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The role of choline on the activity-temperature relationship of brush-border alkaline phosphatase

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We have studied the effect of choline on the activity and temperature dependency of the brush-border alkaline phosphatase isoenzymes from rat intestine (tissue-specific type), and from kidney and placenta (tissue-nonspecific type). The removal of choline with phospholipase D resulted in the loss of enzyme activity in all the membranes, whereas in situ loss in the discontinuity of Arrhenius plots occurred in the kidney and the placental membranes, but not in the intestinal membranes. The lost activity was restored either by addition of free choline or phosphatidylcholine or by the removal of the enzyme from the membrane surface. Intestinal enzyme was removed by papain, while the tissue-nonspecific enzyme was released by subtilisin and by phosphatidylinositol-specific phospholipase C. The enzyme from kidney and placental membranes aggregated ($\rho = 1.13$) upon removal of choline, and addition of choline resulted in disaggregation ($\rho = 1.03$). Conversion of discontinuous to continuous linear plots of alkaline phosphatase in the kidney and placental membranes paralleled the increase in membrane phosphatidic acid content, and the decrease in total phosphatidylcholines. The intestinal enzyme produced plots with break points at all phosphatidic acid/phosphatidylcholine ratios. The change brought about by treatment with phospholipidase D was not due to changes in the half-saturation kinetics (K_m) for the substrate. Based on these studies we conclude (1) that the active site of the tissue-nonspecific phosphatase is approximated to exterior membrane cholines, as in the case of the intestinal isoenzyme [7]; (2) that despite similar effects on the membrane content of phospholipids, phospholipase D treatment caused much greater effects on the tissue-nonspecific enzyme, as assessed by Arrhenius plots and density centrifugation; (3) that these effects are due to different protein structures rather than to a lipid milieu unique to each brush-border membrane.

Introduction

Alkaline phosphatase is an integral membrane protein present in the plasma membranes of several mammalian tissues [1]. The enzymes associated with the brush border have been identified in man with three separate genes expressed in intestine, placenta and kidney or liver (tissue nonspecific) [2–4]. In the rat, however, only two enzymes have been identified, the intestinal and the tissue nonspecific forms [5,6]. Even though considerable work has been carried out on the purification and characterization of the isolated enzyme from these tissues, little is known regarding membrane interactions of the different alkaline phosphatase isoenzymes.

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; DMPC, dimyristoylphosphatidylcholine; DMPA, dimyristoylphosphatidic acid.

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Previous studies from our laboratory [7] have shown that the active site of membrane bound intestinal alkaline phosphatase is located proximally to the polar head group choline, since its removal by phospholipase D treatment resulted in an in situ loss of activity. Full restoration of activity was achieved by the addition of free choline or choline containing phospholipids. We suggested that choline protected the active site of membrane bound alkaline phosphatase from inhibitory interaction with membrane phosphate groups.

Although these studies had suggested that the effect of choline was mediated by a secondary interaction with the enzyme, an additional effect on other membrane components which could alter the activity was possible. One such determinant would be the altered physical state of the membrane due to a decrease in the phosphatidylcholine content and an increase in the content of phosphatidic acid. In order to test this hypothesis, we have constructed the Arrhenius plots of alkaline phosphatase using native, phospholipase D-treated, and phospholipid bilayer membranes.

In the present work we have extended our earlier studies [7] to include brush-border membranes from other rat tissues containing tissue nonspecific alkaline phosphatase (kidney and placenta). Since the two isoenzymes from intestine and placenta are antigenically distinct [6], this comparison could lead to a better understanding of the relative role of tissue specific membrane lipids or the protein itself in or near the membrane on the properties of membrane bound alkaline phosphatase. Our studies show that the presence of choline on the membrane surface is needed for full alkaline phosphatase activity. We propose that this differential response to temperature changes upon the loss of choline may be due largely to isoenzyme structure rather than to changes in the physical state of the lipid bilayer.

Materials and Methods

All synthetic phospholipids (> 99.9% purity as assessed by thin-layer chromatogrphy); phospholipase C (*Bacillus cereus*); phospholipase C (*Clostridium welchii*) and phospholipase D (peanut) were purchased from Sigma Chemical Company, St. Louis, MO. Brush-border membranes from rat intestine, kidney and placenta were isolated by the method of Kessler et al. [8]. The membranes were enriched from 10–20-fold for alkaline phosphatase with a recovery of 40–50%. Phospholipase C which was relatively specific for phosphatidylinositol was partially purified from *Staphylococcus aureus* as described [9].

Alkaline phosphatase was purified from adult male rat kidney and intestine and full-term placenta (21 days) essentially by the procedure of Yedlin et al. [10]. The specific activity (μmoles of *p*-nitrophenyl phosphate hydrolyzed per min per mg protein) was 390, 40, and 20 for intestine, placenta and kidney, respectively. The intestinal enzyme was homogenous while the kidney and placental enzymes showed minor contaminants (< 5%) on both native and denaturing gel electrophoresis after silver staining.

Liposomes were prepared by the cholate dialysis method [11] as described earlier [7]. The proteolipid fraction was separated from lipid free enzyme by subjecting the dialysate to sucrose density centrifugation. 500 μl of the dialysate containing the enzyme and phospholipid was placed on a 10 ml linear sucrose gradient (5–20%) and centrifuged using an SW-41 rotor for 18 h at 100 000 × *g*. At the end of the run fractions (250 μl) were collected from the bottom and were assayed for alkaline phosphatase and inorganic phosphate by the method of Forstner et al. [12] and Bartlett [13], respectively.

Treatment of membrane or liposome bound enzyme with various phospholipases were carried out as follows. Brush-border membranes from rat intestine (10–12 mg protein containing 60–70 units of enzyme activity) and rat kidney and placenta (10–12 mg protein containing 7–8 units were incubated in a total volume of 3 ml. Incubation was carried out at 37°C for 2 h with phospholipase C (15 units, *B. cereus* and *Cl. welchii*) and phospholipase D (15–20 units) according to Sundler et al. [14] and phospholipase C (*S. aureus* according to Low and Finean [9]. The reaction was initiated by the addition of brush-border membranes after a 15 min preincubation of phospholipases with 0.1 mM phenylmethylsufonyl fluoride and *N*-ethylmaleimide to inhibit any possible protease activity

in these enzyme preparations. The reaction mixture was chilled to 4°C after incubation, centrifuged at 150000 × *g* max for 2 h, and the supernatant and particulate fractions assayed for protein and enzyme activity. When brush-border membranes or liposomes were digested with phospholipase D, the sedimentable fraction (150000 × *g* 2 h) was resuspended in 10 mM Tris-HCl buffer (pH 7.4). Phosphoilpase digestion was also carried out with purified alkaline phosphatases from intestine, kidney and placenta bound to DMPC vesicles. One unit of enzyme bound to DMPC was treated with various phospholipases as stated above.

Proteolytic treatment of native and phospholipase D-treated membranes was carried out by incubating 1 mg of membrane protein with either papain or subtilisin (100 μg) for 30 min to 3 h at 37°C in 50 mM Tris-HCl (pH 7.4). Dithiothreitol (1 mM) was included during digestion with papain. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (1 mM). The activity was measured in these digests and in the supernatant obtained by centrifugation of the digested membranes for 1 h at 100000 × *g*.

Sucrose density centrifugation using native and phospholipase D-treated membranes was carried out after an incubation (1 h at 37°C) with choline (1 mg/mg protein) followed by extraction with *n*-butanol (30%) or Triton X-100 (0.25%). These extracts (500 μl) were placed on a linear sucrose gradient (5–35%) prepared using 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Triton X-100 and centrifuged for 18 h at 100000 × *g* using the SW-41 rotor in a Beckman model L-8 ultracentrifuge. Fractions (250 μl) were collected from the bottom and assayed for the enzyme activity.

Temperature dependence on the enzyme activities was carried out essentially as described by Livingstone and Schachter [15]. An aliquot of 1 ml of brush-border membranes or liposomes containing 5–10 miliunits of enzyme activity and 20 μg of protein was used for assay at temperatures from 10° to 48°C, and the results used for construction of Arrhenius plots. The final protein concentration of 20 μg/ml was obtained by adding fatty acid-free bovine serum albumin, since the enzyme protein concentration was were less than 0.15 μl/ml.

Membrane phospholipids from kidney and placenta were estimated both before and after phospholipase D treatment as described earlier [7]. Liposomes containing single phospholipid or mixed phospholipids were prepared essentially as described earlier [7].

Reconstitution of either activity or the construction of Arrhenius plots with solutions of polar head groups were carried out as follows: phospholipase D-treated membranes were prepared in 10 mM Tris-HCl (pH 7.4) and preincubated with 500 μg of choline, inositol, serine, or ethanolamine for 15 min at 22°C. The substrate was then added and further incubated for 15 min at various temperatures.

Results

Effect of phospholipase and protease treatments on the brush-border alkaline phosphatase activity

Incubation of native membranes with phospholipase C did not significantly decrase total enzyme activity (Table I). However, the relatively PI-specific phospholipase C from *S. aureus* solubilized the tissue non-specific enzyme (75–85%) from the kidney and placental membranes, but not the intestinal isoenzyme from intestinal membranes. Similar results were obtained using phospholipase C from *B. cereus*, a source which contains both PI specific and non-specific phospholipase C. *Cl. welchii*, which contains only PI non-specific phospholipase C, was unable to solubilize the enzyme from any of the three membranes. After treatment with phospholipase D there was a loss of nearly 55–60% of total activity in all the three membranes, but the enzymes were not solubilized.

While the intestinal alkaline phosphatase was solubilized with papain, the kidney and placental alkaline phosphatase could be solubilized effectively only with subtilisin. Subtilisin and papain did not cause solubilization of alkaline phosphatase from intestinal and kidney/placental membranes, respectively. Other serine proteases such as trypsin, chymotrypsin and elastase had no effect on either the activity or solubilization in the three membranes. The sulfhydryl protease bromalein did solubilize alkaline phosphatase from

TABLE I

EFFECT OF PHOSPHOLIPASE AND PROTEASE DIGESTIONS ON BRUSH-BORDER ALKALINE PHOSPHATASE

Membranes were incubated as described in Methods. Total activity was detrmined before centrifugation. Soluble activity was determined after centrifugation for 2 h at $105000 \times g_{av}$. All activity measurements after centrifugation for 2 h at $105000 \times g_{av}$. 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. The values represent the mean ± S.D. of three separate experiments.

Additions	Alkaline phosphatase (% of control activity)					
	intestine [a]		kidney		placenta	
	total	soluble	total	soluble	total	soluble
None	100 ±	0	100 ± 1	0	100 ±	0
Phospholipase						
Phospholipase C						
S. aureus	93 ± 3	10 ± 2	95 ± 2	80 ± 4	100 ± 2	75 ± 2
B. cereus	100 ± 1	10 ± 2	96 ± 4	75 ± 3	95 ± 3	70 ± 3
Cl. welchii	98 ± 2	6 ± 2	94 ± 4	5 ± 2	95 ± 4	6 ± 2
Phospholipase D	37 ± 2	3 ± 2	45 ± 4	4 ± 1	46 ± 3	3 ± 1
Protease						
Papain	97 ± 3	100 ± 3	85 ± 5	5 ± 1	90 ± 4	3 ± 1
Subtilisin	100 ± 4	7 ± 3	90 ± 5	70 ± 3	95 ± 4	70 ± 5

[a] Treatment of incubated intestinal brush-border membrane with phospholipases and the results thus obtained are from Seetharam et al. [7].

the intestinal membranes, but it also inactivated the enzyme activity (data not shown).

Restoration of alkaline phosphatase activity following phospholipase D treatment

The in situ loss in enzyme activity observed in membranes from the kidney and placental tissues following phospholipase D treatment could be restored with free choline or choline containing phospholipids, but not with other had groups or phospholipids (Table II). Similar results have been reported previously for intestinal alkaline phosphatase [7]. The activity was also restored by the treatment of these membranes with proteolytic enzymes which solubilized the alkaline phosphatase from the membranes. Thus, the restoration of intestinal alkaline phosphatase activity due to protease digestion was specific with papain, while it was specific for subtilisin with the kidney and placental membranes (Table III). Digestion with *B. cereus* phospholipase C resulted in the solubilization of alkaline phosphatase and restoration of activity from kidney and placental membranes, but not from the intestinal membranes.

These results show that despite the differences in the tissue of origin, the specific alkaline phosphatase isoenzyme, or the nature of the membrane attachment, the response to the loss of choline was a decrease in activity. The loss in the activity in all the three membranes was not due to changes in the pH optima (pH 9.2–9.4), nor was it due to changes in the K_m (0.2–0.3 mM). The only kinetic parameter affected was the V_{max} (data not shown). In order to get more insight into the possible relationship between choline and the activity of alkaline phosphatase from these membranes, we carried out activity measurements both before and after phospholipase D treatment at various temperatures.

When native membranes were assayed with the substrate *p*-nitrophenyl phosphate at temperatures of 10–48° C, the resulting Arrhenius plot showed a brak point at 27 ± 1° C for the three membranes studied (Fig. 1). Treatment of these membranes with phospholipase D resulted in the disappearance of the break point in the Arrhenius plot in the kidney and placental membranes; with intestinal membranes the break occurred at a slightly higher temperature of 29 ± 1° C (Fig. 2). Choline, but not other polar head groups, was able to

TABLE II

THE EFFECT OF PHOSPHOLIPIDS ON THE RESTORATION OF ALKALINE PHOSPHATASE ACTIVITY IN PHOSPHOLIPASE D-TREATED BRUSH BORDERS

Membranes were prepared and treated with phospholipase D as described under Materials and Methods. Sonicated lipids were added to the incubations 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. The values represent the mean ± S.D. of three separate incubations obtained from two separate membranes preparations from each tissue and are expressed as percent of activity present in a control incubation carried out in the absence of phospholipase D.

Chemical class	Additions	Amount added (μg)	Alkaline phosphatase (% of control activity)	
			kidney	placenta
	None	–	45 ± 4	46 ± 3
Polar head groups				
	Choline	500	86 ± 3	88 ± 2
	Inositol	500	47 ± 4	48 ± 3
	Ethanolamine	500	44 ± 3	41 ± 2
	Serine	500	42 ± 2	43 ± 3
Phospholipid				
	Egg PC	50	95 ± 2	96 ± 3
	PI	50	48 ± 3	49 ± 2
	PE	50	50 ± 4	51 ± 4
	PS	50	42 ± 2	40 ± 3
	Sphingomyelin	50	52 ± 3	51 ± 2

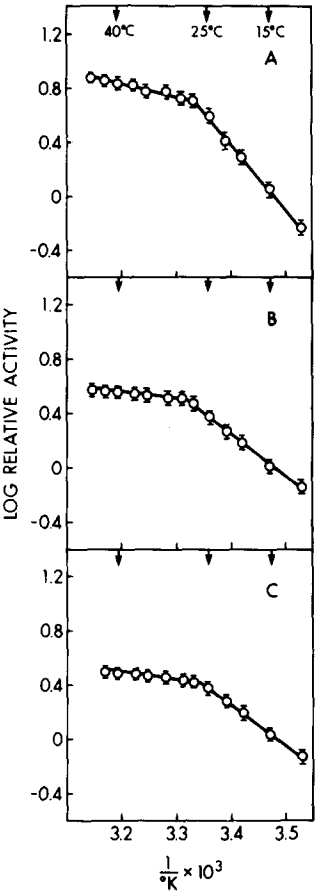

Fig. 1. Arrhenius plots for the brush-border memrbane alkaline phosphatases. (A) Intestine; (B) kidney; (C) placenta. Alkanine phosphatase was assayed over the temperature range of 10–48°C. The value used for the construction of Arrhenius plots are the mean ± S.D. of three different experiments. Other details are discussed in Methods.

TABLE III

EFFECT OF SOLUBILIZATION ON THE ENZYME ACTIVITY OF PHOSPHOLIPASE D-TREATED MEMBRANES

Phospholipase D treated membranes were digested with papain, subtilisin and phospholipase C for 1 h at 37°C. The total activity and soluble activity (105000 × *g* 1 h supernatant) was measured as indicated in Methods. The values represent the mean ± S.D. of three separate phospholipase D-digested membranes and three separate solubilization experiments.

Treatment	Alkaline phosphatase activity (% of control)					
	intestine		kidney		placenta	
	total	soluble	total	soluble	total	soluble
None	100 ± 3	5 ± 1	100 ± 1	3 ± 1	100 ± 3	3 ± 1
Phospholipase D	37 ± 2	3 ± 1	45 ± 4	3 ± 2	46 ± 3	2 ± 1
+ papain	75 ± 5	65 ± 5	45 ± 3	4 ± 1	40 ± 3	4 ± 1
+ subtilisin	37 ± 5	3 ± 1	85 ± 5	75 ± 5	85 ± 3	70 ± 5
+ phospholipase C *(B. cereus)*	35 ± 2	4 ± 2	85 ± 4	80 ± 4	85 ± 3	80 ± 4

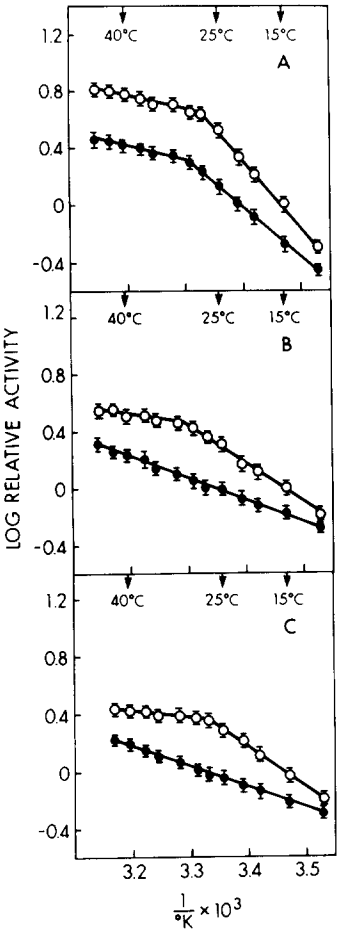

Fig. 2. Effect of choline on the Arrhenius plots of brush-border membrane alkaline phosphatases after phospholipase D treatment. (A) Intestine; (B) kidney; (C) placenta. (●———●) in the absence of choline; (○———○) in the presence of 500 μg of choline. Other details are described in Methods.

decrease the break point temperature with the intestinal membranes and to restore the break points with the kidney and placental membranes.

The ability of choline to affect Arrhenius plots of alkaline phosphatase isoenzymes was not the same for all phosphatases. When another brush-border phosphatase, $(Ca^{2+} + Mg^{2+})$-ATPase, was assayed, phospholipase D treatment altered neither the total activity nor the overall shape of the Arrhenius plots in kidney and placental membranes (date not shown). However, the breaks appeared 2 to 3 Cdeg higher following phospholipase D treatment.

Enzymes bound to phospholipid vesicles

Enzymes from all three tissues were first bound to DMPC vesicles and assayed at various temperatures. The resulting Arrhenius plots revealed a break point between 25 and 27 °C (Fig. 3). However, when the enzymes were bound to DMPA liposomes, the break point was observed only with the intestinal enzyme, but not with the kidney and placental enzymes (Fig. 3). When the kidney enzyme bound to mixed liposomes was analyzed, there was a change from a discontinuous Arrhenius plot with a break point to a continuous linear plot as the ratio of DMPA to DMPC was increased from 0.25 to 0.5 (data not shown). Replacement of PC with sphingomyelin during the preparation of

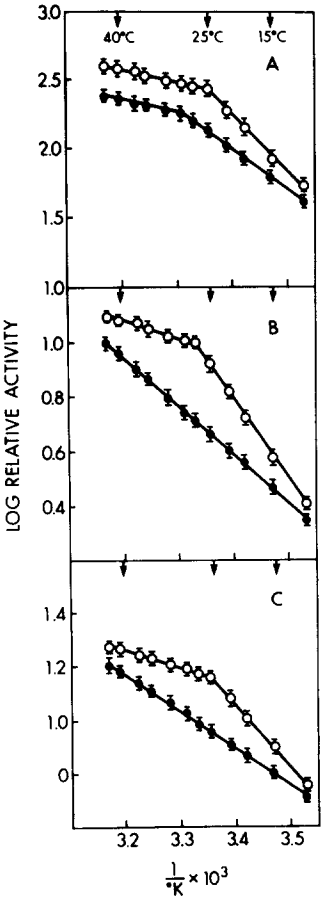

Fig. 3. Arrhenius plots for the purified enzymes from intstinal, kidney, and placenta bound to phospholipid vesicles. (○———○) bound to DMPC liposomes; (●———●) bound to DMPA liposomes. (A) Intestine; (B) kidney; (C) placenta.

mixed liposomes with phosphatidic acid produced Arrhenius plots with straight lines at all ratios tested (data not shown). Liposomes made with egg phosphatidic acid gave similar results to those seen with DMPA. Liposomes made with egg PC and enzyme from all the three membranes gave linear Arrhenius plots (data not shown). When bound to liposomes prepared using PI, the intestinal, kidney and placental enzymes gave a break point at $28 \pm 1°C$, $29 \pm 1°C$ and $31 \pm 1°C$, respectively.

Effect of controlled phospholipase D digestion on the Arrhenius plots

Fig. 4 shows the relative amounts of various kidney brush-border phospholipids as a function of time of phospholipase D digestion. While PC content fellby almost 80% from control levels after a 3 h digestion, there was nearly a 20-fold increase in the phosphatidic acid content. After 30 min of incubation with phospholipase D, the phosphatidic acid/phosphatidylcholine ratio was approximately 0.25, and the Arrhenius plot still was discontinuous (Fig. 5). However, when the ratio increased to 1.5 after 60 min or more of digestion, the Arrhenius plot became a continuous linear curve. Similar results to those shown in Figs. 4 and 5 were noted with placental membranes (data not shown).

The disappearance of the break point in the Arrhenius plot of kidney and placental alkaline phosphatase following phospholipase D treatment was a different effect than that noted in intestinal membranes. To detect any effect on the protein itself, we examined changes in the physical state of the enzyme. In the case of intestinal membranes, the Triton X-100 extracted alkaline phosphatase sedimented on a gradient at a density of 1.03. This value did not change when Triton X-100 extraction was preceded by either phospholipase D treatment or phospholipase D treatment followed by an incubation with choline (Fig. 6A). Phospholipase D treatment of kidney membranes (Fig. 6B) and placental membranes (data not shown) caused nearly 50% of the enzyme to migrate with a density of 1.13, due to aggregation. Enzyme extracted from control membranes or those treated with choline after phospholipase D treatment migrated at a adensity of 1.036.

When a similar experiment in kidney mem-

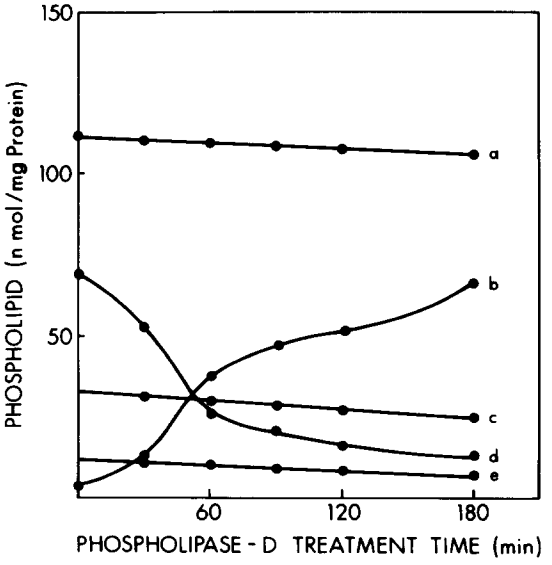

Fig. 4. Changes in the phospholipid content of kidney membranes after phospholipase D treatment. Kidney brush-border memrbanes were incubated with phospholipase D from 0 to 180 min and at each time interval the individual phospholipid concentrations were estimated. Other details of extraction, separation and estimation of phospholipids are given under Methods. a, PE; b, phosphatidic acid; c, PS; d, PC; and e, PI.

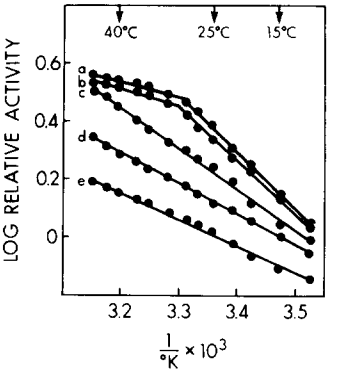

Fig. 5. Arrhenius plot obtained with phospholipase D-digested kidney brush-border membrane. Kidney border membranes were treated with phospholipase D for different time intervals and used for the construction of Arrhenius plots. The lipids (phosphatidic acid and PC) were estimated, as in Fig. 4. The time of digestion with phospholipase D and molar ratios of phosphatidic acid/PC were as follows: a, 0 min = 0.05; b, 30 min = 0.25; c, 60 min = 1.5; d, 120 min = 3.0 and e, 180 min = 4.6.

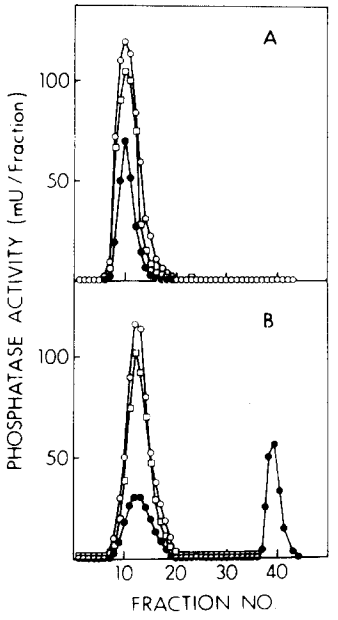

Fig. 6. Sucrose density centrifugations of membrane extracts before and after phospholipase D treatment. 0.5 mg of brush-border memrbane protein from intestine (A) and kidney (B) were treated with phospholipase D for 2 h at 37°C. Half the aliquot from each membrane thus treated was incubated with choline (1 mg) for 1 h at 37°C. Control membranes were also incubated with choline, buth without phospholipase D treatment. Control membranes, those treated with phospholipase D alone, and those incubated with choline following phospholipase D treatment were extracted with 0.25% Triton X-100 for 1 h at 37°C. The supernatant obtained by centrifugation for 1 h at 105000 × *g* was placed on a linear sucrose gradient (5–35%) and centrifuged for 18 h. Other details are provided under Methods. Fractions are numbered in the increasing order from the top of the gradient. Membranes incubated with choline, O——O; membranes treated with phospholipase D, ●——●; membranes incubated with choline subsequent to treatment with phospholipase D, □——□.

branes was carried out with butanol in place of Triton X-100 for the enzyme extraction, the aggregation followin phospholipase D digestion and its reversal by choline did not occur (data not shown). The purified kidney enzyme bound to phosphatidic acid or phosphatidylinositol (data not shown) but not PC liposomes before extraction sedimented at the higher density (1.13) while the intestinal enzyme bound to either PC or phosphatidic acid liposomes sedimented at the same (1.03) density (data not shown).

Discussion

Our previous studies [7] on the intestinal brush-border membrane phosphatase had suggested a secondary interaction between choline and the active site of the enzyme. In the present paper these studies have been extended to include analysis of the tissue nonspecific alkaline phosphatase from kidney and placenta. The interaction between choline and the active site described for the intestinal isoenzyme was confirmed using the tissue nonspecific isoenzyme in kidney and placental membranes (Table II) [7]. In these membranes the ALP expressed is structurally different from that expressed in the intestine [5,6]. This difference in protein structure allows one to ask if the protein itself or the nature and amount of membrane lipids in these various membranes have an effect on the functional orientation of alkaline phosphatase. The loss in the enzyme activity and its reversal by free choine or phosphatidylcholine in the kidney and placental membranes is very similar to the situation with the intestinal membranes [7]. In addition, we observed solubilization of alkaline phosphatase from these membranes by different proteases and by phospholipase C relatively specific for PI from the kidney and placental membranes (Table III). These results are consistent with the previously proposed covalent linkage between PI and tissue non-specific (not intestinal) alkaline phosphatase [11]. Phosphoinositol 'specific' phospholipase C (*B. cereus*) effectively restored the activity lost after phospholipase D treatment to the kidney and placental but not intestinal membranes. This differential effect of the lipase is due to its ability to solubilize alkaline phosphatase from the kidney and placental membranes [11] (Table I) but not intestinal membranes (Table III). The effects of these hydrolases cannot be accounted for by differences in overall membrane lipid composition or structure [7], although local differences could play a role.

Another important difference noted between these membranes was the phosholipase D-induced loss in the break point of the Arrhenius plots constructed with alkaline phosphatase activity in kidney and placental, but not intestinal membranes (Fig. 2). The production of phosphatidic acid in kidney and placental membranes was simi-

lar (50 nmol/mg protein in 2 h) to that found previously in intestinal membranes (49 nmol/mg protein in 2 h) [7]. The switch from a nonlinear Arrhenius plot in kidney membranes occurred when the concentration of phosphatidic acid was at least 1/2 the PC levels (Fig. 5). This effect occurred at the same phosphatidic acid/phosphatidylcholine ratio found to alter activity of the intestinal isoenzyme in DMPC liposomes [7]. Thus, the membrane content of phosphatidic acid could not account for the differences in the alkaline phosphatase activity in the various membranes.

The differential response to temperature and the nature of the Arrhenius plot thus derived following phospholipase D treatment appears to be due to structural differences between the two types of alkaline phosphatase. Other factors, such as release from the membranes [16] and altered kinetics [17], which would affect the interpretation of such a comparative study, have been minimized. First, all the tissue membranes used for the construction of the Arrhenius plots were freshly isolated and used. Second, activity of the membrane bound phosphatases were assessed both before and after phospholipase treatment and at all temperatures by both K_m and V_{max}. Finally, we used both natural and artificial membranes to assess enzyme activity and found similar results in both, common ingredients in enzyme protein and membrane phosphate. This, and the fact that there was no change in the kinetics follwoing treatment with phospholipase D, suggested that orientation of alkaline phosphatase was not altered. Moreover, alkaline phosphatase bound to DMPC vesicles or native membranes can easily be solubilized [7] with papain, again suggesting that alkaline phosphatase orientation with in lipid bilayer-layers did not change when the enzyme was reconstituted with the liposomes.

When the enzymes bound to DMPC liposomes were used for the construction of Arrhenius plots, the purified phosphatase isoenzymes exhibited nonlinear Arrhenius plots (Fig. 3), similar to the profiles obtained when the isoenzymes were bound to native membranes (Fig. 1). However, with the enzyme bound to DMPA liposomes, the kidney and placental enzymes but not the intestinal enzyme exhibited a linear Arrhenius plot (Fig. 3). Thus, the linearization of the plot with the tissue nonspecific alkaline phosphatase occurred in both natural and artificial membranes, and at identical concentration of phosphatidic acid, either added or generated in situ. It seems likely that differences in protein structure, rather than lipid content, account for the more drastic effects of phosphatidic acid on the Arrhenius plot of the tissue nonspecific alkaline phosphatase.

The effect due to increased phosphatidic acid was accompanied by a change in the physical state of the alkaline phosphatase in the kidney and placenta. The enzyme from the kidney membranes aggregated after phospholipase D treatment (Fig. 6B), and following addition of choline the enzyme returned to its original size. Such an enzyme aggregation-disaggregation phenomenon was not seen with the intestinal enzyme following phospholipase D treatment of membranes either before or after the addition of choline (Fig. 6A). The kidney or placental enzymes bound to PI liposomes became aggregated but possessed the break point. This result suggests that a negative charge on the membrane could induce aggregation but the loss in the break point after phospholipase D is due to enzyme–phosphate interaction.

Previously it has been suggested that the break point in the Arrhenius plot of brush-border alkaline phosphatase is regulated by the fluidity of these membranes [18], even though the temperature at which the break appeared did not always coincide with the thermotropic transition temperature of the lipid used [19]. The exact explanation of the effect of temperature on the fluidity induced changes in the slope of the Arrhenius plot is unclear [20,21]. Some studies have suggested the importance of temperature induced conformational changes or alterations in the aggregation of the proteins each case requiring a particular lipid environment [22–24]. Other reports [25] stress the role of the annular lipids and the strength of the interaction with the protein as a determinant of the break point temperature. In contrast to the above studies are reports suggesting that the structure of the protein alone could influence the abrupt changes in the Arrhenius plots independent of changes which could take place within the lipid bilayer interior [26,27]. The present report indicates that an interaction between alkaline phos-

phatase and membrane exterior polar head groups regulates the shape of the Arrhenius plot with the tissue nonspecific type of alkaline phosphatase present in the kidney and placental tissues but not with the tissue specific intestinal alkaline phosphatase present in the intestinal tissue.

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