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The Influence of Dichlorodiphenyltrichloroethane, Polychlorinated Biphenyls and Anionic Amphiphilic Compounds on Stabilization of Sodium- and Potassium-Activated Adenosine Triphosphatases by Acidic Phospholipids

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SUMMARY

SHARP, CHARLES W., HUNT, DOROTHY G., CLEMENTS, SAMUEL T., AND WILSON, WILLIAM E.: The influence of dichlorodiphenyltrichloroethane, polychlorinated biphenyls and anionic amphiphilic compounds on stabilization of sodium- and potassium-activated adenosine triphosphatases by acidic phospholipids. *Mol. Pharmacol.* **10,** 119–129 (1974).

Dichlorodiphenyltrichloroethane (DDT), extensively chlorinated biphenyls, deoxycholate, and phospholipase A inhibited beef brain and rabbit kidney ($Na^+ + K^+$)-ATPases (EC 3.6.1.3). Phosphatidylserine or phosphatidylinositol but not phosphatidylcholine or phosphatidylethanolamine prevented or reversed the inactivation of the enzymes by each of these inhibitors. Albumin protected against and reversed inactivation of ($Na^+ + K^+$)-ATPases by deoxycholate, oleate, or dodecyl sulfate; however, this protein was less effective against inactivation by the extensively chlorinated hydrocarbons. The extent of ($Na^+ + K^+$)-ATPase inactivation by anionic amphiphiles was dependent upon the temperature at which an enzyme-inhibitor mixture was incubated prior to assay, whereas inactivation by chlorinated hydrocarbons was not affected by temperature. Our experiments lead to the hypothesis that acidic phospholipids are necessary for stabilization of the enzyme and that chlorinated hydrocarbons, deoxycholate, and phospholipase A interfere with the stabilization process.

INTRODUCTION

The sodium- and potassium-stimulated adenosine triphosphatase [($Na^+ + K^+$)-ATPase, EC 3.6.1.3], which is generally thought to comprise the membranal sodium-pumping system (1), is inhibited by various chlorinated hydrocarbons such as DDT[1] (2–4) and polychlorinated biphenyls (5, 6). The possible toxicological significance of inhibition of this enzyme by the chlorinated hydrocarbons has received its most substantial support from studies of toxicity in eels. Kinter and co-workers (7–9) observed that DDT and PCBs were capable of inhibiting eel intestinal mucosal and gill ($Na^+ + K^+$)-ATPases to the extent that control of osmoregulation was lost, leading to death.

Since, under the conditions used for enzyme inactivation, the chlorinated hydrocarbons exist as oil-in-water dispersions, the

[1] The abbreviations used are: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; PCBs, polychlorinated biphenyl mixtures such as Aroclor 1221 or Aroclor 1254.

physical state of the inhibitors appears to be of great importance. Because the hydrocarbons are essentially insoluble in water, it is possible that (Na^+ + K^+)-ATPase inactivation results from interactions with either dispersed (micellar) or soluble (presumably monomeric) hydrocarbons. Moreover, enzyme inactivation by chlorinated hydrocarbons should be compared with that caused by oleate (10), dodecyl sulfate, or deoxycholate, as several of these anionic amphiphiles have been reported to effect enzyme inhibition where they exist in micellar form (11). Since phosphatidylserine stimulates enzyme activity following (Na^+ + K^+) ATPase exposure to phospholipase A (12, 13) or to deoxycholate (14–17), we have investigated the selectivity among phospholipids for protection against and reversal of enzyme inactivation by chlorinated hydrocarbons, several amphiphiles and phospholipase A.

MATERIALS AND METHODS

Chemicals. Dodecyl sulfate, ATP, NADH, phosphoenolpyruvate, and deoxycholate were obtained from Sigma Chemical Company. Fraction V albumin (Armour) and defatted, crystalline albumin (Sigma) were used interchangeably. Individual biphenyls were obtained from Aldrich Chemical Company. Aroclor 1221 (biphenyl plus mono- and dichlorinated biphenyls) and Aroclor 1254 (tri-, tetra-, penta-, and hexachlorinated biphenyls) were obtained from Monsanto.

Phosphatidylserine was prepared from bovine brain (18). Brain phosphatidylinositol and Lubrol WX were obtained from General Biochemicals, Inc. Other brain phospholipids were obtained from Supelco, Inc., Bellefonte, Pa. Each phospholipid was at least 96% homogeneous by thin-layer chromatography in three systems (19–21). Phosphate contents were determined, according to Dittmer and Wells (22), to be 1.25 μmoles/mg of phosphatidylserine and 1.20 μmoles/mg of phosphatidylcholine. Dry phospholipids were dispersed in 0.1 M Tris-HCl (pH 7.6), using a Teflon pestle and glass tube homogenizer, followed by 30 sec of sonic oscillation. Phospholipids were also dispersed by the technique of Stahl (23).

Phospholipase A. Phospholipase A (*Vipera russelli*; Sigma Chemical Company) solution in 50% glycerol was heated at 100° for 8 min prior to use (24). The extent of hydrolysis of the various phospholipids was determined by thin-layer chromatography (25) coupled with phosphate analysis (22). Phosphatidylserine was routinely used as a reference substrate for phospholipase A, as this pure phospholipid was hydrolyzed at least 10 times more rapidly than phosphatidylcholine under the conditions indicated in the legend to Fig. 4.

(Na^+ + K^+)-ATPase preparation. Bovine brain (Na^+ + K^+)-ATPase was prepared according to a modification (26) of the method of Skou (27). The enzyme was stored as an aqueous dispersion at −20° at a protein concentration of 30 mg/ml. Prior to assay, the enzyme was thawed and the protein concentration was reduced to 1 mg/ml. Protein was determined by the technique of Lowry *et al.* (28).

Rabbit kidney (Na^+ + K^+)-ATPase was prepared by a modification of the method of Jørgensen and Skou (29). In the deoxycholate activation step 34 mM KCl and 160 mM NaCl were present in addition to 0.6 mg/ml of deoxycholate, 2 mM EDTA, and 25 mM imidazole at pH 7.2. (Na^+ + K^+)-ATPase accounted for 85–90% of the total ATPase activity in these preparations.

(Na^+ + K^+)-ATPase activity assay. Lactate dehydrogenase and pyruvate kinase were obtained from Sigma Chemical Company and were dialyzed against 0.02 M Tris-HCl, pH 7.4, prior to use.

The assay medium was incubated at 37° for 4 min prior to initiation of the enzyme reaction. The reaction was usually started by adding 40 μg (40 μl) of beef brain (Na^+ + K^+)-ATPase protein or 10 μg (10 μl) of rabbit kidney enzyme protein to yield a final assay medium volume of 1 ml. Phospholipid dispersions and/or inhibitor solutions were added in the sequences indicated in the tables and figures. Initial velocities were determined by measuring the rate of NADH oxidation at 340 nm (30). Final concentrations of components of the incubation medium were 3 mM ATP, 3 mM $MgCl_2$, 125 mM NaCl, 25 mM KCl, 1.5 mM phosphoenolpyruvate, 0.25 mM NADH, 2 units

TABLE 1

Brain $(Na^+ + K^+)$-ATPase responsiveness to dispersions of oleate and chlorinated hydrocarbons

Inhibitors were introduced into the assay medium as solutions in dimethyl sulfoxide. At the assay concentration of 1% (v/v), dimethyl sulfoxide had no effect on enzyme activity. $(Na^+ + K^+)$-ATPase specific activity was 40 μmoles of P_i per milligram of protein per hour; enzyme protein concentration in the assay was 40 μg/ml. Assay conditions are described under MATERIALS AND METHODS. Results are means ± standard errors for three determinations.

Inhibitor	Inhibitor concentration during assay		Order of addition of enzyme and inhibitor to assay medium			
			Inhibitor added 15 sec after initiation of $(Na^+ + K^+)$-ATPase reaction	$(Na^+ + K^+)$-ATPase reaction initiated by adding enzyme at indicated intervals after inhibitors		
				0.1 min	20 min	120 min
	μM	ppm	% control activity			
None			100			
Tris-oleate	100		40 ± 4	43 ± 3	42 ± 4	43 ± 4
DDT	20		44 ± 4	44 ± 4	46 ± 4	43 ± 4
Aroclor 1254		10	42 ± 4	47 ± 4	53 ± 3	55 ± 4
Aroclor 1221		15	48 ± 4	49 ± 4	48 ± 3	57 ± 4

of lactate dehydrogenase, 7 units of pyruvate kinase, and 30 mM Tris-HCl, pH 7.4.

In order to ascertain the validity of the results obtained using the coupled lactate dehydrogenase–pyruvate kinase system, we initially determined that the rate of NADH oxidation was unaltered when 0.15 mM ADP was used as substrate in the absence of $(Na^+ + K^+)$-ATPase and in the presence of each amphiphilic compound, hydrocarbon, phospholipid, and combination thereof. Also, most of the data in Tables 3 and 4 were corroborated by results from our phosphate analysis technique (26).

RESULTS

$(Na^+ + K^+)$-ATPase responsiveness to water-insoluble substances. Inactivation resulted from interaction of $(Na^+ + K^+)$-ATPase with oil-in-water dispersions, rather than solutions, of fatty acids or chlorinated hydrocarbons. The extent of $(Na^+ + K^+)$-ATPase inactivation by chlorinated hydrocarbons or by oleate was not appreciably influenced by the time at which the enzyme was exposed to inhibitors (Table 1). Clear aqueous phases obtained by centrifugation of oleate, PCBs, or DDT dispersions (Table 1) for 30 min at 100,000 × *g* were not inhibitory.

Addition of ethanol solutions of DDT (31), 2-chlorobiphenyl, PCB mixtures, or Tris-oleate to water resulted in the formation of dispersions whose particle sizes and/or numbers appeared to change continuously for several hours (31) (Fig. 1). The spectral alterations accompanying the change of phase of 2-chlorobiphenyl (Fig. 1A) reflected contributions from hydrocarbon in solution and from light scattering by micelles; however, the alterations accompanying the change of phase of oleate (Fig. 1B) reflected only the time-dependent increases in micelle number and/or size (32).

In a concentration dependence study of $(Na^+ + K^+)$-ATPase inactivation, an increase in the chlorinated hydrocarbon concentration resulted only in an increase in amount of inhibitor in the dispersed phase (Fig. 2). However, the extent of enzyme inactivation increased as the extent of chlorine substitution of biphenyl was increased (Fig. 2).

Concentration and temperature dependence of $(Na^+ + K^+)$-ATPase inactivation by anionic amphiphilic compounds. The curve for concentration dependence of brain $(Na^+ + K^+)$-ATPase inactivation by oleate had a different shape from the curve for inactivation by dodecyl sulfate(Fig. 3). This may be due to the occurrence of enzyme inactivation at assay concentrations below the critical micelle concentration for dodecyl sulfate (calculated to be about 0.17 mM from ref.

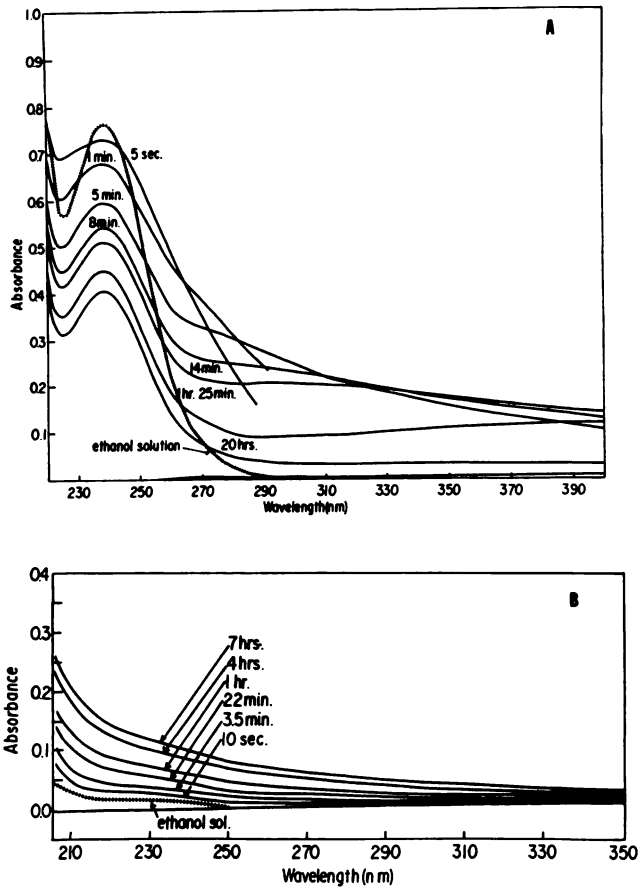

FIG. 1. *Absorption spectra of 67 μM 2-chlorobiphenyl (A) and 25 μM Tris-oleate (B) in ethanol (- - -) and in 1% ethanol in water (——)*

Spectral alterations in the ethanol–water solutions were determined at the indicated intervals after preparation. Spectra were recorded with a Beckman Acta V spectrophotometer.

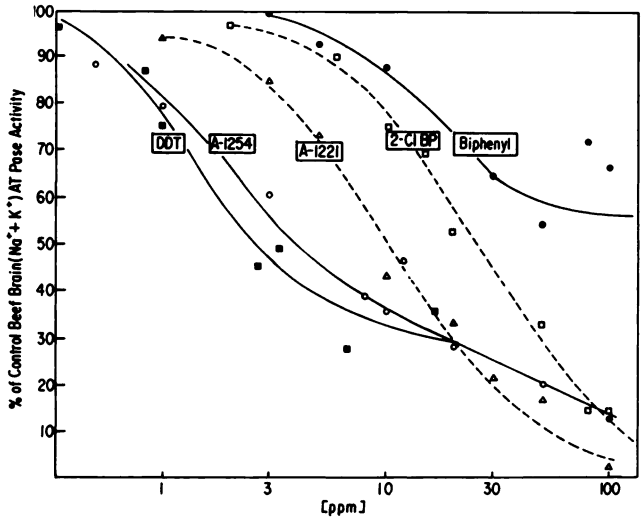

FIG. 2. *Concentration dependence of brain ($Na^+ + K^+$)-ATPase inhibition by DDT, Aroclor 1254, Aroclor 1221, 2-chlorobiphenyl and biphenyl*

Ten microliters of a dimethyl sulfoxide solution of each chlorinated hydrocarbon were added 3 sec after addition of 40 μg of enzyme protein to initiate the ATPase assay.

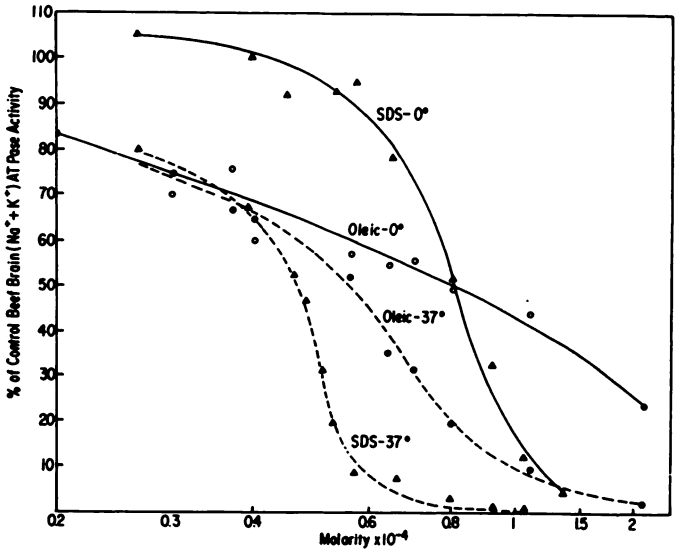

FIG. 3. *Concentration dependence of brain (Na+ + K+)-ATPase inhibition by Tris-oleate and sodium dodecyl sulfate (SDS)*

Dispersions of enzyme plus inhibitor, both at 12.5-fold higher concentrations than during subsequent assay, were incubated in a medium containing 5 mM KCl, 24 mM NaCl and 17 mM Tris-HCl, pH 7.5. Incubation was conducted for 10 min at 0° or 37°; then 80 μl of this mixture were added to 920 μl of the assay mixture (see MATERIALS AND METHODS) in order to initiate the (Na+ + K+)-ATPase reaction.

11), whereas micellar oleate was required to effect inactivation.

Following prior incubation the anionic amphiphiles (but not the hydrocarbons) inactivated (Na+ + K+)-ATPases in a temperature-dependent fashion (Fig. 3). In contrast with results obtained with dodecyl sulfate, accentuated enzyme inactivation by oleate was observed only at an inhibitor concentration greater than that required to effect 40% inactivation.

The extent of (Na+ + K+)-ATPase inactivation by deoxycholate depended upon the absolute quantities of enzyme and inhibitor subjected to prior incubation at 37° (Table 2). Temperature-dependent enzyme inactivation was only observed following prior incubation at deoxycholate concentrations appreciably in excess of the critical micelle concentration (calculated to be about 1.2 mM from data in ref. 11); however, deoxycholate concentrations during the assay were always below this level.

Protection of (Na+ + K+)-ATPase against inactivation by anionic amphiphilic compounds. Phosphatidylserine and phosphatidylinositol[2] (not shown) protected both (Na+ + K+)-ATPases against inactivation by deoxycholate when prior incubation was conducted at 37°; however, phosphatidylcholine and phosphatidylethanolamine (not shown) provided no protection (Table 3). By contrast, each phospholipid protected the enzyme against inactivation by oleate or dodecyl sulfate. The indicated stoichiometric relationships among enzyme, inhibitor, and protective phospholipid were optimal for the observation of phospholipid selectivity; however, identical results were obtained over a 4-fold range of concentrations of either phosphatidylcholine or phosphatidylserine (i.e., from one-half to twice the

[2] In each instance when protection or reversal by phosphatidylinositol or phosphatidylethanolamine is mentioned only in the text of RESULTS, at least two analyses were performed. In no case did the results using phosphatidylethanolamine differ significantly from those using phosphatidylcholine. The commercial bovine brain phosphatidylinositol consistently provided results similar to those obtained with our brain phosphatidylserine; however, commercial preparations of plant phosphatidylinositol have yielded variable results.

TABLE 2

Influence of prior incubation on extent of brain ($Na^+ + K^+$)-ATPase inactivation by deoxycholate

Deoxycholate concentration during assay	Standard assay[a]	Prior incubation at multiples of [deoxycholate] in standard assay[b]							
		×3.1		×6.2		×12.5		×25	
		0°	37°	0°	37°	0°	37°	0°	37°
μM		% control activity							
0	100	92	86	89	91	88	83	87	91
64	88	84	74	74	64	73	72	79	62
128	85	73	58	66	65	71	61	46	21
255	67	64	56	67	61	51	28	20	10
510	48	45	42	44	29	17	8	14	7
1020	20	21	10	12	4				

[a] As described under MATERIALS AND METHODS, 40 μg/ml of enzyme protein and the designated amount of deoxycholate were added separately to the cuvette in order to obtain the results in this column. Control ($Na^+ + K^+$)-ATPase specific activity was 36 μmoles of P_i per milligram of protein per hour. Results are averages of three determinations.

[b] For example, the multiple ×12.5, at 255 μM deoxycholate, indicates that 500 μg of enzyme protein, 3200 μM deoxycholate, and 17 mM Tris-Cl, pH 7.5, were mixed and incubated for 6 min at 0° or 37°; then an aliquot was diluted 12.5-fold into the assay cuvette, containing a reaction mixture such that assay conditions were identical with those of the standard assay. Results below the short lines represent the temperature dependence effect following prior incubation.

TABLE 3

Phospholipid protection of mammalian ($Na^+ + K^+$)-ATPases against inactivation by several anionic amphiphilic compounds

See the legend to Fig. 3 for a general description of the incubation technique. Albumin or phospholipid was added to the ($Na^+ + K^+$)-ATPase dispersion prior to addition of the inhibitor solution. The concentrations of each component of the incubation mixture was 12.5-fold greater (beef brain enzyme) or 50-fold greater (rabbit kidney enzyme) than during assay. Respective specific activities for beef brain and rabbit kidney ($Na^+ + K^+$)-ATPases were 40 and 140 μmoles of P_i per milligram of protein per hour, while corresponding protein concentrations were 40 and 10 μg/ml during assay. Enzyme activity assay conditions are described under MATERIALS AND METHODS. The values are means of six determinations (SEM ≤ ±10%).

Enzyme source and inhibitor	Temperature	No protective compound	Phosphatidylcholine (1.6 mg/ml)	Phosphatidylserine (1.6 mg/ml)	Albumin (6.25 mg/ml)
	°C	% control activity			
Brain (beef)					
No inhibitor	0	100	100 (92)	100 (129)	100 (95)
	37	100 (98)[a]	100 (96)	100 (140)	100 (91)
Oleate, 1.25 mM	0	39	90	93	94
	37	7	89	84	70
Dodecyl sulfate, 0.84 mM	0	71	75	80	94
	37	11	67	60	88
Deoxycholate, 3.4 mM	0	54	71	83	75
	37	19	15	62	60
Kidney (rabbit)					
No inhibitor	0	100	100	100	100
	37	100 (93)	100 (93)	100 (93)	100 (95)
Dodecyl sulfate, 0.84 mM	0	96	100	100	102
	37	23	100	100	92
Deoxycholate, 3.4 mM	0	58	92	99	97
	37	22	18	93	90

[a] Values in parentheses are percentages of the 100% values at the top of the column headed "No protective compound" for each enzyme preparation. All other values are relative to the appropriate 100% value (i.e., the ones obtained with no inhibitor present during prior incubation at 0° or at 37°).

TABLE 4

Phospholipid and albumin protection of brain $(Na^+ + K^+)$-ATPase against inhibition by chlorinated biphenyls and DDT

Incubation techniques are described in the legend to Fig. 3. The $(Na^+ + K^+)$-ATPase protein concentration was 40 μg/ml during the assay. Values are means ± standard errors of four determinations.

Inhibitor	Temperature during initial incubation	No protective compound	Phosphatidylcholine (133 μg/ml)	Phosphatidylserine (133 μg/ml)	Phosphatidylinositol (133 μg/ml)	Albumin (1000 μg/ml)
	°C			*% control activity*		
None	0	100	100 (92)[a]	100 (135)	100 (110)	100 (99)
	37	100 (98)	100 (96)	100 (145)	100 (108)	100 (95)
DDT, 3.5 ppm	0	46 ± 8	70 ± 8	95 ± 9	90 ± 3	46 ± 5
	37	45 ± 3	70 ± 2	72 ± 6	76 ± 5	49 ± 6
Aroclor 1221, 10 ppm	0	44 ± 7	68 ± 7	103 ± 7	98 ± 9	66 ± 5
	37	42 ± 6	66 ± 8	98 ± 4	93 ± 5	65 ± 4
Aroclor 1254, 5 ppm	0	48 ± 4	67 ± 6	95 ± 9	100 ± 9	67 ± 5
	37	46 ± 7	70 ± 8	88 ± 8	95 ± 5	66 ± 4

[a] Values in parentheses are percentages of the control $(Na^+ + K^+)$-ATPase activity shown at the top of the column headed "No protective compound."

concentrations of phospholipid indicated in Table 3).

Albumin, at 4 times the mass of the phospholipids, effectively protected the brain enzyme against each of the anionic amphiphiles (Table 3).

Protection of $(Na^+ + K^+)$-ATPases against inhibition by chlorinated hydrocarbons. The prior incubation technique was not optimal for observing selective phospholipid protection against $(Na^+ + K^+)$-ATPase inactivation by chlorinated hydrocarbons (Table 4). Both albumin and phosphatidylcholine protected against the chlorinated hydrocarbons. Furthermore, both phosphatidylserine and phosphatidylinositol activated the controls (Tables 3 and 4). Since it was not possible to determine the extent of chlorinated hydrocarbon influence on phospholipid activation of the controls, the validity of our arbitrary correction of the values in the vertical columns of Table 4 could not be determined.

In contrast with the above results, acidic phospholipids did not increase control $(Na^+ + K^+)$-ATPase activity when both enzyme and phospholipid were added directly to the assay medium (Table 5). Acidic phospholipids selectively protected the two $(Na^+ + K^+)$-ATPases against inactivation by deoxycholate, DDT, and Aroclor 1254 (Table 5).

Albumin protected against enzyme inactivation by deoxycholate but not against that caused by DDT or Aroclor 1254 (Table 5).

Reversal of $(Na^+ + K^+)$-ATPase inactivation by hydrocarbons or by amphiphiles. Albumin and both acidic and neutral phospholipids reversed brain $(Na^+ + K^+)$-ATPase inactivation resulting from enzyme exposure to dodecyl sulfate, oleate, Aroclor 1221, or 2-chlorobiphenyl (Table 6). Only phosphatidylserine or phosphatidylinositol (not shown) effectively reversed inactivation of the enzyme by DDT or Aroclor 1254. Albumin, at 2000 μg/ml, reversed inactivation by deoxycholate, DDT, or Aroclor 1254.

The conditions described in Table 6 were nearly optimal for demonstrating selectivity among different compounds that could reverse inactivation by the chlorinated hydrocarbons. For instance, a 3-fold increase in phosphatidylcholine concentration gave only 15% greater reversal of inactivation by DDT. Phosphatidylserine, at 80 μg/ml during assay, was essentially as effective as it was at 100 μg/ml.

Phospholipid selectivity in reversing and protecting against $(Na^+ + K^+)$-ATPase inactivation by phospholipase A. Phosphatidylserine and phosphatidylinositol reversed the inactivation resulting from $(Na^+ + K^+)$-ATPase exposure to phospholipase A (Fig.

TABLE 5

Protection of mammalian ($Na^+ + K^+$)-ATPases with inhibitor and/or protective compound present only during assay

Brain and kidney enzyme protein concentrations were 40 and 10 μg/ml, respectively, during the assay; respective specific activities were 40 and 140 μmoles of P_i per milligram of protein per hour. The reaction was initiated by adding enzyme to the assay mixture, which contained protective compound, at 37°. Inhibitor solution (10 μl) was added immediately after the enzyme. Values are means of four to six determinations (SEM ≦ ±10% for each value).

Enzyme source and inhibitor	No protective compound	Phosphatidylcholine (100 μg/ml)	Phosphatidylserine (100 μg/ml)	Albumin (800 μg/ml)
		% control activity		
Brain (beef)				
No inhibitor	100	97	104	95
Deoxycholate, 600 μM	42	49	65	68
Dodecyl sulfate, 100 μM	37	48	52	79
Oleate, 100 μM	41	65	73	86
Aroclor 1221, 20 ppm	34	54	84	34
Aroclor 1254, 10 ppm	38	45	84	41
DDT, 3.5 ppm or 10 μM	46	47	72	47
Kidney (rabbit)				
No inhibitor	100	98	101	97
Deoxycholate, 600 μM	34	36	56	60
Dodecyl sulfate, 100 μM	24	51	64	90
Oleate, 100 μM	42	64	92	99
Aroclor 1254, 10 ppm	71	69	99	67
DDT, 10 μM	66	63	96	65
DDE, 10 μM	69	72	95	67

TABLE 6

Reversal of enzyme inactivation by phospholipids and albumin after exposure of brain ($Na^+ + K^+$) ATPase to anionic amphiphiles and chlorinated hydrocarbons

Six minutes after initiation of the enzyme reaction in the presence of inhibitor (see MATERIALS AND METHODS), 10 μl of a phospholipid dispersion or a protein solution were added to the cuvette. Reaction velocities were measured for an additional 6 min. Control ($Na^+ + K^+$)-ATPase specific activity was 40 μmoles of P_i per milligram of protein per hour; protein concentration during assay was 40 μg/ml. Values are means ± standard errors of six determinations.

Inhibitor	Concentration		No reversing compound	Phosphatidylcholine (100 μg/ml)	Phosphatidylserine (100 μg/ml)	Albumin	
						800 μg/ml	2000 μg/ml
	μM	*ppm*		*% control activity*			
None			100	95 ± 7	105 ± 8	97 ± 8	
Deoxycholate	600		38 ± 5	40 ± 5	45 ± 5	48 ± 5	65 ± 5
Dodecyl sulfate	83		50 ± 4	69 ± 5	80 ± 4	85 ± 4	
Oleate	100		34 ± 4	68 ± 6	74 ± 7	71 ± 8	
DDT	10	3.5	44 ± 5	50 ± 3	76 ± 4	47 ± 5	59 ± 6
Aroclor 1254		10	41 ± 6	45 ± 3	71 ± 5	51 ± 6	64 ± 6
Aroclor 1221		10	44 ± 5	66 ± 2	95 ± 4	62 ± 2	
2-Chlorobiphenyl		20	45 ± 6	68 ± 4	83 ± 5	69 ± 2	

4). After the 8-min exposure to phospholipase A, both phosphatidylcholine and phosphatidylserine in the brain ($Na^+ + K^+$)-ATPase preparation were hydrolyzed at least 90%, in agreement with the observations of Taniguchi and Tonomura (12).

Acidic phospholipids selectively protected the ($Na^+ + K^+$)-ATPases against inactiva-

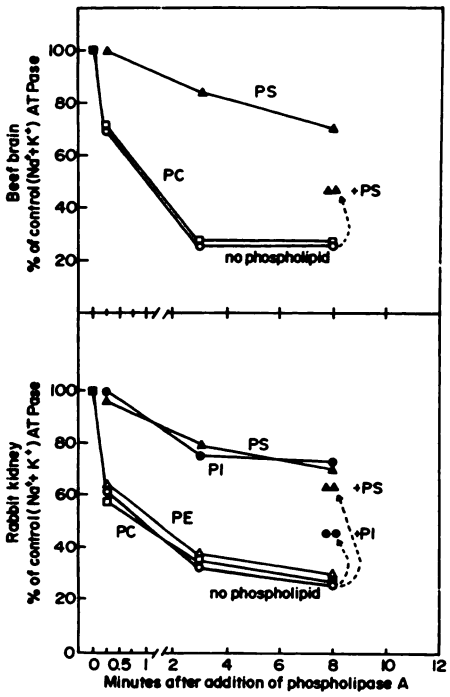

FIG. 4. *Protection by acidic phospholipids against and reversal of inactivation of* $(Na^+ + K^+)$-*ATPase preparations exposed to phospholipase A*

Beef brain or rabbit kidney $(Na^+ + K^+)$-ATPases, at protein concentrations of 0.5 mg/ml, were incubated at 37° in 0.5 ml of a medium consisting of 50 mM Tris-Cl (pH 7.0), 2 mM $CaCl_2$ and 4 mg of albumin. Phospholipid concentration was 0.6 mg/ml of incubation mixture. After each mixture (with or without phospholipid) was incubated for 6 min, phospholipase A was added to yield a final protein concentration of 2.5 μg/ml. At the indicated times 24 volumes of the phospholipase A-treated mixture were withdrawn and mixed with 1 volume of 0.1 M EDTA at 0° to stop the hydrolysis by phospholipase A. Reversal of $(Na^+ + K^+)$-ATPase inactivation (indicated by dashed arrows), resulting from 8 min of enzyme exposure to phospholipase A, was effected by mixing 5 volumes of the EDTA-inactivated reaction mixture with 1 volume of the appropriate phospholipid dispersion to give a final phospholipid concentration of 1.67 mg/ml. (Although the results are not shown in this figure, phosphatidylcholine and phosphatidylethanolamine were also added after the 8-min exposure to phospholipase A. Neither neutral phospholipid effected reversal of enzyme inactivation.) Assays for $(Na^+ + K^+)$-ATPase activity were performed as described under MATERIALS AND METHODS, using aliquots containing 40 μg of brain enzyme protein or 10 μg of kidney enzyme protein per milliliter of assay mixture. Each point is the average of six determinations. PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

tion by phospholipase A (Fig. 4). However, under identical assay conditions, about 40% of the pure phosphatidylserine and less than 5% of the pure phosphatidylcholine were hydrolyzed in 8 min by phospholipase A. When it was mixed with 5% Lubrol WX (w/w), phosphatidylcholine hydrolysis by phospholipase A was increased to 8–12%, but the extent of protection against phospholipase A inactivation was only slightly higher than indicated for pure phosphatidylcholine in Fig. 4.

The selectivity among phospholipids for protection of brain $(Na^+ + K^+)$-ATPase against inactivation by deoxycholate or for reversal of inactivation by phospholipase A was essentially identical whether the phospholipids were dispersed in Tris-Cl (MATERIALS AND METHODS) or in Tris–ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), followed by dialysis against Tris-Cl, according to the technique of Stahl (23). The only obvious difference was that phosphatidylserine prepared according to Stahl's technique (23) was somewhat more efficient than our usual preparations in reversing inactivation by phospholipase A.

DISCUSSION

Inactivation of $(Na^+ + K^+)$-ATPases subsequent to exposure to oil-in-water dispersions of chlorinated hydrocarbons (Figs. 1 and 2; Table 1) or of oleate (Figs. 1 and 3; Table 1) presumably occurred as a consequence of formation of mixed micelles comprising the enzyme and each of the inhibitors. No attempt was made to study the kinetics of the inhibition phenomena, as such efforts would have required additional (unavailable) information relative to micelle dimensions, composition, and stabilities.

The conditions of prior incubation with micellar deoxycholate accentuated the extent of $(Na^+ + K^+)$-ATPase inactivation even though a constant enzyme-inhibitor ratio was maintained (Table 2). However, neither deoxycholate nor dodecyl sulfate needed to exist in micellar form to effect $(Na^+ + K^+)$-ATPase inactivation—a rela-

tionship implied by Jørgensen and Skou (11).

In view of its binding capacity for dodecyl sulfate (33–35), albumin was anticipated to protect against (Tables 3 and 5) and reverse (Table 6) inactivation of the $(Na^+ + K^+)$-ATPases by dodecyl sulfate and the other anionic amphiphiles.

The selective protection by acidic phospholipids against enzyme inactivation by deoxycholate (Tables 3 and 5) suggested that inactivation involved either extraction or dissociation of phospholipids required for normal stabilization. The phospholipid selectivity was obviously related to some property of the steroid ring of deoxycholate rather than to its carboxyl group. Although neither phosphatidylserine nor albumin reversed (Table 6) $(Na^+ + K^+)$-ATPase inactivation by deoxycholate under conditions in which these compounds could provide protection (Table 5), the reversal by a higher concentration of albumin indicated that the enzyme had not been irreversibly inactivated.

The nonselective phospholipid protection against, and reversal of, inactivation by oleate or dodecyl sulfate (Tables 3, 5, and 6) suggested that the inactivation was unrelated to interference with $(Na^+ + K^+)$-ATPase stabilization by acidic phospholipids. The lack of selectivity among phospholipids for reversal of $(Na^+ + K^+)$-ATPase inactivation by these two amphiphiles resembled observations on enzyme inactivation by phospholipase C (23). Stahl observed that maximum inactivation of brain $(Na^+ + K^+)$-ATPase resulted when phospholipase C hydrolyzed 95% of the enzyme-associated phosphatidylcholine and 65% of the phosphatidylserine plus phosphatidylinositol (23). Although pure phospholipids were hydrolyzed at markedly different rates by phospholipase C (36), they were equally effective in reversing $(Na^+ + K^+)$-ATPase inactivation by phospholipase C (23).

The selectivity among phospholipids in protecting against $(Na^+ + K^+)$-ATPase inactivation by phospholipase A (Fig. 4) could be explained, in part, on the basis of apparent substrate preference of phospholipase A for the pure phospholipids. Hokin and Hexum (13) had observed that pure phosphatidylcholine protected their highly purified $(Na^+ + K^+)$-ATPase against phospholipase A inactivation, but did not report the effects of other phospholipids. Since many factors, including detergents (37) and organic solvents (38), can influence the rates of hydrolysis of pure phospholipids by phospholipase A or phospholipase C (23), the selectivity among phospholipids for protection against $(Na^+ + K^+)$-ATPase inactivation was considered to be of limited usefulness for this investigation.

The responsiveness of $(Na^+ + K^+)$-ATPase to DDT and the more extensively substituted biphenyls differed from that to anionic amphiphilic compounds in several important respects. Albumin provided very little protection against or reversal of enzyme inactivation by DDT or Aroclor 1254 (Tables 5 and 6), presumably because of a relatively low affinity for extensively chlorinated hydrocarbons. Anionic amphiphilic compounds could accentuate $(Na^+ + K^+)$-ATPase inactivation in a temperature-dependent fashion (Fig. 3; Tables 2 and 3), but the chlorinated hydrocarbons were unable to do so (Table 4). Temperature dependence was assumed to reflect the influence of variations in membranal phospholipid hydrocarbon phase fluidity on the ease of enzyme inactivation.

The structure of chlorinated hydrocarbon dispersions probably differs from the micellar structure of the anionic amphiphiles in that no ionizable groups can exist at the surface of hydrocarbon dispersions. However, it has not been possible to define any unusual properties of oil-in-water dispersions of DDT (31). The X-ray diffraction pattern of DDT sedimented from an oil-in-water dispersion, such as the one in Table 1, was identical with that of crystalline DDT.[3] The packing pattern in the crystal lattice of DDT did not reveal any unanticipated potential for intermolecular dipole-dipole or dipole-induced dipole interactions (39).

In regard to the molecular association capabilities of the hydrocarbons, DDT can associate to significant extents with indole (40), phenyl (40), and phosphate groups (41). However, hydrogen bonding of chlorinated hydrocarbons to phospholipid phos-

[3] N. Morosoff, personal communication.

phate groups must be unrelated to $(Na^+ + K^+)$-ATPase inactivation, since DDE (Table 5) and the PCBs are incapable of such interactions (i.e., these inhibitors do not possess benzhydryl hydrogens).

The acidic phospholipid selectivity for protection against and reversal of $(Na^+ + K^+)$-ATPase inactivation by DDT, Aroclor 1254, and deoxycholate obviously mimics that for reversal of enzyme inactivation by phospholipase A. Since this selectivity cannot be explained on the basis of structural or other properties of the inhibitors, it must reflect properties of the $(Na^+ + K^+)$-ATPases. These studies support the hypothesis that the extensively chlorinated aromatic hydrocarbons DDT and Aroclor 1254 inactivate membrane $(Na^+ + K^+)$-ATPases by interfering with the stabilizing function of acidic phospholipids.

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