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Requirement for Negatively Charged Dispersions of Phospholipids for Interaction with Lipid-Depleted Adenosine Triphosphatase

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The basis of the requirement for a net negative charge on phospholipid dispersions able to re-activate lipid-depleted ($Na^+ + K^+$)-dependent adenosine triphosphatase was studied. The origin and density of the charge in phospholipid dispersions were varied before interaction with the adenosine triphosphatase protein, and the charge density on restored phospholipid–adenosine triphosphatase complexes was changed after interaction. The results indicated that: (*a*) re-activation requires a lamellar arrangement of the lipid molecules with sufficient density of negative charge, but not necessarily negatively charged phospholipid molecules; (*b*) the net charge appears to be necessary for the correct interaction between the enzyme protein and the phospholipids, although the amount of phospholipid that binds to the protein is also a function of the nature of the acyl chains; (*c*) it is not possible on the basis of these findings and those in the literature to decide unequivocally if the charge is also required for the enzyme reaction itself. The possible relevance of the findings to the situation *in vivo* is discussed in terms of the charge being concerned only with lipid–protein interaction.

When phospholipids are removed from the ($Na^+ + K^+$)-dependent ATPase it is inactivated; but, under appropriate conditions, it is sometimes possible to re-activate the enzyme simply by mixing it with phospholipid dispersions. In general, although there have been exceptions, the results of many such re-activation studies have been interpreted as showing that only those phospholipids bearing a net negative charge are capable of significant re-activation, and that the extent of re-activation is also dependent on the nature of their fatty acyl chains (see Walker & Wheeler, 1975, and references therein). It is obviously invalid to extrapolate from such re-activation findings to conclude that a similar requirement for a negatively charged phospholipid exists *in vivo*, although that might be so. On the other hand, where such specificity in re-activation ability is demonstrated, it is clearly important to try to find out why it exists; that information might throw light on the situation *in vivo* and, equally as important, it might help eventually to explain the molecular basis of the ($Na^+ + K^+$)-dependent ATPase reaction.

The negative charge on the phospholipid could be required merely for the initial interaction between the ATPase protein and the lipid, or it could be necessary for the actual enzymic reaction (Walker & Wheeler, 1975). The former possibility would lead to the question of how the protein and lipids 'recognize' one another, both *in vitro* and *in vivo*, whereas the latter possibility has implications for the enzyme reaction mechanism. We have tried to distinguish between these possibilities by examination of the re-activation of lipid-depleted ($Na^+ + K^+$)-dependent ATPase by several different methods, coupled with manipulation of the net charge on the restored lipid–protein complex. A part of this work was reported in a preliminary manner previously (Isern de Caldentey & Wheeler, 1977; Wheeler, 1978).

Abbreviation used: ATPase, adenosine triphosphatase.

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Experimental

Chemicals

All chemicals used were obtained from the sources noted previously and all solutions were similarly prepared (Wheeler *et al.*, 1975; Walker & Wheeler, 1975). The phospholipids were grade I products from Lipid Products, Surrey, U.K., that had been extracted from chicken egg yolk (phosphatidylcholine and phosphatidylethanolamine) or bovine brain (phosphatidylserine).

Lipid dispersions

Dispersions of pure phospholipids were prepared exactly as described by Wheeler *et al.* (1975). Disper-

sions of mixtures of either different kinds of phospholipids or phospholipid plus other compounds were prepared by first mixing appropriate volumes of the components in solution in chloroform/methanol (2:1, v/v) and then evaporating the solution to dryness under a stream of N_2. Thereafter the procedure was exactly the same as for the pure phospholipids. Mixed liposomes containing phospholipid with cholate and deoxycholate were prepared by addition of the required amount of the bile salts in 0.1M-Tris/HCl (pH8 at 20°C) to the dried phospholipids and then the mixture was sonicated, and further treated in the usual manner (Wheeler *et al.*, 1975).

ATPase preparations

These were made from rabbit kidney and were partly depleted of lipids by the procedures described by Goodman *et al.* (1978). The lipid-depleted preparations were 're-activated' or 'restored' with exogenous lipids by the methods described in the same paper. The ability of phospholipids to bind to the lipid-depleted ATPase was also measured by application of the same 'restoration' process.

Treatment with phosphatidylserine decarboxylase

A sample (1mg of protein) of lipid-depleted ATPase that had been 'restored' with phosphatidylserine was incubated with 0.3ml of phosphatidylserine decarboxylase (Kanfer & Kennedy, 1964) in 0.1M-Tris/HCl (pH7 at 20°C) containing 0.1% Triton X-100 in a final volume of 1.3ml. The mixture was incubated at 22°C for 5h. A control sample of enzyme was incubated in exactly the same way without the decarboxylase. After the incubation samples (0.05ml) of the mixture were tested for ATPase activity and the rest was used for analysis of the phospholipid composition.

Lipid substitution

This was attempted by application of the method devised by Warren *et al.* (1974*a*,*b*). A sample (10mg of protein) of the ATPase was mixed on ice with a dispersion of cholate and phosphatidylcholine (each 20mg/ml) in 1M-KCl, 0.25M-sucrose, 1mM-EDTA, 1mM-cysteine and 30mM-imidazole/HCl (pH7 at 20°C). The mixture was sonicated for 20s (MSE Ultrasonic Disintegrator operated to give a peak-to-peak amplitude of 12μm) at 0°C and incubated on ice for 1h with constant stirring. A sample (2ml) of the resulting solution was added to the top of a discontinuous sucrose gradient formed with 0.2ml of 50% (w/v) sucrose and 1.0ml of 20% (w/v) sucrose. The sucrose solutions and the overlay contained 1M-KCl, 20mM-NaCl, 1mM-EDTA, 1mM-cysteine and 30mM-imidazole/HCl (pH7 at 20°C) (Hilden & Hokin, 1976). The samples were centrifuged at 160000*g* ($r_{av.}$ 7.3cm) for 22h at 4°C. The ATPase was collected either from the interphase or from the bottom of the tubes and resuspended in a solution containing 0.1M-KCl, 20mM-NaCl, 1mM-EDTA, 1mM-cysteine and 30mM-imidazole/HCl (pH7 at 20°C). Finally, the samples were dialysed against the same solution for 8h at 4°C. When required the substitution treatment was repeated in exactly the same way, but starting with the samples obtained before the dialysis step.

All other methods and measurements were carried out exactly as described previously (Wheeler *et al.*, 1975; Walker & Wheeler, 1975; Goodman *et al.* 1978).

Results

Phospholipid dispersions containing charged molecules

Various charged molecules were dispersed together with either phosphatidylcholine or phosphatidylethanolamine to see if these normally non-reactivating phospholipids could be made to re-activate the lipid-depleted ATPase. To compare the efficacies of the various different dispersions and ATPase preparations used, the results were normalized by comparison with the $(Na^+ + K^+)$-dependent ATPase activities recorded in the presence of 0.1mM-phosphatidylserine, which generally produced maximum re-activation. Mean values (±S.E.M.) for the activities of normal, lipid-depleted and maximally re-activated preparations were 1.02±0.08 (26), 0.09±0.02 (11) and 0.77±0.11 (11) μmol of P_i/min per mg of protein respectively for the numbers of preparations in parentheses.

Dispersions containing phosphatidylserine. Mixed dispersions of either phosphatidylethanolamine plus phosphatidylserine or phosphatidylcholine plus phosphatidylserine always produced some re-activation, the magnitude of which depended on the nature of the phospholipid with the phosphatidylserine (Fig. 1). For example, when the phosphatidylethanolamine was derived from phosphatidylserine, by decarboxylation, the mixed dispersion was a poor activator, whereas the same phosphatidylserine plus phosphatidylethanolamine derived from egg proved to be a good activator. This observation stresses the importance of the acyl chains of the phospholipids in the interaction between the lipids and protein. However, when these and other results were plotted as a function of the phosphatidylserine content of the dispersions, it became clear that the amount of re-activation produced was never greater than that expected from the concentration of phosphatidylserine present: that is, the effects of the mixed dispersions were never greater than the sum of the effects of the individual components, and often it was less (Fig. 2).

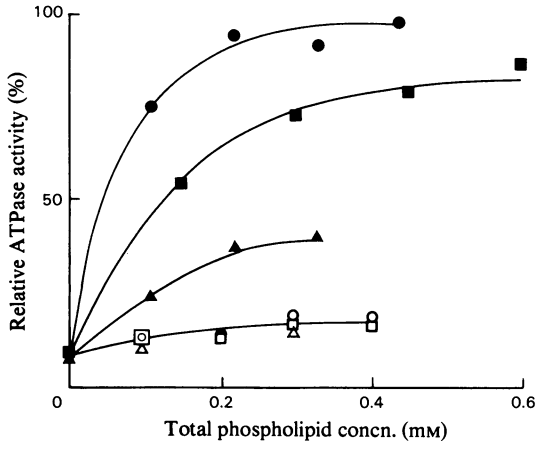

Fig. 1. *Relative re-activation by various phospholipid dispersions containing phosphatidylserine*
The $(Na^+ + K^+)$-dependent ATPase activity of samples of lipid-depleted ATPase was measured during incubation with dispersions of phospholipids containing the indicated proportions of pure phosphatidylserine. Comparison of values obtained from different experiments and different preparations has been facilitated by expressing the activities relative to that measured in the presence of 0.1 mM-phosphatidylserine alone, which generally produced maximum activation. Symbols: □, phosphatidylcholine alone; ■, phosphatidylcholine+phosphatidylserine, molar ratio 2:1; ○, phosphatidylethanolamine from egg alone; ●, phosphatidylethanolamine from egg+phosphatidylserine, molar ratio 10:1; △, phosphatidylethanolamine from phosphatidylserine by decarboxylation alone; ▲, phosphatidylethanolamine from phosphatidylserine by decarboxylation+phosphatidylserine, molar ratio 10:1.

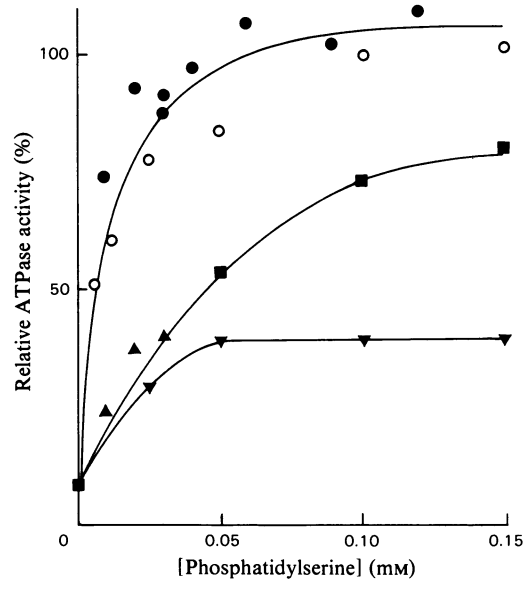

Fig. 2. *Relative re-activation by mixed phospholipid dispersions as a function of their content of phosphatidylserine*
The results of the experiments shown in Fig. 1 plus those from other experiments are plotted as a function of the phosphatidylserine concentration in the various mixtures, and are expressed as in the legend to Fig. 1. Symbols: ○, phosphatidylserine alone; ■, phosphatidylcholine+phosphatidylserine, molar ratio 2:1; ●, phosphatidylethanolamine from egg+phosphatidylserine, molar ratios 10:1 and 10:3; ▼, cholesterol+phosphatidylserine, molar ratio 1:1; ▲, phosphatidylethanolamine from phosphatidylserine by decarboxylation+phosphatidylserine, molar ratio 10:1.

Dispersions containing dicetyl phosphate. Dicetyl phosphate dispersions also re-activated the ATPase but, in contrast with the findings described above, mixed dispersions of either phosphatidylethanolamine+dicetyl phosphate or phosphatidylcholine +dicetyl phosphate produced higher activation than did the dicetyl phosphate alone (Fig. 3). Hence the effects of the mixtures were greater than the sum of the effects of their separate components.

Dispersions containing oleic acid or bile salts. Unlike the charged compounds described so far, neither oleic acid nor cholate plus deoxycholate were able to re-activate the ATPase by themselves. However, mixed dispersions of phosphatidylethanolamine together with either oleic acid or cholate plus deoxycholate did re-activate (Fig. 4). Similar, though slightly less pronounced, activation was observed when phosphatidylcholine was used in place of phosphatidylethanolamine (results not shown). Thus the combination of two types of compound, which separately had no effect, provided a mixture capable of significant re-activation of the lipid-depleted ATPase.

Dispersions containing cetyltrimethylammonium bromide. This positively charged compound did not re-activate the ATPase and mixed dispersions of it with phosphatidylethanolamine were similarly ineffective.

Binding of phospholipids to the lipid-depleted ATPase

The possibility that the negative charge on the re-activating phospholipids was required for binding of the latter to the lipid-depleted protein was tested by direct measurement of the amount of phospholipid associated with the ATPase after incubation with either phosphatidylserine or phosphatidylethanolamine that had been obtained from the phosphatidylserine by enzymic decarboxylation. This comparison thus eliminated any differences in binding caused by differences in the nature of the fatty acyl chains of the phospholipids. The ATPase–

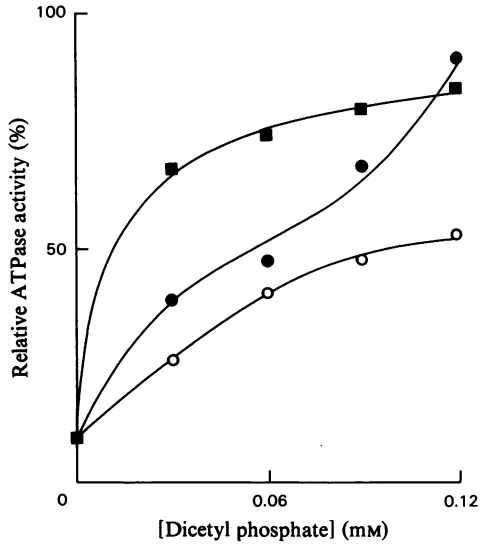

Fig. 3. *Relative re-activation by dispersions containing dicetyl phosphate*

Results are expressed as in the legend to Fig. 1, but as a function of the concentration of dicetyl phosphate present in the dispersions. Symbols: ○, dicetyl phosphate alone; ●, phosphatidylethanolamine from egg+dicetyl phosphate, molar ratio 10:3; ■, phosphatidylcholine+dicetyl phosphate, molar ratio 10:3.

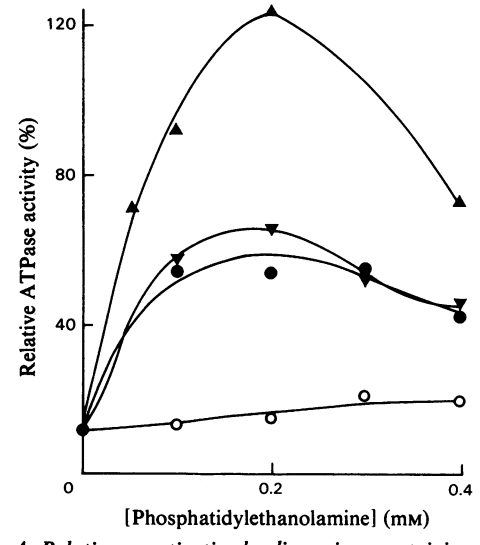

Fig. 4. *Relative re-activation by dispersions containing oleic acid or bile salts*

Mixed dispersions of phosphatidylethanolamine (from egg) with either oleic acid or cholate and deoxycholate were used, as described in the text. The values given are again expressed as in the legend to Fig. 1, but as a function of the phosphatidylethanolamine concentration present. Symbols: ○, phosphatidylethanolamine alone; ●, phosphatidylethanolamine+oleic acid, molar ratio 10:7; ▼, phosphatidylethanolamine+cholate+deoxycholate, molar proportions 5:3:6; ▲, phosphatidylethanolamine+cholate+deoxycholate, molar proportions 5:6:12.

phospholipid complexes were separated from the mixtures by centrifugation, as described in the Experimental section, and both their $(Na^+ + K^+)$-dependent ATPase activities and their phospholipid contents were measured. Fig. 5 shows that there was no detectable difference in the amounts of phosphatidylserine and phosphatidylethanolamine bound to the enzyme, but that only the product containing the added phosphatidylserine was substantially re-activated. Hence, within the limits of the assay method, the failure of phosphatidylethanolamine to re-activate cannot be attributed to its failing to bind to the enzyme protein. In fact, further experiments indicated that the nature of the acyl chains was

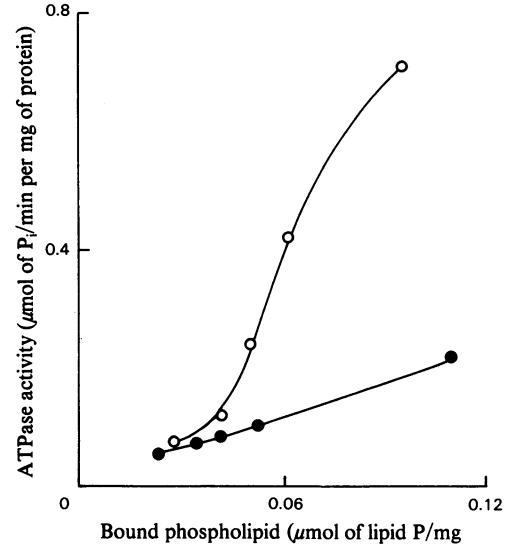

Fig. 5. *Comparison of binding of phosphatidylserine and phosphatidylethanolamine to lipid-depleted ATPase and extent of re-activation*

Lipid-depleted ATPase was incubated with dispersions of either phosphatidylserine or phosphatidylethanolamine obtained from phosphatidylserine by enzymic decarboxylation and both the amounts of the phospholipids bound to the enzyme and the $(Na^+ + K^+)$-dependent ATPase activities of the lipid–ATPase complexes were measured as described in the text. Symbols: ○, restoration with phosphatidylserine; ●, restoration with phosphatidylethanolamine.

probably most important in determining the binding ability, since comparison of binding with the use of the two different kinds of phosphatidylethanolamine available, derived from egg or the phosphatidylserine, revealed that the latter bound preferentially (Fig. 6).

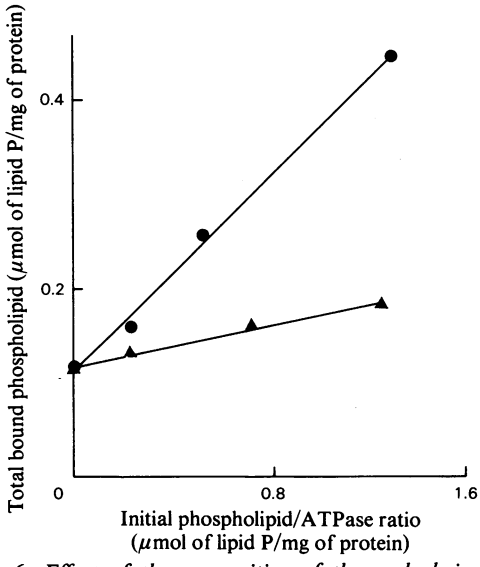

Fig. 6. *Effect of the composition of the acyl chains of phosphatidylethanolamine on its ability to bind to lipid-depleted ATPase*

Lipid-depleted ATPase was incubated with dispersions of phosphatidylethanolamine extracted from egg (▲) or obtained from phosphatidylserine by enzymic decarboxylation (●) at the indicated concentrations, and the amounts of phospholipid firmly bound to the ATPase were determined as described in the text.

Effect of removal of the negative charges after restoration and re-activation

Another approach to the question of whether the negative charge is required for something more than just the initial interaction between lipid and protein was to try to remove the negative charge, but not phospholipid, from the complex after restoration and re-activation. The information available from the results outlined above suggested two ways in which that could be done.

Removal of bile salts by dialysis. The finding that mixed dispersions of phosphatidylethanolamine plus cholate plus deoxycholate re-activated the ATPase (Fig. 4) provided one very convenient way of removing the negative charge after re-activation, because the cholate and deoxycholate could be separated from the protein and phospholipids by dialysis (Wheeler *et al.*, 1975). An initial experiment showed that if the lipid-depleted ATPase was re-activated by addition of phosphatidylethanolamine plus cholate plus deoxycholate, as in Fig. 4, and the mixture was then dialysed against 10mM-Tris/HCl (pH7.5 at 20°C) overnight at 4°C, the ($Na^+ + K^+$)-dependent ATPase activity fell to the initial value, before re-activation.

To confirm this finding a more elaborate experiment was carried out with a different lipid-depleted enzyme preparation. Essentially, the effect of the dialysis process was tested also on two other samples, one simply suspended in the buffer solution and the other mixed with phosphatidylserine and buffer solution. Full details are given in the legend to Table 1. Initially the addition of phosphatidylserine produced a 5-fold increase and the addition of phosphatidylethanolamine+cholate+deoxycholate a 2.3-fold increase in activity (Table 1). After dialysis the activity of the test sample had fallen to that of the control sample, which remained unchanged, whereas the activity of the sample containing phos-

Table 1. *Effect of dialysis on re-activation by phospholipid dispersion containing bile salts*

Samples (0.1ml, containing 0.09mg of protein) of lipid-depleted ATPase were separately mixed with: (*a*) 2ml of 10mM-Tris/HCl (pH7.5 at 20°C); (*b*) 2ml of a dispersion of 2mM-phosphatidylserine in 10mM-Tris/HCl (pH7.5 at 20°C); or (*c*) 2ml of a dispersion containing 2mM-phosphatidylethanolamine, 1.2mM-cholate and 2.4mM-deoxycholate in 10mM-Tris/HCl (pH7.5 at 20°C). Four 0.1ml samples were taken from each mixture for assay of ATPase activities and the remainder of each mixture was separately dialysed at 4°C against 10mM-Tris/HCl (pH7.5 at 20°C) as follows: (i) 250ml for 1h; (ii) 100ml for 1h; (iii) 250ml overnight. The non-diffusible material in each sample was then assayed for both protein content and ATPase activity.

Mixture	Ouabain-sensitive ATPase activity (μmol of P_i/min per mg of protein)		
	Initial	After dialysis	After dialysis plus 0.1mM-phosphatidylserine
(*a*) Enzyme+buffer	0.14	0.14	0.32
(*b*) Enzyme+phosphatidylserine	0.68	0.47	—
(*c*) Enzyme+phosphatidylethanolamine+cholate+deoxycholate	0.32	0.14	0.25

phatidylserine fell only by about 30%. When phosphatidylserine was added to the dialysed samples the activity of the control increased 2.3-fold and that of the sample containing phosphatidylethanolamine 1.8-fold. Hence the enzyme had clearly deteriorated during the dialysis, but both the control and the test samples could still be re-activated significantly by the added phosphatidylserine. Removal of the cholate plus deoxycholate by dialysis therefore resulted in removal of the activation brought about by the phosphatidylethanolamine plus cholate plus deoxycholate. The presence of phosphatidylserine throughout the dialysis process in the third sample also obviously afforded some protection of the activity during the dialysis.

Conversion of phosphatidylserine into phosphatidylethanolamine after restoration. The availability of the phosphatidylserine decarboxylase provided another means of converting a negatively charged activating phospholipid, into a neutral non-activating phospholipid, after the lipid-depleted enzyme had been restored with phosphatidylserine. Samples of the lipid-depleted enzyme were incubated with phosphatidylserine dispersions, the resulting phosphatidylserine–protein complexes separated and washed by centrifugation, and then treated with phosphatidylserine decarboxylase, as described in the Experimental section, to try to convert the bound phosphatidylserine into phosphatidylethanolamine. The results of four such experiments with two different lipid-depleted and 'restored' preparations are shown in Table 2. After 'restoration' the phosphatidylserine content of the preparations accounted for about 73% of the total phospholipid, whereas after exposure to the decarboxylase that was decreased to about only 14%. But the $(Na^+ + K^+)$-dependent ATPase activities remained unchanged at 0.72 ± 0.04 (mean ± S.E.M. for four measurements) μmol of P_i/min per mg of protein. Since the amount of phosphatidylethanolamine present increased in proportion to the decrease in phosphatidylserine, it was clear that the enzyme remained active in the presence of relatively large amounts of phosphatidylethanolamine with small amounts of phosphatidylserine, a mixture that could

Table 2. *Effect of phosphatidylserine decarboxylase on the phospholipid composition of the restored ATPase*

Lipid-depleted ATPase was restored by incubation with pure phosphatidylserine and separation of the phospholipid–enzyme complex from excess of phosphatidylserine by centrifugation. Samples of the complex were then incubated with phosphatidylserine decarboxylase to try to convert the bound phosphatidylserine into phosphatidylethanolamine. Both the $(Na^+ + K^+)$-dependent ATPase activity and the phospholipid content of the preparations were monitored throughout. Full details are given in the Experimental section. Four experiments with two different ATPase preparations were carried out and the ranges of values obtained are given. The $(Na^+ + K^+)$-dependent ATPase activity of the preparations was unaffected by the treatment, the mean value being 0.72 ± 0.04 (4) (S.E.M.) μmol of P_i/min per mg of protein.

	Phospholipid content of ATPase (μmol of lipid P/mg of protein)		
	Phosphatidylserine	Phosphatidylethanolamine	Total
Initial	0.35–0.53	0.09–0.07	0.52–0.68
After exposure to decarboxylase	0.07–0.10	0.50–0.38	0.53–0.68

Table 3. *Results of attempts to replace endogenous phospholipids by phosphatidylcholine*

The replacement procedure was carried out as described in the text by incubation of ATPase preparations with dispersions containing phosphatidylcholine and cholate, followed by separation of the enzyme from the mixture by density-gradient centrifugation and dialysis. Control samples were put through the same procedure, but without the initial incubation with phosphatidylcholine and cholate. Abbreviation: N.D., not determined.

	Once-treated enzyme		Twice-treated enzyme	
	Control	Experimental	Control	Experimental
Ouabain-sensitive ATPase activity (μmol of P_i/min per mg of protein)	0.29	0.26	0.27	0.31
Phospholipid composition (% of total recovered)				
Sphingomyelin	22	11	21	7
Phosphatidylcholine	37	73	34	80
Phosphatidylserine	8	6	9	6
Phosphatidylethanolamine	33	10	36	7
Total phospholipid content (μmol of lipid P/mg of protein)	0.73	0.68	0.67	0.45
Total cholesterol content (μmol/mg of protein)	0.14	0.05	N.D.	0.01
Protein recovery (% of initial)	45		33	

not re-activate the lipid-depleted enzyme very well initially (cf. Fig. 1). On the other hand, complete conversion of the bound phosphatidylserine into phosphatidylethanolamine could not be achieved, so that significant amounts of negatively charged phospholipid always remained bound to the enzyme.

Substitution of endogenous phospholipids with exogenous phosphatidylcholine

A different approach to the problem was to try to replace all the endogenous phospholipids of the ATPase preparations with an exogenous neutral phospholipid by application of the method of Warren *et al.* (1974*a*,*b*), which avoids the isolation of an inactive lipid-depleted enzyme. This procedure, which was originally developed for the sarcoplasmic Ca^{2+}-dependent ATPase, consists essentially of incubation of the enzyme in the presence of high concentrations of exogenous phospholipid dispersed in cholate, followed by separation of the phospholipid–protein complex by centrifugation through a sucrose gradient and subsequent dialysis. The process is repeated to obtain virtually complete replacement of the endogenous lipids by the exogenous phospholipid.

The results obtained with our ATPase preparation are summarized in Table 3, which shows that, although the added exogenous phosphatidylcholine increased the phosphatidylcholine content to about 80% of the total phospholipids, the small (6%) proportion of phosphatidylserine initially present remained unchanged, even after the enzyme had been subjected to the substitution process twice. Also, although the $(Na^+ + K^+)$-dependent ATPase activities of the test and control samples were about the same, the absolute activity had decreased from 0.82 to about 0.3 μmol of P_i/min per mg of protein. It appeared therefore that the loss of ATPase activity must have resulted from some aspect of the method unrelated to the partial replacement of the endogenous lipids by the added phosphatidylcholine. Repeated use of this method with different ATPase preparations gave similar results.

Discussion

Re-activation of lipid-depleted ATPase

While this work was in progress a report of partly similar experiments was published by Palatini *et al.* (1977). In so far as they overlap, our results are generally in agreement. For example, we both observed re-activation of the lipid-depleted ATPase by liposomes containing negatively charged molecules, such as dicetyl phosphate and oleic acid, together with phospholipids such as phosphatidylcholine, which alone did not re-activate. Similarly, we found that the positively charged cetyltrimethylammonium was completely ineffective, just as Palatini *et al.* (1977) showed that liposomes containing ethylhexadecyldimethylammonium with phosphatidylcholine did not re-activate. On the other hand, our finding that dicetyl phosphate alone produced significant re-activation (Fig. 3) does not agree with their observations, but it could be more in keeping with their explanation that 'a lamellar organization with appropriate density of negative charges is sufficient to allow a correct interaction between the lipids and protein and to activate the ATPase', since dicetyl phosphate dispersions might fulfil those conditions (Tanford, 1973).

We were prompted to test the effect of the incorporation of cholate plus deoxycholate into phosphatidylcholine and phosphatidylethanolamine liposomes when we realized that this mixture of bile salts was used as a routine during the re-activation experiments of Ottolenghi (1975) and Jensen & Ottolenghi (1976), in which no phospholipid specificity for re-activation was detected. Our observation that the addition of the bile salts to the phospholipids rendered the resulting dispersions effective activators of the lipid-depleted ATPase not only adds more weight to the general thesis, quoted above, proposed by Palatini *et al.* (1977), but also provides an explanation for the apparent absence of the need for negatively charged phospholipids to re-activate the ATPase preparations of Ottolenghi (1975). These findings also raise the possibility that the cholate used in experiments in which endogenous phospholipids were directly replaced by exogenous phospholipids (Hilden & Hokin, 1976), and in which the ATPase was incorporated into phospholipid liposomes such that active movements of Na^+ into and K^+ out of the liposomes were demonstratable (Sweadner & Goldin, 1975; Hilden & Hokin, 1975, 1976), might similarly have provided a negative charge important for such reconstitution processes.

Since this work was completed Mandersloot *et al.* (1978) have reported that mixed liposomes containing phosphatidylcholine with cholate were able to re-activate an $(Na^+ + K^+)$-dependent ATPase that had been partly depleted of lipids by exposure to phospholipase A_2. Although they are not identical, our findings with phosphatidylcholine plus cholate plus deoxycholate serve to confirm that report. They also suggest that the state of the ATPase after lipid depletion by extraction with detergent cannot be completely different from the state after treatment with the phospholipase.

Basis of the requirement of a net negative charge

Although the direct measurement of the amounts of added phospholipid that became firmly bound to the lipid-depleted ATPase seemed to provide clear results, both in this work and in that of Palatini *et al.* (1977), the two sets of results apparently suggest opposite interpretations. Thus Palatini *et al.* (1977)

showed that maximum binding occurred with liposomes containing negatively charged molecules and concluded that the latter were important in governing the extent of lipid–protein interaction. In contrast, we showed that phosphatidylserine and phosphatidylethanolamine containing the same mixture of acyl chains bound equally well (Fig. 5), whereas the two kinds of phosphatidylethanolamine with different acyl chains did not bind to the same extent (Fig. 6), and concluded that the acyl chains were the limiting factor. There are at least two possible explanations of this apparent anomaly. One is that the relative impurity of the ATPase preparations used renders both sets of results inconclusive, because they did not distinguish between lipid binding to the ATPase protein and to the excess of extraneous, non-ATPase, protein. If only a small fraction of the bound lipid was directly associated with the ATPase protein, then clear interpretation of the results is difficult. (The 4–10-fold difference in the specific activities of the preparations used in the two sets of experiments also makes direct comparison unreliable.) The second possibility is that the negative charge is important for permitting the 'correct' interaction between the ATPase protein and the lipid. Thus certain fatty acyl chains in the phospholipids might bind to the proteins more readily than other acyl chains are able to, but such binding in the absence of the negative charges would be 'incorrect' and so would produce inactive complexes. This would explain our common finding that only in the presence of a net negative charge were the lipid–protein complexes enzymically active.

The results of the experiments with phosphatidylserine decarboxylase (Table 2) can also be interpreted in two ways, but, again, each suggests that the negative charge is involved directly in the interaction between lipid and ATPase protein. Since 80% of the bound phosphatidylserine could be converted into phosphatidylethanolamine without affecting the ATPase activity, either the phosphatidylserine was not required for the activity, so that it must have been necessary for the correct lipid–protein interaction to start with, or it was required for the ATPase reaction and the decarboxylase must have selectively hydrolysed those phosphatidylserine molecules that were bound to other proteins. That would suggest that the remaining phosphatidylserine molecules were bound to the ATPase, at least in part, via their polar groups, thus rendering them less susceptible to attack by the decarboxylase.

The fact that the re-activation caused by phosphatidylethanolamine plus cholate plus deoxycholate was abolished by removal of the bile salts by dialysis (Table 1) is most simply interpreted as showing that the negative charges were required for the ATPase reaction itself. But it is also possible to argue that, unlike the conversion of phosphatidylserine into phosphatidylethanolamine *in situ* by the decarboxylase, removal of the bile salts resulted in a change in the nature of the interaction between the remaining phosphatidylethanolamine and the ATPase protein, such that the binding was no longer 'correct'. This argument is difficult to dismiss and could not be avoided even if the experiments were repeated with a pure ATPase preparation.

Our failure with the use of the method of Warren *et al.* (1974*a*,*b*) to obtain complete replacement of endogenous lipids by the added phosphatidylcholine, coupled with the marked loss of ATPase activity in the control preparations (Table 3), renders those experiments of little value. Hilden & Hokin (1976) reported that, with the same method applied to a purified ATPase from dogfish rectal gland, they were able to obtain 100% replacement of endogenous phospholipids by added phosphatidylcholine. (Their final preparation obviously retained some ATPase activity, but its value was not recorded.)

We must conclude, therefore, that the net negative charge required for re-activation of the lipid-depleted ATPase is necessary for the correct interaction between phospholipid and protein, but that neither our results nor those of Palatini *et al.* (1977) and Mandersloot *et al.* (1978) are sufficient to decide unequivocally whether or not the charge is also required for the ATPase reaction itself.

Relevance to the situation in vivo

Although we have been concerned here primarily with the question of the requirements for re-activation of the ATPase after it has been removed from its normal lipid environment, the situation *in vivo* is obviously most important, and two very recent publications bear on this question and on our results. Both Mandersloot *et al.* (1978) and De Pont *et al.* (1978) have studied the phospholipid requirement of rabbit kidney ($Na^+ + K^+$)-dependent ATPase by selective hydrolysis of endogenous phospholipids with the aid of various phospholipases. The former group used a simple microsomal fraction, whereas the latter group used a highly purified ($Na^+ + K^+$)-dependent ATPase. Mandersloot *et al.* (1978) concluded that the enzyme has a specific requirement for phosphatidylinositol as the essential endogenous phospholipid, whereas De Pont *et al.* (1978) concluded that there is no absolute requirement for either phosphatidylinositol or phosphatidylserine.

If we assume that the trace of anionic dodecyl sulphate remaining in the preparations used by De Pont *et al.* (1978) was insufficient to provide any necessary negative charge for the measured ATPase activity after removal of phosphatidylserine and phosphatidylinositol, and that sulphatides (Karlsson *et al.*, 1968, 1971) were absent, then the weight of evidence must favour the conclusion that a negative charge is not required for the ATPase reaction.

However, De Pont *et al.* (1978) did also show that the ATPase activity was markedly decreased when both phosphatidylserine and phosphatidylinositol were removed, so that the possibility remains that these negatively charged phospholipids are normally intimately associated with the ATPase in the membrane. Hence it seems reasonable to speculate that these, or other negatively charged lipids, are involved in the interaction between the ATPase protein and the lipid bilayer *in vivo*. They could serve in some kind of recognition role, to ensure insertion of the protein both in the required part of the membrane and in the necessary alignment. This interpretation would thus also explain the requirement for the negative charge in the reactivation process described above.

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