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ALKALINE PHOSPHATASE IN HeLa CELLS

STIMULATION BY PHOSPHOLIPASE A_2 AND LYSOPHOSPHATIDYLCHOLINE

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Summary

Treatment of homogenates and plasma membrane preparations from HeLa cells with phospholipase A_2 (EC 3.1.1.4) caused a 50% increase in activity of membrane-associated alkaline phosphatase. Lysophosphatidylcholine, dispersed in 0.15 M KCl, affected alkaline phosphatase in a similar fashion by releasing the enzyme from particulate fractions into the incubation medium and by elevating its specific activity. Higher concentrations of lysophosphatidylcholine solubilized additional protein from particulate fractions but did not further increase the specific activity of the released alkaline phosphatase.

Particulate fractions from HeLa cells were exposed to the effects of liposomes prepared from lysophosphatidylcholine and cholesterol. The ratio of particulate protein/lysophosphatidylcholine (by weight) required for optimal activation of alkaline phosphatase was one. Kinetic studies indicated that phospholipase A_2 and lysophosphatidylcholine enhanced the apparent V of the enzyme but did not significantly alter its apparent K_m. The increased release of alkaline phosphatase from the particulate matrix by lysophosphatidylcholine was confirmed by disc electrophoresis. The release of the enzyme by either phospholipase A_2 or by lysophosphatidylcholine appeared to be followed by the formation of micelles that contained lysophosphatidylcholine. The new complexes had relatively less cholesterol and more lysophosphatidylcholine than the native membranes. The possibility that lysophosphatidylcholine formed a lipoprotein complex with the solubilized alkaline phosphatase was indicated by a break point in the Arrhenius plot which was evident only in the lysophosphatidylcholine-solubilized enzyme but could not be demonstrated in alkaline phosphatase that had been released with 0.15 M KCl alone.

Introduction

It is well known that removal of membrane phospholipids either by treatment with phospholipases or by extraction with organic solvents frequently results in changes in enzyme activity [1,2]. Studies of membrane-bound Ca^{2+}-dependent ATPase have demonstrated that digestion of membrane lipids by phospholipase A_2 (EC 3.1.1.4) and the removal of digestion products was followed by a decrease in enzyme activity to less than 10% of the original level. The enzyme, treated in this fashion, could be reactivated by the addition of oleate or lysophosphatidylcholine or both [3,4]. Fiehn and Hasselbach [3] have also shown that treatment with phospholipase A_2 enhanced Ca^{2+}-dependent ATPase activity of sarcoplasmic vesicles more than 2-fold, provided the reaction products remained in the reaction mixture. Studies of plasma membrane-bound (Na^+ + K^+)-ATPase had shown that phospholipase A_2 removed almost all membrane phospholipids causing a loss of enzyme activity and its ability to form phosphoryl enzyme [5]. The ATPase, both in plasma membrane and in mitochondria appears to require phosphatidylserine or other negatively charged phospholipids for activity [6,7].

The function of cholesterol as one of the major components of biological membranes has been studied both in isolated membranes and phospholipid membrane models. Järnefelt [8] demonstrated the importance of cholesterol for (Na^+ + K^+)-ATPase. Papahadjopoulus and coworkers [9] showed that the presence of cholesterol interfered with the ability of proteins to increase permeability of phospholipid vesicles and also inhibited activity of a reconstituted preparation of (Na^+ + K^+)-ATPase. Lysophosphatidylcholine, the product of phospholipase A_2 digestion, has been shown to enhance the activity of UDP-glycuronyltransferase [10], glycoprotein-glycosyltransferase [11] and glucose-6-phosphatase [12]. All of this evidence seems to point out that the requirement of lipids in some of the membrane enzyme activities is not specific and that the ability of phospholipids to restore activity of delipidated membrane-bound enzymes or to enhance some activities of the untreated membrane-bound enzyme is also regulated by cholesterol. In this paper, we describe studies of the effects of lipid on membrane-bound alkaline phosphatase in HeLa cells. The membrane-bound alkaline phosphatase was stimulated following treatment with phospholipase A_2 or lysophosphatidylcholine, suggesting that phospholipids might be involved in the regulation of alkaline phosphatase activity in vivo.

Experimental

Cells and media. Two different strains of HeLa cells, S3G and S3K, were used. The S3G cells (courtesy of Dr. M. Griffin, Oklahoma Research Foundation) have a low level of alkaline phosphatase which is increased in response to glucocorticoids. The S3K strain (courtesy of Dr. K. Kajiwara, University of Wisconsin) has a high level of this enzyme which is suppressed if the cells are grown in the presence of these steroids. Both cell strains were passed twice weekly by detaching the cells with trypsin and splitting each culture 4—5-fold. Eagle's minimal essential medium was supplemented with 10% calf serum. Peni-

cillin and streptomycin were added at a final concentration of 100 μg/ml each.

Radioactive materials and method. [*Me*-^{14}C]Choline chloride, 5.1 Ci/mol and [^{14}C]cholesterol, 20—30 Ci/mol were purchased from New England Nuclear, Boston, Mass. The samples were solubilized in Soluene 100 (Packard) and were added to a scintillator consisting of 4 g of PPO and 0.1 g of POPOP (Packard)/l of toluene. Samples were counted in a Packard Tri-Carb Model 3375 liquid scintillation spectrometer. The counting efficiency was from 84 to 87% for carbon as calculated by the method of internal standards.

Preparation of plasma membranes. Plasma membranes were isolated on the aqueous two phase polymer system of Brunette and Till [13]. The two-phase system consisted of polyethylene glycol (Carbowax 600, Union Carbide) and Dextran 500 (Pharmacia). The membrane fraction was concentrated at the interphase and was collected with a Pasteur pipette.

Preparation of particulate fraction. Cells were removed from glass by scraping with perforated cellophane, washed twice with cold 0.85% NaCl and suspended in cold deionized water. They were homogenized with a tight fitting Dounce homogenizer. The homogenization was monitored by means of a phase contrast microscope. It was stopped when 95% or more of the cells were ruptured. The homogenate was pelleted at 10 000 × *g* for 30 min and the pellet was collected and resuspended in cold deionized water.

Preparation of liposomes. Liposomes were prepared in accordance with the method of Shinitzky and Inbar [14]. Various proportions of lysophosphatidylcholine (chromatographically pure from Sigma Chemical Co., St. Louis, Mo.) and cholesterol (Sigma Chemical Co.) were dissolved in chloroform/methanol (2 : 1, v/v) and were evaporated to dryness under N_2 gas and then dispersed in 0.15 M KCl. The dispersions were then subjected to ultrasonic irradiation in a Sonic Dismembrator 300 (Fisher Scientific Co.). The sonication was carried out in iced water for total of 20 min with four 2-min interruptions. The sonicated dispersions were centrifuged at 30 000 × *g* for 20 min. The insoluble portion was discarded, and the concentrations of cholesterol and lysophosphatidylcholine in the supernatant fluid were measured.

Treatment of particulate preparations with liposomes. Liposomes prepared either from lysophosphatidylcholine alone or from proportions of lysophosphatidylcholine and cholesterol were used to treat the particulate fraction. The treatment was usually carried out by mixing liposomes and the particulate preparation and incubating at 37°C for 20 min with constant shaking. After the incubation, the mixtures were centrifuged at 30 000 × *g* for 30 min to separate the supernatants from the pellets.

Pretreatment of membrane preparation with phospholipase A_2. Purified phospholipase A_2 from *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo., 1200—2400 units/mg protein) was used to treat the membrane preparations. The treatment was carried out at 37°C, usually for 20 min in 15 mM histidine buffer, pH 6.4, containing 0.07 M KCl and 4.3 mM $CaCl_2$. 10 μg of phospholipase A_2 per mg of membrane protein was used. The details of the treatment are given in the legends of the tables and figures.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were made according to the standard gel system of Davis [15] and Ornstein [16]. The system was stacked at pH 8.9 and run at pH 9.5. The final concentration of separating gel

was 7%. After the electrophoresis was finished, the gels were stained with indigo for alkaline phosphatase in Tris/borate buffer at pH 9.0 [17].

Analytical methods. The measurement of alkaline phosphatase activity was based on the hydrolysis of *p*-nitrophenylphosphate at 37°C in 0.75 M 2-amino-2-methyl-1-propanol buffer, pH 10.1. Alkaline phosphatase activity was expressed in terms of units, each unit being equal to the number of μmol *p*-nitrophenyl liberated in 2 h [18]. Protein was determined by the Folin-phenol method of Lowry et al. [19] with bovine serum albumin (Sigma Chemical Co.) as standard. Total lipids were extracted by the method of Folch et al. [20] employing a chloroform/methanol (2 : 1, v/v) mixture. Phospholipid was determined by the Bartlett [21] modification of Fiske-SubbaRow procedure for the determination of inorganic phosphorus. Total cholesterol was extracted according to the method of Sperry and Webb [22] and determined by the method of Kabara [23].

Results

Distribution of alkaline phosphatase activity of S3K cells

About 87% of the enzyme activity was present in the pellet obtained after centrifugation at 10 000 × *g* which consisted of various membrane fragments. The microsomal fraction pelleted at 105 000 × *g* had only about 11% of the total activity. There was less than 2% of the total activity present in the supernatant fraction which could have been released from the particulate fraction during the preparation.

Effect of phospholipase A_2 on plasma membrane-bound alkaline phosphatase activity

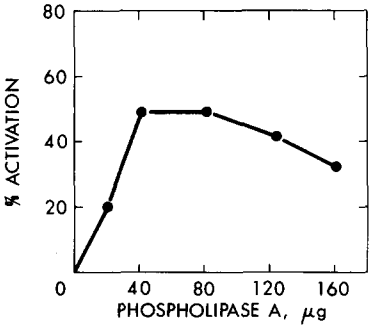
Treatment of both S3G and S3K cell membrane preparations and whole cell

TABLE I

EFFECT OF PHOSPHOLIPASE A_2 (*NAJA NAJA*) ON PLASMA MEMBRANE-BOUND ALKALINE PHOSPHATASE ACTIVITY

Plasma membranes were isolated according to the method of Brunette and Till [13]. Phospholipase A_2 treatment was carried out in 15 mM histidine buffer, pH 6.4, containing 0.07 M KCl and 4.3 mM $CaCl_2$ at 37°C for 20 min. 10 μg purified phospholipase A_2 from *Naja naja* venom per mg of membrane protein was used. The treatment was stopped by transferring the tubes to ice-cold water and alkaline phosphatase activity was measured directly in the reaction mixture. Slight 5′-nucleotidase activity present in the phospholine preparation was subtracted by using appropriate blanks.

	Alkaline phosphatase activity		Activation (%)
	Control	Phospholipase A_2	
Membrane			
S3G	5.3 ± 0.2	6.5 ± 0.4	23
S3K	284.5 ± 10.2	357.2 ± 12.5	26
Whole cell homogenate			
S3G	3.0 ± 0.1	3.9 ± 0.2	30
S3K	84.2 ± 5.2	120.0 ± 6.3	41



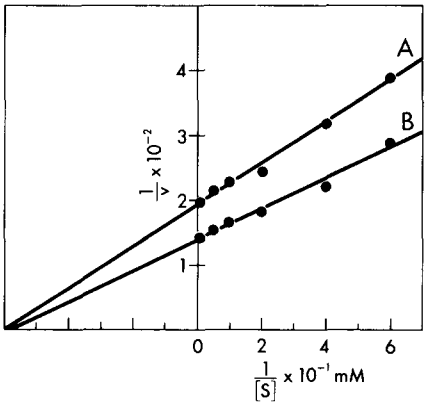


Fig. 1. Effect of phospholipase A_2 concentration on the membrane-bound alkaline phosphatase activation. Phospholipase A_2 treatment was carried out in 15 mM histidine buffer, pH 6.4, at 37°C for 20 min. Alkaline phosphatase activity was measured directly in the mixture.

Fig. 2. Effect of phospholipase A_2 (*Naja naja* venom) on particulate alkaline phosphatase activity. The range of substrate concentrations was between 2 and 0.017 mM. Samples were preincubated in 2.5 ml for 0.75 M 2-amino-2-methyl-1-propanol buffer containing 2 mM Mg^{2+}, pH 10.1, at 37°C for 15 min with constant shaking. Then 0.5 ml of substrate was added to bring the reaction mixture to 3 ml. The reaction was terminated by adding 3 ml of 1 M NaOH, and the absorbance was read at 410 nm in a spectrophotometer. A, untreated particulate preparation alkaline phosphatase activity; B, phospholipase A_2 treatment.

homogenates with phospholipase A_2 stimulated alkaline phosphatase activity by about 25% in the membrane preparations and more than 30% in the whole cell homogenates (Table II). As shown in Fig. 1 the maximum stimulation by phospholipase A_2 in the membrane preparation under the conditions used in this study, was about 50%. Further increases in phospholipase A_2 concentration (Fig. 1) or extending the time of digestion past 20 min (not shown) resulted in a decline of the stimulatory effect. Kinetic studies (Fig. 2) also revealed that under optimal conditions, phospholipase A_2 treatment increased

TABLE II

EFFECT OF LYSOPHOSPHATIDES AND DETERGENTS ON PARTICULATE ALKALINE PHOSPHATASE ACTIVITY

Lysophosphatides and detergents were dispersed in 0.15 M KCl separately by sonication. The dispersions were then used to treat the same amount of particulate preparation (1 mg/ml, 10 000 × *g* pellet) at 37°C for 20 min with constant shaking. Alkaline phosphatase activity was measured directly in the reaction mixture. The results were the average of three determinations in each treatment.

Treatments	Amount added (mg/ml)	Units of alkaline phosphatase	Activation (%)
Control	0	95.2 ± 4.0	0
Lysophosphatidylcholine	1	136.4 ± 6.2	43
Lysophosphatidylethanolamine	1	114.0 ± 3.4	20
Triton X-100	1	106.2 ± 5.1	11
	5	111.3 ± 2.4	17
Sodium deoxycholate	1	114.6 ± 2.4	20
	5	120.3 ± 1.9	26

the apparent V, whereas the apparent K_m of the enzyme activity remained unchanged, suggesting that phospholipase A_2 modified the membrane microenvironment without changing the apparent affinity of the enzyme molecule for the substrate.

Effect of lysophosphatidylcholine on the activity of particulate alkaline phosphatase

When exogenous lysophosphatidylcholine dispersed in 0.15 M KCl by ultrasonication was incubated with particulate preparation from HeLa cells for 30 min at 37°C with constant shaking, the alkaline phosphatase activity increased to about the same extent as in preparations treated with phospholipase A_2 (Table II). When the incubation mixtures were centrifuged at 30 000 × g for 30 min, the enzyme specific activity in the supernatant after lysophosphatidylcholine treatment was higher than in either complete incubation mixture or in the supernatant of the control sample. The amount of protein released from the particulate fraction after incubation with lysophosphatidylcholine was proportional to the amount of lysophosphatidylcholine added. The enzyme specific activity reached its maximum when the ratio of lysophosphatidylcholine to the particulate protein was one (Fig. 3). With this ratio, lysophosphatidylcholine extracted about 80% of particulate protein into the supernatant whereas in the control tube about 40% or less of particulate protein was released into the supernatant fraction. Further increasing the lysophosphatidylcholine concentration caused enzyme specific activity to decline slightly. The results of polyacrylamide gel electrophoresis showed that lysophosphatidylcholine solubilized membrane enzyme protein (Fig. 4). As the amount of lysophosphatidylcholine increased, the alkaline phosphatase protein band in polyacrylamide gel electrophoresis became wider and more dense. A slightly higher mobility of alkaline phosphatase was evident in gel electrophoresis with increased lysophosphatidylcholine. Possibly, some of the membrane alkaline phosphatase protein was deeply embedded within the membrane and was solu-

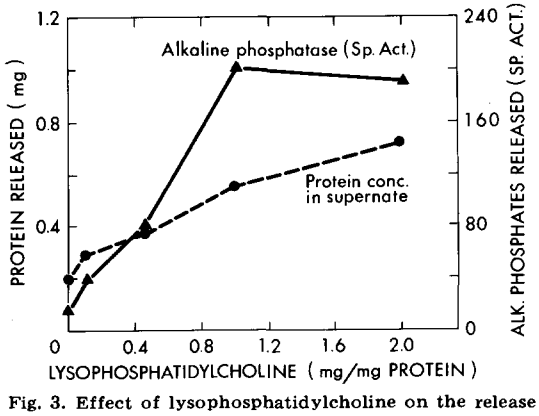


Fig. 3. Effect of lysophosphatidylcholine on the release of alkaline phosphatase from particulate fraction of HeLa S3K cells. Pellet obtained from 10 000 × g centrifugation was treated with various amounts of lysophosphatidylcholine in 0.15 M KCl at 37°C for 20 min. After treatment, mixtures were centrifuged at 30 000 × g for 30 min and supernatants were separated. Protein concentration and alkaline phosphatase activity were measured in the supernatant fluids.

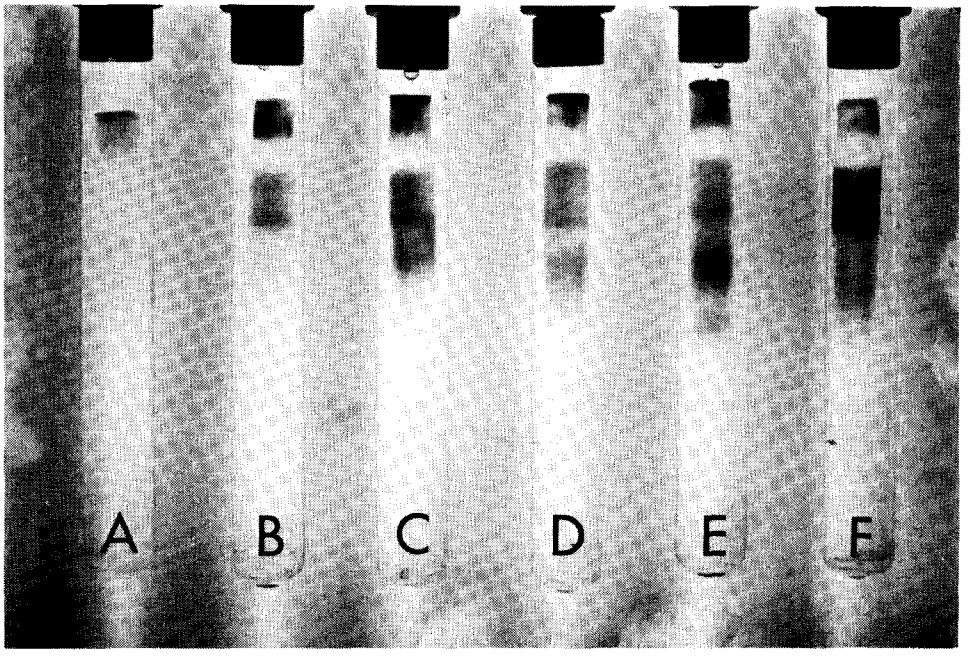


Fig. 4. Polyacrylamide gel electrophoresis of alkaline phosphatase solubilized by different concentrations of lysophosphatidylcholine. The gels were stained with indigo specifically for alkaline phosphatase in Tris/borate buffer at pH 9.0. A, Control: Alkaline phosphatase protein from particulate fraction was solubilized by 0.15 M KCl at 37°C with constant shaking for 20 min. B, C, D, E and F were alkaline phosphatase proteins solubilized by lysophosphatidylcholine in ratios 0.5, 1, 1.5, 2 and 3, respectively, of lysophosphatidylcholine to particulate protein.

bilized when lysophosphatidylcholine was added. Lysophosphatidylethanolamine, Triton X-100 or sodium deoxycholate were much less effective than either phospholipase A_2 or lysophosphatidylcholine (Table II). The kinetic studies revealed that lysophosphatidylcholine treatment increased the V without significantly changing the apparent K_m of the membrane-bound alkaline phosphatase suggesting that the effect of lysophosphatidylcholine treatment was identical to phospholipase A_2 digestion.

Effect of cholesterol on the solubilization and activation of particulate alkaline phosphatase by lysophosphatidylcholine liposomes

The results in Tables III and IV show that when liposomes made by ultrasonicating various proportions of cholesterol and lysophosphatidylcholine were incubated with particulate preparations, the effect of cholesterol on the solubilization and activation of particulate alkaline phosphatase by lysophosphatidylcholine was very prominent. The liposomes prepared by mixing cholesterol and lysophosphatidylcholine were less effective in solubilizing membrane or particulate preparations than those made from lysophosphatidylcholine alone. The amount of particulate protein solubilized into the 30 000 × g supernatant fraction decreased as the proportion of cholesterol present in the liposomes increased. Moreover, alkaline phosphatase activity present in the 30 000 × g supernatant fraction from liposomes made with equimolar concentrations of

TABLE III

EFFECT OF CHOLESTEROL ON THE SOLUBILIZATION AND ACTIVATION OF MEMBRANE-BOUND ALKALINE PHOSPHATASE BY LYSOPHOSPHATIDYLCHOLINE LIPOSOMES (30 000 × *g* CENTRIFUGATION)

Particulate fractions (10 000 × *g*) from S3K cells (3.12 mg/tube) were treated with liposomes consisting of various proportions of cholesterol and lysophosphatidylcholine. After incubation (20 min at 37°C) the mixtures were centrifuged at 30 000 × *g* for 30 min and the supernatants were collected. The treatments were as follows: A, control (0.15 KCl); B, 1.4 mM lysophosphatidylcholine liposomes; C, D and E, liposomes made from cholesterol and lysophosphatidylcholine in molar ratios of 0.9, 1.7 and 2.1, respectively; F, equimolar dispersions of lysophosphatidylcholine and phosphatidylcholine. Each value was the average of three determinations.

Treatments	30 000 × *g* supernatant fraction			30 000 × *g* pellet fraction			Total specific activity *
	Total protein (mg)	Total alkaline phosphatase (units)	Specific activity	Total protein (mg)	Total alkaline phosphatase (units)	Specific activity	
A	1.06	38.7	36.5	2.04	307.2	150.0	111.6
B	2.92	663.5	227.3	0.40	38.0	95.0	211.3
C	2.32	559.5	241.1	0.88	67.6	76.4	196.0
D	1.60	222.5	138.6	1.24	198.2	158.8	148.1
E	1.26	107;6	85.3	1.68	286.0	170.0	133.8
F	2.50	590.4	231.5	0.64	64.6	101.0	208.6

* Total specific activity = Total alkaline phosphatase activity from both fractions/total protein from both fractions.

cholesterol and lysophosphatidylcholine was slightly enhanced, whereas the liposomes of higher proportions of cholesterol suppressed the enzyme specific activity. The solubilization of particulate protein by lysophosphatidylcholine, affected by the presence of cholesterol in the liposomes, was also examined by treating the particulate preparation with liposomes made by mixing equimolar concentrations of lysophosphatidylcholine and phosphatidylcholine. The results (Tables III and IV) indicate that incorporating phosphatidylcholine into the lysophosphatidylcholine liposomes decreased the release of protein into the

TABLE IV

EFFECT OF CHOLESTEROL ON THE IMMOBILIZATION AND ACTIVATION OF MEMBRANE-BOUND ALKALINE PHOSPHATASE SOLUBILIZED BY LYSOPHOSPHATIDYLCHOLINE LIPOSOMES (120 000 × *g* CENTRIFUGATION)

The 30 000 × *g* supernatant fractions obtained from the previous experiment shown in Table III were further centrifuged at 120 000 × *g* for 2 h and the supernatants were separated from pellets. The protein concentrations and enzyme activities were measured from both fractions. The treatments were given in detail in Table III.

Treatments	Total initial protein (mg)	120 000 × *g* supernatant fraction			120 000 × *g* pellet fraction		
		Total protein (mg)	Total alkaline phosphatase (units)	Specific activity	Total protein (mg)	Total alkaline phosphatase (units)	Specific activity
A	1.06	0.80	22.0	27.5	0.136	6.7	49.4
B	2.92	2.15	467.0	233.4	0.640	78.6	122.5
C	2.32	1.75	277.0	158.3	0.424	122.6	289.1
D	1.60	1.25	91.5	73.3	0.296	79.6	235.1
E	1.26	0.90	16.0	17.5	0.208	39.6	190.4

supernatant but did not affect the enzyme activity, suggesting that cholesterol may suppress the detergent effect of lysophosphatidyl choline liposomes. This suggestion is further strengthened by the fact that as the proportion of cholesterol in the liposomes increased, less protein was released by the liposomes. However, the slightly higher alkaline phosphatase specific activity present in the supernatant fractions from both treatment with equimolar concentrations of cholesterol-phosphatidylcholine and phosphatidylcholine-lysophosphatidylcholine suggests that the inhibitory effect of cholesterol at higher concentrations on the activation of alkaline phosphatase by lysophosphatidylcholine liposomes was not due to dilution of the effective level of lysophosphatidylcholine. With the available information concerning the physical properties of cholesterol-phospholipid interactions, this appears to reflect an increased rigidity of the micellar structures containing cholesterol.

Effect of lysophosphatidylcholine on the solubilization of membrane cholesterol, phospholipids and alkaline phosphatase protein

It has been established (Table III and Fig. 4) that lysophosphatidylcholine can solubilize and enhance the membrane alkaline phosphatase activity. The

TABLE V

EFFECT OF LYSOPHOSPHATIDYLCHOLINE ON THE SOLUBILIZATION OF MEMBRANE CHOLESTEROL, PHOSPHOLIPID AND ALKALINE PHOSPHATASE

Cells were grown in the presence of either [^{14}C]cholesterol or [^{14}C]choline (3 μCi/100 ml) for 72 h. The medium was discarded and the cells were washed three times with cold 0.85% NaCl and twice with cold deionized water. Cells were then removed and suspended in deionized water. The preparation of particulate fraction and lysophosphatidylcholine treatment were followed by the method mentioned in Experimental. The amount of particulate protein used in each tube was 2 mg for Exp. I and 1.3 mg for Exp. II. After the treatment the mixtures were centrifuged at 30 000 × *g* for 30 min to separate the supernatants from the pellets. The protein concentration, alkaline phosphatase activity and radioactivity present in the supernatants were measured. For radioactivity, 25 μl of supernatant was dissolved in 0.5 ml Soluene 100 (Packard) and 10 ml of scintillation fluid in a scintillation vial. The result in each tube was the average of two determinations.

Tube	Lysophosphatidylcholine added (mg)	30 000 × *g* supernatant fraction			
		Protein concentration (mg/ml) (2 ml)	cpm/μg protein	Percent of total cholesterol	Alkaline phosphatase specific activity
Expt. I: [^{14}C]Cholesterol-labeled particulate					
1	0	0.2	16.8	7.0	25.2
2	0.7	0.6	116.6	52.2	195.2
3	1.33	0.67	111.1	50.3	206.4
4	2.0	0.71	107.2	47.3	225.6
5	2.5	0.80	114.6	51.6	212.5
6	3.0	0.85	101.1	45.7	200.4
Expt. II: [^{14}C]phosphatidylcholine					
1	0	0.12	38.3	7.7	28.3
2	0.5	0.34	253.2	51.2	220.4
3	1.0	0.41	270.0	54.6	252.4
4	1.5	0.47	240.9	48.7	215.9
5	2.0	0.50	249.7	51.0	218.7
6	3.0	0.52	218.0	44.1	190.6

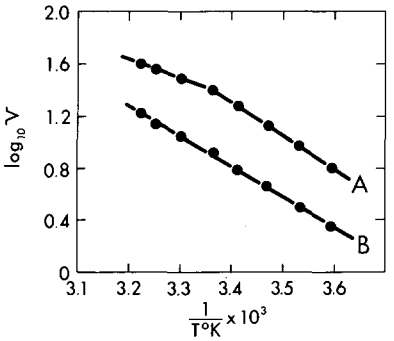


Fig. 5. Arrhenius plots of lysophosphatidylcholine-solubilized alkaline phosphatase and soluble alkaline phosphatase extracted in 0.15 M KCl. Lysophosphatidylcholine dispersed in 0.15 M KCl was used to treat particulate preparation at 37°C for 20 min with constant shaking. After the treatment the mixture was centrifuged at 30 000 × *g* for 30 min, and the supernatant was collected. Soluble alkaline phosphatase was extracted in 0.15 M KCl with constant shaking at 37°C for 2 h. After the extraction the mixture was centrifuged at 105 000 × *g* for 1 h, and the supernatant was saved. The ranges of temperature used for this study were between 40 and 50°C with 5°C apart in each assay. Each point was the average of three determinations. A, lysophosphatidylcholine-solubilized alkaline phosphatase; B, soluble alkaline phosphatase.

question then arises as to whether the alkaline phosphatase protein is present as a lipoprotein complex in the supernatant after lysophosphatidylcholine treatment. Two approaches were taken to resolve this question. The first approach was to grow the cells in the presence of radioactive [^{14}C] cholesterol or [^{14}C]-choline for 3 days to label the endogenous cholesterol or phosphatidylcholine. The radioactive materials in the particulate fractions prepared from the cells were measured after lysophosphatidylcholine treatment. The results shown in Table V indicate that lysophosphatidylcholine solubilizes both membrane alkaline phosphatase protein and membrane lipids. In a given amount of membrane protein treated with various amounts of lysophosphatidylcholine, the amount of membrane protein solubilized was proportional to the amount of lysophosphatidylcholine used, whereas the amount of membrane cholesterol and phosphatidylcholine released into the supernatants were relatively constant representing about 50% of the total lipid. Without exogenous lysophosphatidylcholine, only about 7% of membrane radioactive [^{14}C] cholesterol and 7.7% ^{14}C-labeled phosphatidylcholine were solubilized by the salt alone. The second line of evidence showing that alkaline phosphatase was solubilized into the 30 000 × *g* supernatant fraction as a lipoprotein complex comes from the observation that a break point occurs at 22°C in the Arrhenius plot (Fig. 5). The alkaline phosphatase activity solubilized by salt retained about 7% of total membrane lipid and showed a straight line in the same plot with an activation energy of about 10 cal. In contrast, the lysophosphatidylcholine-solubilized enzyme had a break point at 22°C with activation energy of 6 cal calculated from the plot, whereas below 22°C it increased to about 10 cal.

Discussion

It appears that the activation of membrane-bound alkaline phosphatase by either phospholipase A_2 digestion or directly by lysophosphatidylcholine treat-

ment is the result of solubilization of membrane protein by lysophosphatidylcholine. Phospholipase A_2 digestion increases lysophosphatidylcholine content of the membrane fractions whereas lysophosphatidylcholine treatment displaces more than 50% of membrane phospholipid and cholesterol. The content of lysophosphatidylcholine in the membrane increased by either mechanism can bring about a change in membrane structure and in membrane lipid microenvironment. Haydon and Taylor [24] have shown that lysophosphatidylcholine which may be formed by endogenous phospholipase A_2 reduces the stability of the lipid bilayer and favors the formation of globular lipid micellar structure. Increased lysophosphatidylcholine in the membrane should concomitantly increase membrane lipid fluidity which has been shown to correlate with the activation of some membrane-bound enzymes [1]. Further increases in lysophosphatidylcholine either by prolonging phospholipase A_2 digestion or by exogenous lysophosphatidylcholine treatment can lead to a disruption of membrane structure [26]. Following the dissolution of membrane bilayer structure, the new lipid micelles and some small lipid vesicles may be formed from lysophosphatidylcholine and from the released endogenous phospholipid. During this process, some of the membrane alkaline phosphatase protein, originally deeply buried within the membrane and so inaccessible to the substrate, might be released and reassociated with the new lipid micellar structures. This newly exposed enzyme would account for the total increase in enzyme activity.

There may be several mechanisms by which lysophosphatidylcholine could enhance the membrane-bound alkaline phosphatase activity. One possibility is the immobilization of the solubilized enzyme proteins by lysophosphatidylcholine micelles. Biological membrane lipids usually form lamellar structures having a repeating bilayer pattern. Extensive ultrasonication brings about formation of liposomes having only one bimolecular pattern separating two aqueous systems [26]. However, lysophosphatidylcholine usually forms true micellar structures. Recently, Barratt et al. [27] showed that C_{14}, C_{16} and C_{18} acyl lysophosphatidylcholine formed stable lipid micelle-β_1 casein protein complexes whereas C_{14} or higher fatty acyl phosphatidylcholine failed to do so, suggesting that with the same fatty acyl length lysophosphatidylcholine has a stronger protein binding capacity than the phosphatidylcholine which usually constitutes the major fraction of phospholipids in biological membranes. The membrane-bound alkaline phosphatase solubilized by lysophosphatidylcholine may reassociate with lysophosphatidylcholine micellar structures. Our data (Tables III—V) show that alkaline phosphatase protein released from the particulate preparation by lysophosphatidylcholine treatment is reassociated with liposomes as a lipoprotein complex having a higher specific activity than that which remains as a free enzyme. The formation of a lipoprotein complex is further evidenced by the results shown in the Arrhenius plot (Fig. 5). That immobilization of alkaline phosphatase activates the enzyme was demonstrated first by Poltorak and Vorobeva [28] who showed that the activity of purified alkaline phosphatase from *Escherichia coli* adsorbed on the hydrophobic surface of charcoal or of a charcoal and phosphatidylcholine mixture was enhanced as much as six times when compared to the native enzyme. Later Goldman et al. [29] used porous collodion sheets made of nitrocellulose in a mixture of ethanol/ether/water to adsorb the alkaline phosphatase isolated

from *E. coli*. They showed that the collodion membrane-adsorbed alkaline phosphatase activity increased by about 65% at pH 11. The enzyme solubilized by KCl alone may be present as free protein since our data show that KCl alone extracted only about 7% of total membrane lipid whereas lysophosphatidylcholine solubilized more than 50% of total membrane lipid. In addition, in the presence of cholesterol the binding capacity of lysophosphatidylcholine liposomes to protein was decreased slightly. In view of this evidence, it is reasonable to conclude that alkaline phosphatase protein solubilized by lysophosphatidylcholine treatment and then immobilized in lysophosphatidylcholine liposomes that have a high degree of fluidity may become more active. Although immobilization of the enzyme protein modifies the enzyme activity by limiting the freedom of molecules and sterically constraining its catalytic action, it can induce a conformational change which may favor the enzyme's catalytic activity.

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