thus, a 10% increase in activity needed 0.007 and 0.3 μmol of diheptanoyl-PC and choline, respectively. Moreover, the restoration of activity could be correlated with the binding of a lecithin (diheptanoyl-PC) to phospholipase D treated membranes (Figure 2).

It was possible that phospholipase D treatment altered membranous alkaline phosphatase activity by exposing the enzyme to either adjacent lipids (or proteins) or to the phosphate group of the degraded lecithins. Therefore, we examined the effect of phospholipase D treatment and its reversal by lipids in liposomes containing only purified alkaline phosphatase and a single lecithin, DMPC. Table III demonstrates that results were obtained that were similar to those in intact brush borders, except that the degree of inhibition by phospholipase D was not as great.

In order to test whether the loss of activity was due to changes in the kinetics of the enzyme, we determined the $K_m$ and $V_{max}$ of alkaline phosphatase in both treated and untreated membranes. The $K_m$ for the hydrolysis of *p*-nitrophenyl phosphate was 0.3–0.4 mM for both control and phospholipase D treated membranes. The $V_{max}$ decreased from 5.4 to 1.5 units/mg of protein upon treatment with phospholipase D. The addition of 0.07 μmol of dioctanoylphosphatidylcholine restored the $V_{max}$ to the untreated value of 4.8 units/mg of protein.

Since choline could act as a cofactor binding to alkaline phosphatase or could exert its effect only in the presence of intact membrane fragments, the effect of phospholipase D treatment on solubilized and delipidated membranous alkaline phosphatase was examined. Loss of activity upon treatment with phospholipase D and its restoration by lecithins were only seen when crude extracts of membrane obtained with Triton X-100 or papain were used and not after delipidation with 30% butanol (Table IV). Moreover, the activity of the pure enzyme (prepared with initial solubilization with butanol) was not affected by phospholipase D treatment. The pure enzyme did not appear to contain any endogenous phospholipids when

Table IV: Effect of Phospholipase D Digestion on Solubilized Membranous Alkaline Phosphatase Activity[a]

| solubilizing agent | stage of purification | alkaline phosphatase activity (% control activity) phospholipase D treated | +egg PC | +dioleoyl-PC |
|---|---|---|---|---|
| Triton X-100 | 105000*g* supernatant | 45 ± 5 | 90 ± 2 | 95 ± 3 |
| papain | 105000*g* supernatant | 55 ± 4 | 90 ± 4 | 94 ± 4 |
| 30% butanol | 105000*g* supernatant | 100 ± 1 | 98 ± 2 | 96 ± 4 |
| 30% butanol | homogeneous, pure | 98 ± 2 | 100 ± 4 | 100 ± 2 |

[a] Treatment with phospholipase D, addition of lipid, and assay of the enzyme activity were similar to those of the experiment outlined in Table III and under Materials and Methods. Fifty micrograms of egg PC and dioleoyl-PC were added. The enzyme activity is reported as the mean ± SD of three separate treatments and is expressed as the percent of total activity present in control membranes incubated in the absence of phospholipase D.

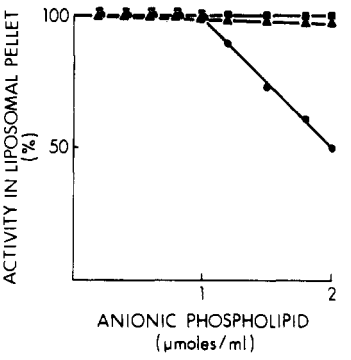


FIGURE 3: Effect of anionic lipid addition on phosphatase activity in DMPC liposomes. Mixed liposomes were prepared as described under Materials and Methods. The enzyme activity was assayed in the vesicle sedimenting after contrifugation for 4 h at 105000$g_{av}$ and expressed as percent of activity present in the liposomal pellet obtained at zero anionic phospholipid. Total phospholipid content was kept at 2 μmol/mL. PI, ■; PS, ▲; PA, ●.

the lipid extract prepared from 200 μg of protein was analyzed by phosphate estimation, either before or after fractionation by thin-layer chromatography.

*Effect of Anionic Phospholipids on the Activity of Purified Alkaline Phosphatase.* The loss of alkaline phosphatase activity following phospholipase D treatment could be related to electrogenic repulsion of the substrate by exposure of free phosphate groups and creation of an anionic microenvironment. Therefore, we tested the phosphatase activity after its incorporation into liposomes containing a variable amount of anionic lipids (Figure 3). Loss of enzyme activity occurred only when the phosphatidic acid concentration exceeded 50% of the total phospholipid (2 μmol/mL). Inhibition was maximal (50%) at a phosphatidic acid concentration of 100 μmol % (2 μmol/mL). Neither PI nor PS had any effect on the enzyme activity (Figure 3).

When the enzyme bound to 100% PA liposomes was separated on a Bio-Gel A-0.5m column from the lipid-free enzyme, only the activity eluting with vesicles ($V_e/V_0$ of 0.9–1.15) was lower than that found in PC vesicles. The amount of activity eluting with free enzyme ($V_e/V_0$ of 1.25–1.45)) was the same when the lipid used to make liposomes was either PA or PC. When PA–phosphatase liposomes were assayed in the presence of PC (50 μg), only the enzyme activity in the vesicle fraction

increased 2-fold, while the activity eluting as free enzyme was not affected (data not shown). In the presence of 0.3 μmol of PA, alkaline phosphatase showed a $K_m$ for *p*-nitrophenyl phosphate of 0.37 mM and a $V_{max}$ of 180 units/mg of protein. When diheptanoyl-PC (1 μmol) was added to this system, the $K_m$ was the same (0.4 mM) but the $V_{max}$ was restored to 375, similar to the control value of 390 units/mg of protein. There was no change in either $K_m$ or $V_{max}$ when either PS or PI was substituted for PA.

The above results suggested that loss of enzyme activity observed after phospholipase D treatment could result from formation of PA following the removal of choline. In fact, PA content rose 10-fold after treatment of brush border membranes (Table II). Only solubilization with butanol seemed able to spatially separate the enzyme from surrounding phospholipids, especially PA (Table IV). To confirm this possibility, we examined phosphatase activity in phospholipase D treated brush borders after the addition of 1-butanol. The aqueous phase recovered after 30% butanol treatment contained all the initial enzyme activity (101 ± 1), consistent with the fact that removal of a lipid-soluble inhibitor could explain the phospholipase D effect. Triton X-100 (1%) treatment did not restore phosphatase activity in phospholipase D treated brush borders, although it released the enzyme into the aqueous phase.

## Discussion

The primary interaction of alkaline phosphatase with the membrane does not require charged groups. First, the enzyme could not be solubilized with chaotropic salts. Second, phospholipase C specific for phosphatidylinositol (*S. aureus*) and nonspecific phospholipase C failed to release the enzyme bound either to brush border membranes or to liposomes (Table I), even though extensive hydrolysis of both phosphatidylcholine and phosphatidylinositol had occurred (Table II). Third, the enzyme bound well to liposomes prepared with phosphatidic acid (Figure 1A). Thus, the polar head group was not needed for binding.

Alkaline phosphatase does seem to interact with choline head groups in the membrane, even though these groups are not required for binding the enzyme to the membrane. Removal of the polar head group from brush border or liposomal lipids by phospholipase D treatment resulted in a loss of activity and hydrolysis of PC and PI (and probably PS and PE). The restoration of activity was achieved with free choline, or with lecithins that form micelles (egg lyso-PC, diheptanoyl- and dioctanoyllecithin) or bilayers (egg PC, dioleoyl-PC, dimyristoyl-PC), but not with serine-, inositol-, and ethanolamine-containing phospholipids or other amines (Table III). To achieve maximal restoration of activity, free choline was effective only when used at 40 times molar excess compared to diheptanoyl-PC (Figure 2 and Table III). The effect was not due to neutralization of the surface charge alone because hydrophobic amines, ethanolamine, and one quaternary ammonium salt were not effective in the restoration of the activity. Moreover, the generation of an anionic microenvironment itself was not responsible for the observed loss in activity, since only the addition of phosphatidic acid but not other anionic lipids was effective (Figure 3).

There are three lines of evidence suggesting that PA is the inhibitor of phosphatase activity that is produced by phospholipase D action. First, addition of PA (200–300 μg) but not other anionic lipids to the enzyme assay system decreased activity by 50% with no change in $K_m$. Fifty percent of enzyme activity was also inhibited when PA was added to make liposomes (Figure 3). Second, the membrane concentration of PA was markedly increased after phospholipase D treatment (Table II). Finally, the results using brush border membranes were found to be similar to those using DMPC liposomes (Table III), a system in which PA is the only possible product of phospholipase D treatment. These results suggest that PA is located at a site near the active center of the membrane-bound phosphatase.

Phospholipase D decreased alkaline phosphatase activity when the enzyme was solubilized by Triton X-100 or papain but not by butanol (Table IV). Treatment of the membrane with Triton X-100 or papain might be expected to release the enzyme with phosphatidylcholine still attached to it. The inability to restore the activity by Triton X-100 is probably due to solubilization of membrane fragments by detergent micelles without complete delipidation of the proteins (Lichtenberg et al., 1983). After phospholipase D treatment of membranes, subsequent addition of butanol probably removes phosphatidic acid, the proposed inhibitor of alkaline phosphatase activity, and also restores phosphatase activity. Since we were not able to detect endogenous phospholipids bound to the butanol-purified enzyme, this result is consistent with extensive delipidation of the enzyme by butanol.

Unlike other enzymes such as D-β-hydroxybutyrate dehydrogenase (Grover et al., 1975; Gazzotti et al., 1975) where choline is required for activity of the pure enzyme, pure alkaline phosphatase activity was neither lost upon treatment with phospholipase D nor were lecithins required for activity (Table IV). The absence of any role of inositol or choline in the insertion of rat intestinal alkaline phosphatase is interesting, since such a role for inositol phosphate has been suggested for kidney and liver enzyme from rabbit (Low & Finean 1977a; Yusufi et al., 1983) and kidney enzyme from dog (Low & Zilversmit, 1980). Our own studies on rat kidney brush border enzyme have confirmed that the enzyme is not only released from brush borders by PI-specific phospholipase C but also integrates with anionic rather than cationic liposomes (B. Seetharam, unpublished observations). It is interesting to note that the lipid interactions with alkaline phosphatase could be so different in the plasma membranes of two different tissues of the same animal species.

Since intestinal alkaline phosphatase is difficult to solubilize without using detergents, it was thought that it might include a large part of its mass (subunit $M_r$ 68 000) within the membrane. However, the bulk of the protein mass is external to the membrane surface since the $M_r$ of the membrane insertion piece has been estimated to be between 3000 and 4000 (Colbeau & Maroux, 1978). This orientation of alkaline phosphatase in brush borders is not unique. Other functional proteins such as the intrinsic factor–cobalamin receptor (Seetharam et al., 1981), disaccharidases (Brunner et al., 1979), and γ-glutamyltranspeptidase (Frielle & Curthoys, 1983) are also asymmetrically oriented with an anchor piece comprising less than 5% of their total mass. Perhaps the varying membrane interactions of intestinal and kidney phosphatases are due to intramembranous anchor peptides of different length or composition.

**Registry No.** DMPC, 18194-24-6; *p*-$NO_2C_6H_4OPO_3H_2$, 330-13-2; alkaline phosphatase, 9001-78-9; choline, 62-49-7; diheptanoyl-PC, 39036-04-9; dioctanoyl-PC, 19191-91-4; distearoyl-PC, 816-94-4; diarachidonoyl-PC, 17688-29-8; dioleoyl-PC, 4235-95-4.

## References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Brunner, J., Hauser, H., Braun, H., Wilson, K. J., Wacker, H. O., O'Neill, B., & Semenza, G. (1979) *J. Biol. Chem. 254*, 1821–1828.

Colbeau, A., & Maroux, S. (1978) *Biochim. Biophys. Acta 511*, 39–51.
Fishman, W. H. (1974) *Am. J. Med. 56*, 617–649.
Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.
Frielle, T., & Curthoys, N. P. (1983) *Biochemistry 22*, 5709–5714.
Gazzotti, P., Bock, H. G., & Fleischer, S. (1975) *J. Biol. Chem. 250*, 5782–5790.
Grover, A. K., Slotboom, A. J., De Haas, G. H., & Hammes, G. G. (1975) *J. Biol. Chem. 250*, 31–38.
Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., & Semenza, G. (1978) *Biochim. Biophys. Acta 506*, 136–154.
Lichtenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285–304.
Low, M. G., & Finean, J. B. (1977a) *Biochem. J. 167*, 281–284.
Low, M. G., & Finean, J. B. (1977b) *Biochem. J. 162*, 235–240.
Low, M. G., & Zilversmit, D. B. (1980) *Biochemistry 19*, 3913–3918.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 293*, 265–275.
Rouser, G., Fleischer, S., & Yamamoto, A. (1969) *Lipids 5*, 494–496.
Seetharam, B., Bagur, S. S., & Alpers, D. H. (1981) *J. Biol. Chem. 256*, 9813–9815.
Stein, S., & Koshland, D. E. (1952) *Arch. Biochem. Biophys. 39*, 229–230.
Sundler, R., Alberts, A. W., & Vagelos, P. R. (1978) *J. Biol. Chem. 253*, 5299–5304.
Yedlin, S. T., Young, G. P., Seetharam, B., Seetharam, S., & Alpers, D. H. (1981) *J. Biol. Chem. 256*, 5620–5626.
Yusufi, A. N., Low, M. G., Turner, S. T., & Dousa, T. P. (1983) *J. Biol. Chem. 258*, 5695–5701.

# Phase Behavior of Galactocerebrosides from Bovine Brain†

William Curatolo*
*Molecular Biophysics Group, Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*
Firoze B. Jungalwala
*E. K. Shriver Center for Mental Retardation, Waltham, Massachusetts 02254*
*Received April 19, 1985*

ABSTRACT: Bovine brain cerebrosides (BOV-CER) were separated by high-performance liquid chromatography into cerebroside fractions with a single acyl chain type or with a relatively homogeneous acyl chain distribution. The thermal behavior of these isolated cerebroside fractions was studied by differential scanning calorimetry. Nonhydroxy (*n*-acyl) fatty acid cerebrosides (NFA-CER) possessing a saturated acyl chain (C16:0, C18:0, C24:0) exhibit their major order–disorder transition temperature $T_M$ at 83 °C, independent of chain length. NFA-CER possessing primarily unsaturated acyl chains (C24:1) exhibits $T_M$ at 70 °C. 2-Hydroxy fatty acid cerebrosides (HFA-CER), which possess a saturated hydroxyacyl chain (C18:0h, C24:0h), exhibit $T_M$ at 70–72 °C. Thus, naturally occurring cerebrosides exhibit high $T_M$'s that do not depend significantly on acyl chain length and that depend only to a small degree on unsaturation and the presence of a 2-hydroxy branch in the amide-linked chain. Isolated NFA-CER's each exhibit metastable polymorphism of the type previously described for unfractionated NFA-CER [Curatolo, W. (1982) *Biochemistry 21*, 1761]. Polymorphism in HFA-CER is complex, with a different type of thermal behavior observed for each isolated acyl chain fraction studied. On prolonged storage at low temperature, unfractionated HFA-CER and unfractionated BOV-CER reach a highly ordered gel state similar to that which is readily reached by NFA-CER's. These results indicate that all cerebrosides exhibit metastable polymorphism. However, the kinetic barriers to reaching the stable gel state are greater for HFA-CER and BOV-CER than for NFA-CER.

Cerebroside, the simplest mammalian glycosphingolipid, is found in large quantities in the myelin membrane and in the brush border membrane of the intestinal wall (Norton, 1975; Hauser et al., 1980; Hansson, 1983). The order–disorder transition temperature ($T_M$) of bovine brain cerebroside model membranes is extremely high (67 °C) relative to body temperature (Curatolo, 1982), and it is likely that this property provides the basis for a major function: imparting increased order to membranes. In the case of myelin, this would decrease permeability to ions, thus facilitating saltatory conduction. In the case of the brush border, decreased ionic permeability assures proper ion gradients for ion gradient driven pumps for active transport. In addition, some special stabilization of the brush border may be necessary to prevent dissolution by bile salts. The involvement of interlipid hydrogen bonding in this membrane stabilization by sphingolipids has been emphasized by Pascher (1976). While more complex glycolipids probably

† This work was supported by National Institutes of Health Grants GM/NS-28149, NS-10437, and HD-05515.
* Address correspondence to this author at Pfizer Central Research, Groton, CT 06340.

0006-2960/85/0424-6608$01.50/0 