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Lipid Requirement of the Membrane Sodium-plus-Potassium Ion-Dependent Adenosine Triphosphatase System

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The dependence of the $(Na^+ + K^+)$-dependent ATPase (adenosine triphosphatase) (EC 3.6.1.3) on lipid has been examined in a number of different ways, with the use of various preparations from kidney tissue. The main findings were as follows. (1) The ATPase activities of the preparations examined were closely correlated with their total phospholipid content. (2) Extraction of the ATPase with deoxycholate or Lubrol W, combined with suitable salt-fractionation and washing procedures, removed phospholipid, cholesterol and enzymic activity in parallel; but activity was completely lost before all lipid had been removed. (3) The loss of activity could not be attributed to inhibition by residual detergent. (4) No selective removal of any particular phospholipid class by detergent could be detected. (5) Consistent reactivation of the Lubrol-extracted enzymes was obtained by adding dispersions of exogenous phospholipid, but only some, bearing a net negative charge, such as phosphatidylserine and phosphatidylglycerol, were effective. (6) The degree of reactivation was correlated with the amount of residual activity remaining after lipid depletion. (7) Partial purification of the ATPase, giving a 50-fold increase in specific activity, was not accompanied by selective enhancement of any particular class of phospholipid. We conclude that although the ATPase is dependent on phospholipid, only the reactivation results provide evidence for specificity.

The membrane ATPase* (EC 3.6.1.3) that requires Na^+ and K^+ as cofactors and is inhibited by ouabain is generally thought to consist of a lipid–protein complex. The strongest evidence for this concept is the loss of enzymic activity after exposure to detergents, degradation with phospholipases, or extraction with organic solvents, and the subsequent reactivation of the enzyme by added exogenous lipid. However, the exact nature of the lipid, or lipids, required is uncertain because the literature contains several apparently conflicting reports. [See Roelofsen & van Deenen (1973) for a summary and references.] No doubt some of these discrepancies arise from the use of exogenous lipids of various degrees of purity (Fenster & Copenhaver, 1967; Wheeler & Whittam, 1970) and possibly from variable chelating effects (Wheeler, 1971); but others could result from unestablished differences in the composition and physical state of the enzyme after the different treatments used to remove lipids. It seemed necessary therefore to combine such inactivation and reactivation studies with a quantitative analysis of the main lipid constituents of the ATPase preparations before and after the treatment designed to remove lipids. A clear discrimination between the two possibilities of the enzyme's requiring either a specific type of lipid, or merely a certain amount of lipid in general, would provide a clue as to how the lipid functions and why it is necessary.

We have used detergents rather than enzymes to remove lipid from the ATPase, partly to avoid problems associated with phospholipase impurities, particularly proteinase activity. Treatment with detergents, however, introduces another possible artifact, that residual detergent inhibits the enzyme and addition of exogenous lipid merely relieves this inhibition, rather than reactivating by replacing essential lipid. Hence we have considered the following questions. (1) To what extent is loss of ATPase activity correlated with removal of lipids by the detergent? (2) Is complete removal of lipid associated with complete loss of activity? (3) Is enzyme inactivation caused by the presence of residual detergent? (4) What types of residual lipids are present in partly depleted enzyme preparations? (5) Is there any selective removal of particular lipid classes? (6) Can reactivation to the original specific activity be achieved, or is only partial restoration possible? (7) Is reactivation possible after complete loss of ATPase activity, or must there be some residual activity?

To answer these questions it was necessary to have a reasonably reproducible method of removing lipid

* Abbreviation: ATPase, adenosine triphosphatase.

from the ATPase without completely denaturing it. Treatment with deoxycholate, based on the procedure originally described by Tanaka & Strickland (1965), was not satisfactory because of extreme variability in results, particularly with respect to reactivation of the depleted enzyme (see also Kimelberg & Papahadjopoulos, 1972). After preliminary experiments with the deoxycholate method, therefore, we adopted a modified version of the procedure described by Palatini *et al.* (1972), which uses the non-ionic detergent Lubrol. This slightly modified method produced much more consistent results, and the specific activities of the ATPase preparations were considerably higher than those recorded in earlier work with the use of the deoxycholate method (Wheeler & Whittam, 1970; Wheeler, 1971; Kimelberg & Papahadjopoulos, 1972).

Finally it seemed reasonable to suppose that if any particular class of lipid is specifically required for the $(Na^+ + K^+)$-dependent ATPase, then that lipid might be selectively retained, relative to other lipids, during purification of the enzyme. To check this possibility quantitative analysis of the lipid constituents of the enzyme in different states of purification was also carried out.

Experimental

Materials

Pure phospholipids were obtained from Lipid Products Ltd., Redhill, Surrey, U.K. All other chemicals were normal commercial products of analytical-reagent grade where possible. Glass-distilled water was used throughout.

ATPase preparations. Rabbit kidney was used as a routine as the enzyme source. Occasionally fresh tissue was used but more often the kidneys were immersed in 0.25M-sucrose immediately after removal from the animal and then stored at −20°C until required. Pig kidney was used in one series of experiments (see below). The capsule and adhering fat was removed from the kidney before it was homogenized in a Potter–Elvehjem-type homogenizer.

Methods

Microsomal fraction (method I). After homogenization in 0.25M-sucrose containing 5mM-EDTA, pH7.5, a microsomal fraction was isolated from the rabbit kidney by the procedure described for bovine brain by Tanaka & Strickland (1965): it was resuspended in water and stored frozen.

Microsomal fraction (method II). The kidney was homogenized in a solution containing 0.25M-sucrose, 1mg of deoxycholate/ml, 5mM-EDTA and 0.03M-histidine, pH6.8 (Skou, 1962) and then centrifuged for 15min at about 6000*g* and 4°C. The supernatant was decanted and centrifuged again at 4°C, either for 30min at 48000*g* or for 40min at 33000*g*. The sediment was resuspended in water by gentle homogenization and stored frozen.

Microsomal fraction (method III). This was prepared from the outer medulla of pig kidney by the method of Grisham & Barnett (1972).

Sodium iodide treatment. Microsomal fractions prepared by method II were incubated with 2M-NaI and washed with EDTA solutions by the procedure of Nakao *et al.* (1965). The final sediment was resuspended in water and either used immediately for extraction with Lubrol, or stored frozen. This treatment with NaI not only removed most of the ouabain-insensitive ATPase activity but also increased the specific activity of the ouabain-sensitive component (see Fig. 1).

Lipid-depletion with deoxycholate. Microsomal fractions prepared by method I were extracted with deoxycholate and fractionated with $(NH_4)_2SO_4$ by the methods outlined by Wheeler & Whittam (1970).

Lipid-depletion with Lubrol. (a) Single extraction. [This method was adopted because the original method of Palatini *et al.* (1972), designed for bovine heart preparations, failed to give a product that could be reactivated in preliminary experiments with the kidney enzymes.] All procedures were carried out at 0–4°C. A sample of the suspension obtained by the NaI procedure was sonicated for 2min in an MSE Ultrasonic Disintegrator (settings: power, medium; amplitude, 1) with the small probe. The specific activity of the enzyme decreased by about 12% during sonication. The sonicated enzyme suspension was then mixed for 60min in a solution containing 0.2mM-EDTA (pH7.5), 2.5mM-ATP (pH7.5), 1mM-dithiothreitol, 35% (v/v) glycerol and 20–100mg of Lubrol W/ml: the final protein concentration was 2mg/ml. Usually the Lubrol concentration was 30–40mg/ml, the higher concentrations being used only for certain experiments (see the Results section). After centrifugation at about 120000*g* for 60min the clear supernatant was decanted, solid $(NH_4)_2SO_4$ added to it (0.21g/ml) and the mixture stirred until all the salt had dissolved. Centrifugation at about 40000*g* for 30min produced a floating disc, which was removed and resuspended by homogenization in 0.2M-NaCl. The mixture was centrifuged at 120000*g* for 30min to give a sediment that was washed by resuspension and centrifugation first in 0.6M-NaCl and then in 1M-NaCl. When Lubrol concentrations of 50–100mg/ml had been used, the final pellet was washed once more in 1M-NaCl. Finally, the pellet from the last centrifugation was resuspended by homogenization in the glycerol-containing 'buffer A20' described by Towle & Copenhaver (1970) and stored at −20°C. [This procedure has more recently been slightly modified by carrying out the initial sonication step after suspending the protein in the Lubrol solution.

Better dispersal of the enzyme results and the final yield of protein is improved.]

(b) Multiple extractions. (1) The enzyme was sonicated in the Lubrol solution, exactly as described above, and the fractionation procedure followed to the floating-disc stage. The disc was washed once in 1M-NaCl and the resulting sediment resuspended in water. For a double-extraction procedure (a), above, was then repeated. For a triple extraction, procedure (1) was repeated and then procedure (a).

Protein concentrations. The method of Miller (1959) was used, with crystalline bovine serum albumin as standard; correction for interference by the 'A20 buffer' solution was made when necessary.

Dialysis. Samples (1ml; 2–3mg of protein) of the enzymes that had been extracted with deoxycholate were dialysed overnight at 4°C against 200ml of 0.1M-NaCl containing 0.01M-imidazole–HCl, pH7.0.

Gel filtration. A column (approx. 26cm×1.5cm) of Sephadex G-75 was equilibrated at 4°C with a solution containing 0.1M-NaCl and 0.01M-imidazole–HCl (pH7.4). Samples (0.5ml) of the deoxycholate-'solubilized' ATPase were applied to the top of the column and eluted with the same buffered solution of NaCl at 4°C. The eluate was collected in fractions of about 1ml and those constituting the void volume were pooled and analysed for protein concentration, deoxycholate concentration and ATPase activity. Control samples of the enzyme were similarly diluted with the same NaCl–imidazole solution.

Measurement of deoxycholate concentrations. The following method is based on that described by Mosbach *et al.* (1954). A sample (0.3–1.0ml) of the lipid-depleted enzyme was added to 5ml of ethanol and the mixture heated to boiling before filtering it through a glass-fibre filter under reduced pressure. The filter was washed twice with 5ml portions of hot ethanol and the combined filtrate evaporated to dryness at 60°C under a current of air. The residue was dissolved in 6ml of 2:1 (v/v) H_2SO_4 and incubated at 60°C for 15min. After the solution had been cooled to laboratory temperature the E_{383} was measured in a 2cm-light-path cuvette. The measurements were repeated three times for each sample of enzyme, with different sample volumes each time, and the deoxycholate content was calculated by the use of a standard curve, which was constructed with the deoxycholate solution used to extract the ATPase.

Lubrol concentrations. A sample (0.1–0.4ml) of the lipid-depleted enzyme was extracted once with 20vol. and twice with 4vol. of chloroform–methanol (1:1, v/v) and the combined extract evaporated to dryness under a stream of N_2. The residue was dissolved in a small volume of chloroform–methanol and a known quantity used for t.l.c. on silica gel, with a pre-washing mixture of acetone–light petroleum (1:3, v/v) and a developing solvent composed of chloroform–methanol–acetic acid–water (50:30:6:3, by vol.). Iodine vapour was used to detect the separated lipid spots: Lubrol ranwith the solvent front. The Lubrol spots were assayed by the dichromate oxidation method, as described by Skipski & Barclay (1969). (Note that the Lubrol was probably contaminated with lipids that also ran at the solvent front, so that this non-selective oxidation may have overestimated the Lubrol concentration.)

Phospholipid dispersions. The lipid solution (chloroform–methanol) was evaporated to dryness under a stream of N_2 and then water or Tris buffer (pH8), previously saturated with N_2 at 0°C, added. The mixture was sonicated for 20min in an MSE Ultrasonic Disintegrator (power, medium; amplitude, 1 or 2), with either the medium or small size probe, depending on the volume of the mixture. The vessel was maintained at 20°C throughout by means of a water-jacket and additional buffer was added if necessary to keep the pH around 7.5. The resulting dispersion was briefly centrifuged at about 2000*g*, to remove most of the titanium dust, and then centrifuged at 120000*g* for 30min, to remove any undispersed lipid and final traces of titanium. The supernatant was stored at 4°C. The concentrations of these dispersions were determined by ashing samples and measuring their total phosphorus content by the method of Chen *et al.* (1956).

Lipid analyses. Phospholipids were extracted from the various enzyme preparations, separated by t.l.c. on silica gel, identified by specific sprays and comparison with pure standards, and assayed by measuring total phosphorus content, by the procedures described by Radin (1969), Skipski & Barclay (1969) and Rouser *et al.* (1970). When only the total phospholipid content was required, a sample of the enzyme preparation was precipitated with 7% (w/v) trichloroacetic acid, sedimented by centrifugation, and the clear supernatant removed. The sediment was than ashed and assayed for phosphorus by the method of Chen *et al.* (1956). Cholesterol was assayed by a colorimetric method based on the Lieberman–Burchard reaction. First, the material running at the solvent front during thin-layer separation of phospholipids was eluted from the silica gel and the solvent removed by evaporation under N_2. The residue was mixed with 5ml of 7M-acetic anhydride in 6.4M-acetic acid and, after cooling, 1ml of conc. H_2SO_4 added. The solution was mixed and left for 30min, when its E_{570} was measured. A solution of cholesterol in acetic acid was used as a standard.

ATPase activity. The initial rate of P_i release from ATP was measured as follows. Samples of the enzyme preparations were added to tubes containing

3mM-Na_2ATP, 3mM-$MgSO_4$, 120mM-NaCl, 20mM-KCl, 0.5mg of bovine serum albumin/ml, 0.5mM-EGTA, 30mM-Tris–HCl (pH7.5) and any other compounds required, as indicated in the legends to Tables and Figures, giving a total volume of 2ml. The mixtures were incubated at 37°C, usually for 20min, and then the reaction was stopped by adding 1ml of 25% (w/v) trichloroacetic acid. The precipitated protein was sedimented by centrifuging at about 2000*g* for 15min at 4°C and the concentrations of P_i in samples of the clear supernatants were measured by the method of Fiske & SubbaRow (1925). Ouabain-sensitive or '(Na^++K^+)-dependent' ATPase activity was determined as the difference in the activity measured in the presence and absence of 0.1mM-ouabain.

Results

Correlation between (Na^++K^+)-dependent ATPase activity and phospholipid content

The total phospholipid contents of many different ATPase preparations were determined and compared with their ATPase activities. The preparations were all made from kidney homogenates and included microsomal fractions obtained by two different procedures, deoxycholate-treated microsomal fractions, NaI-treated microsomal fractions, and the latter extracted with Lubrol, as described in the Experimental section. In spite of some scatter, the combined measurements show that there was a marked correlation between the total phospholipid content of those preparations and their ouabain-sensitive ATPase activity (Fig. 1) but not their ouabain-insensitive activity. Thus the treatment of microsomal fractions with NaI generally was associated with an increase in both phospholipid content and ouabain-sensitive activity, but with a very pronounced decrease in ouabain-insensitive activity. Similarly, exposure of these kinds of preparations to relatively high concentrations of deoxycholate or Lubrol decreased both phospholipid content and ouabain-sensitive ATPase activity, with a smaller effect on the ouabain-insensitive component. It is apparent from Fig. 1, however, that the (Na^++K^+)-dependent ATPase activity of detergent-treated preparations tended to zero, and frequently was completely abolished, before all the phospholipid had been removed from them. In fact, no preparation examined, after any treatment, had been completely depleted of phospholipid, whereas several had lost all ouabain-sensitive ATPase activity.

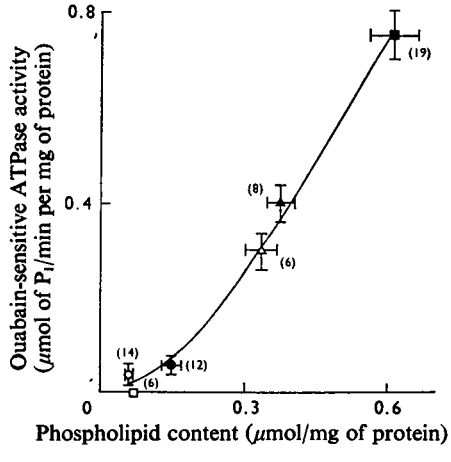

Fig. 1. *Correlation between ATPase activity and phospholipid content*

Microsomal fractions were isolated from homogenates of rabbit kidney by methods I (△) and II (▲) described in the Experimental section. The former (I) preparations were partly depleted of lipid by extraction with deoxycholate (○) whereas the latter (II) were treated with NaI (■) before being extracted with Lubrol, either once (●) or several (□) times. Both ouabain-sensitive ATPase activities and total phospholipid contents of all preparations were measured as detailed in the Experimental section. Mean values±S.E.M. are given for the numbers of preparations shown in parentheses.

Hence the answers to questions (1) and (2), above, are that, in the specific activity range covered, there is a general correlation between (Na^++K^+)-dependent ATPase activity and the total phospholipid content of enzyme preparations; that extraction with deoxycholate or Lubrol does remove the phospholipid and the activity in parallel; but that enzymic activity is completely lost before all the lipid has been removed.

Relation between phospholipid and cholesterol contents

Since we have never observed any sign of reactivation of lipid-depleted ATPase by added cholesterol, alone or together with phospholipid, we have not examined so many of the enzyme preparations for cholesterol content. Fig. 2 shows the relationship between cholesterol and the total phospholipid contents of a few representative preparations. In general the cholesterol content increased and decreased after the treatments with NaI and Lubrol respectively, in much the same way as the phospholipid, though the correlation was not exact.

Residual detergents

Inhibition of ATPase activity by protein-bound detergent is an obvious possibility in these kinds of experiments, and one that is not easy to discount. The literature contains two, apparently contradictory, reports about deoxycholate, and none about Lubrol. Tanaka & Strickland (1965) showed that gel filtration of their 'solubilized' ATPase from bovine

brain removed residual deoxycholate but did not affect the reactivation caused by added phospholipid. In contrast, Towle & Copenhaver (1970) found that the ratio of ATPase activity with phospholipid to activity in its absence decreased when the residual deoxycholate in their kidney preparation was lowered by dialysis. To check the possibility that added phospholipid activated the ATPase merely by removing protein-bound deoxycholate, those experiments have been repeated with the use of both dialysis and gel filtration to try to remove the residual detergent from several enzyme preparations made from kidney and extracted with deoxycholate. The results in Table 1 show that dialysis or gel filtration removed more than 90% of the residual deoxycholate remaining in the depleted ATPase, but that neither the ATPase activity nor its reactivation by added phospholipid was affected.

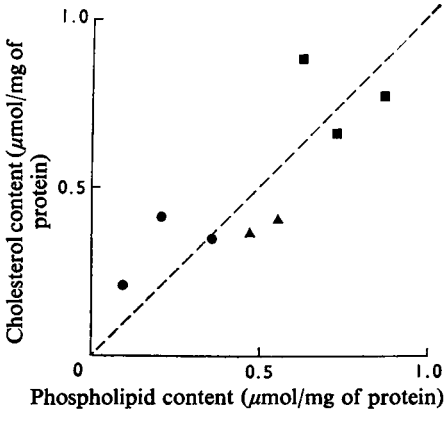

Fig. 2. *Relation between cholesterol and phospholipid contents of ATPase*

Both the cholesterol and the total phospholipid contents of several of the preparations described in Fig. 1 were measured. Symbols have the same meaning as in Fig. 1 and the broken line indicates an exact one-to-one relationship.

The amount of residual Lubrol was estimated in two of the ATPase preparations that had been extracted with Lubrol at a concentration of 30mg/mg of protein, as described in the Experimental section. The actual values measured for the two preparations were 0.84 and 0.76mg of 'Lubrol'/mg of protein, but those must represent upper limits because of the analytical method used (see the Experimental section). That such contamination was unlikely to have inhibited the enzyme significantly was shown by the fact that incubation of the original preparation with 10mg of Lubrol/mg of protein caused the ouabain-sensitive activity to fall by only 10%, from 0.42 to 0.38 μmol of P_i/min per mg of protein.

The answer to question (3) is, therefore, that residual protein-bound detergent almost certainly could not account for the lack of ATPase activity in the lipid-depleted enzymes whether deoxycholate or Lubrol was used to prepare them.

Nature of residual phospholipids after extraction with detergents

The phospholipids present in several ATPase preparations before and after depletion with both the deoxycholate and the Lubrol method were extracted and analysed by quantitative t.l.c. The relative proportions of the various lipid classes in the preparations are shown in Tables 2 and 3. In the earlier experiments, with the deoxycholate method, one-dimensional chromatography was used and it is probable that some of the minor phospholipid classes, such as the cardiolipids and various lyso compounds, were present but not resolved, as indicated in Table 2. It seems clear, however, that neither the deoxycholate nor the Lubrol selectively removed any particular kind of phospholipid: the overall decrease in phospholipid content brought about by both treatments (Fig. 1) appeared to result from a more or less general depletion of the various types present.

The data given in Tables 2 and 3 also reveal that these enzyme preparations varied sufficiently in their quantitative content of the various phospho-

Table 1. *ATPase activity after removal of residual deoxycholate*

Samples of deoxycholate-extracted ATPase preparations from rabbit kidney were dialysed or filtered through Sephadex G-75 and their deoxycholate contents and ATPase activities measured as described in the Experimental section. Mean values are given for the numbers of experiments indicated.

Preparation	Deoxycholate content (μmol/mg of protein)	Ouabain-sensitive ATPase activity (μmol of P_i/min per mg of protein)		Number of preparations
		Control	+Phosphatidylserine	
Initial	0.12	0.04	0.19	8
Dialysed	0.01	0.03	0.16	4
Gel-filtered	<0.01	0.04	0.22	4

Table 2. *Relative proportions of phospholipids in ATPases before and after extraction with deoxycholate*

Phospholipids were extracted from rabbit kidney ATPase preparations and assayed by quantitative t.l.c., before and after the enzymes had been treated with deoxycholate, as described in the Experimental section. Mean values for two or three assays are given.

	Phospholipids in ATPase			
	Before extraction		After extraction with deoxycholate	
Phospholipid	% of total	Range	% of total	Range
Phosphatidic acid	Trace	(0–1)	4	(3–7)
Phosphatidylethanolamine	28	(27–28)	24	(21–26)
Phosphatidylserine+phosphatidylinositol	13	(11–15)	8	(7–8)
Phosphatidylcholine (+lysophosphatidylethanolamine)	30	(25–34)	33	(29–40)
Sphingomyelin (+lysophosphatidylcholine)	24	(20–28)	27	(17–32)
Unknown*	5	(4–6)	4	(2–6)

* Material remaining at origin and running with the solvent front.

Table 3. *Relative proportions of phospholipids in ATPases before and after extraction with Lubrol*

Data were obtained as for Table 2, except that the initial ATPase preparations had been treated with NaI and extraction was with Lubrol, as described in the Experimental section. Mean values from three analyses are given.

	Phospholipids in ATPase			
	Before extraction		After extraction with Lubrol	
Phospholipid	% of total	Range	% of total	Range
Diphosphatidylglycerol	14	(13–15)	9	(0–16)
Unknown*	5	(3–6)	3	(0–6)
Phosphatidylethanolamine	22	(19–27)	22	(12–34)
Phosphatidylcholine	22	(18–27)	22	(8–28)
Phosphatidylserine	9	(3–18)	8	(3–17)
Phosphatidylinositol	Trace	(0–1)	2	(0–4)
Sphingomyelin	14	(10–18)	9	(4–17)
Lysophosphatidylethanolamine	7	(3–16)	4	(0–7)
Lysophosphatidylserine	1	(0–1)	5	(3–6)
Lysophosphatidylcholine	3	(2–4)	6	(0–11)
Unknown	1	(0–2)	4	(0–11)
Unknown†	2	(1–2)	6	(2–10)

* Reacted with Schiff's reagent.

† Material remaining at origin and running with solvent front.

lipids to make detection of significant selectivity during detergent extraction very difficult. For detection in this way the individual type of lipid would have had to be either one of the major classes present and subject to a large percentage removal, or a minor class that was always completely removed. Clearly, neither of those conditions was fulfilled and the answer to question (5) is that we could find no evidence of selective removal of a particular class of phospholipid during extraction with either of the two detergents used; and hence we could not attribute loss of ATPase activity to removal of any special lipid species.

Reactivation by exogenous phospholipids

The general pattern of reactivation of kidney ATPase after extraction with deoxycholate has been extensively documented elsewhere (Fenster & Copenhaver, 1967; Towle & Copenhaver, 1970; Wheeler, 1971; Kimelberg & Papahadjopoulos, 1972). The lack of reproducibility of that method made it unsuitable for trying to answer questions (6) and (7) posed in the introduction. In contrast, the most impressive aspect of the modified Lubrol method was the consistency of reactivation by exogenous lipid, so that it seemed to be much more suitable for examining those questions.

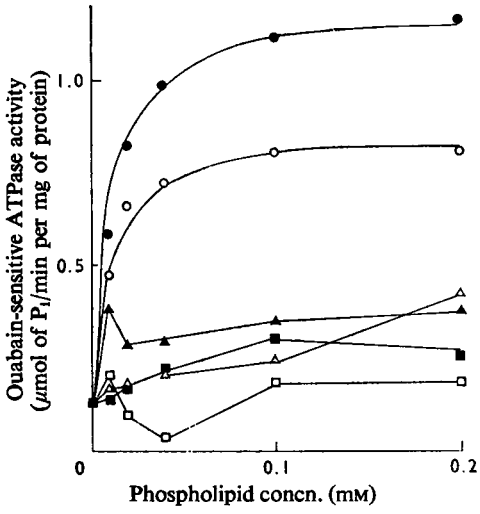

Fig. 3. *Reactivation of lipid-depleted ATPase by exogenous phospholipids*

ATPase preparations were obtained from rabbit kidney and partly depleted of lipid by extraction with Lubrol, as described in the Experimental section. Pure phospholipids were dispersed by sonication and incubated at the indicated concentrations with the depleted ATPases (0.015mg of protein/ml) for measurement of ouabain-sensitive ATPase activity. Mean values from two or three experiments are given. ●, Phosphatidylserine; ○, phosphatidylglycerol; ▲, phosphatidylinositol; △, phosphatidylethanolamine; ■, diphosphatidylglycerol; □, phosphatidylcholine.

Fig. 3 illustrates the general pattern that we have found for reactivation of the Lubrol-treated preparations by endogenous lipids. In those particular experiments phosphatidylserine was easily the most effective phospholipid but in others phosphatidylglycerol proved to be equally as good. Note, however, that even higher concentrations (up to 2mM) of the other phospholipids tested produced either no or very little and variable reactivation. (Such high concentrations of phospholipid tended to interfere with the ATPase assay, causing turbidity, even with albumin present.) Nor did higher concentrations of phosphatidylserine and phosphatidylglycerol show signs of producing less activation: both gave maximal effects at about 0.1mM with the protein concentrations normally used. Several checks of the effect of adding such phosphatidylserine dispersions to the original enzymes, not extracted with Lubrol, under exactly the same conditions showed that the ATPase activity remained unchanged over the entire concentration range of lipid used for reactivation experiments.

In view of these results phosphatidylserine at a concentration of 0.1–0.2mM was used as a routine to test for reactivation and the data obtained in this way from 27 different enzyme preparations are summarized in Fig. 4. Here the extent of reactivation, relative to the original, untreated, enzyme is plotted against the residual activity in the depleted preparations, also relative to the original. Several important points are revealed by this plot. First, restoration of the original specific activity by the added phosphatidylserine was frequently observed; in fact, the specific activities of several of the preparations after reactivation were significantly higher than those of the original enzyme. Secondly, in spite of the obvious scatter, the two functions plotted are quite highly correlated, showing that in general the extent of reactivation varied directly with the amount of residual activity. (The correlation coefficient for all the points shown was 0.65. If only those values obtained with the use of a single Lubrol extraction are considered, the coefficient falls to 0.57. These two values show significant correlation at the 0.001 and 0.01 levels respectively.) Thirdly, reactivation of lipid-depleted enzymes devoid of ouabain-sensitive activity clearly was possible, although it was not consistently observed.

Several attempts were made to obtain preparations

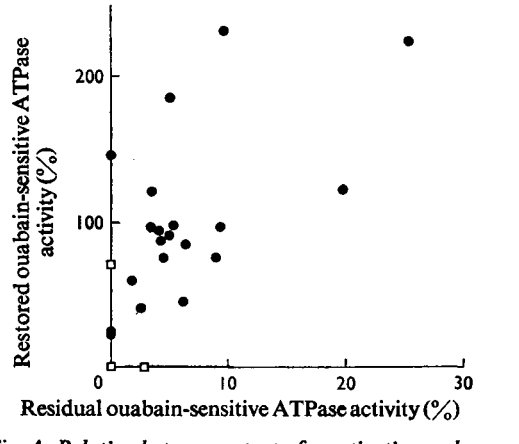

Fig. 4. *Relation between extent of reactivation and amount of residual ATPase activity*

ATPase preparations were obtained from rabbit kidney, partly depleted of lipid by extraction with Lubrol, and 'restored' by incubation with sonicated dispersions of phosphatidylserine (0.1–0.2mM), as described in the Experimental section. Ouabain-sensitive ATPase activity was measured for each kind of preparation and both the residual activity (after lipid-depletion) and the 'restored' activity (in the presence of phosphatidylserine) expressed as percentages of the activity of the original preparation. ●, Enzymes extracted once with Lubrol; □, enzymes extracted two or three times with Lubrol.

devoid of ATPase activity, both by increasing the concentration of Lubrol used to extract the lipid and by using multiple extractions, as described in the Experimental section. However, the effect of a given concentration of Lubrol varied from one enzyme preparation to another and consistent removal of all activity was not obtained with a single extraction, even with Lubrol concentrations in the range 50–100mg/ml. On the other hand, a double or triple extraction with Lubrol almost invariably not only abolished all ATPase activity but also produced lipid-depleted enzymes that could not be reactivated. Of seven enzymes subjected to multiple extractions, only one could be reactivated. Thus two of the four preparations that were reactivated from zero (Fig. 4) were obtained with single Lubrol extractions at 30mg/ml, one with 100mg/ml, and the other by extracting twice with 50mg of Lubrol/ml. The reactivations observed were 20, 24, 147 and 71% respectively. The mean absolute ATPase activities (μmol of P_i/min per mg of protein, ±S.E.M.) of the 21 preparations that could be reactivated were: initial, 0.77±0.06; depleted, 0.05±0.01; restored, 0.69±0.02.

Phospholipid content during purification of the ATPase

Several attempts were made to obtain highly active ATPase from kidney outer medulla, as described in the Experimental section, so that the variation in phospholipid content during purification could be determined to see if any selective enhancement of a particular type of lipid occurred. The results for the best purification obtained are given in Table 4; other, slightly less active, preparations gave essentially the same results. The ouabain-sensitive ATPase activity of the original microsomal fraction was increased about 50-fold (from 0.17 to 8.97μmol of P_i/min per mg of protein) during the purification process, which always increased the total phospholipid content of the enzyme preparations from about 0.2 to greater than 1.0μmol/mg of protein. However, apart from some increase in the content of lysophospholipids, and a fall in the content of phosphatidylcholine, the relative proportions of the various phospholipids were remarkably similar at the two stages of purification, with no sign of a specific enhancement.

Table 4. *Relative proportions of phospholipids in ATPases with low and high specific activities*

A microsomal fraction was isolated from an homogenate of pig kidney outer medulla, treated with deoxycholate and fractionated by density-gradient centrifugation, to give a partially purified ATPase preparation. The phospholipid contents of these low and high activity preparations were assayed as described in Table 2 and the Experimental section. The specific activities of the microsomal fraction and the purified fraction were 0.17 and 8.97μmol of P_i/min per mg of protein respectively.

Phospholipid	Phospholipids in ATPase (% of total)	
	Microsomal fraction	Purified fraction
Diphosphatidylglycerol	4	4
Unknown	4	3
Phosphatidylethanolamine	13	11
Phosphatidylcholine	39	28
Phosphatidylinositol	3	3
Phosphatidylserine	12	12
Sphingomyelin	8	8
Lysophosphatidylethanolamine	6	11
Lysophosphatidylserine	2	5
Lysophosphatidylcholine	6	12
Unknown*	3	3

* Material remaining at origin and running with solvent front.

Discussion

When detergent is used to extract an enzyme it is always possible that any inhibition of the enzyme stems from the presence of residual protein-bound detergent, rather than from the removal of lipid. Apparent reactivation by added lipid could then result from the removal of that residual detergent. Some of the results presented above exclude that possibility as far as extraction of the $(Na^+ + K^+)$-dependent ATPase with either deoxycholate or Lubrol is concerned, so that the other results are best interpreted as evidence supporting the concept of an essential role for phospholipid in the functioning of the enzyme. The general parallelism between the specific activity and the phospholipid content of the enzyme preparations, coupled with the consistent restoration of the activity of lipid-depleted preparations by exogenous phospholipid, is difficult to interpret in any other way. Having accepted this concept, however, we are left with the problems of whether there is a requirement for a specific kind of phospholipid and what the lipid is required for. We have used three different approaches to tackle the question of phospholipid specificity: enzyme purification, enzyme inactivation by extraction with detergents, and reactivation by exogenous phospholipids. The results of these three approaches are discussed in turn below.

Our failure to observe enhancement of the content of a particular type of lipid during purification of the ATPase contrasts with the findings of Hokin & Hexum (1972). They reported that the phosphatidylserine content of their ATPase increased from 13 to 24% concomitant with an increase in specific activity from 1.3 to 2.2 (μmol of P_i/min per mg of

protein), whereas we found no consistent change when the activity increased from 0.2 to 9.0. The enzymes used in these two studies were obtained from different tissues and the methods of purification were also different: but both the initial phosphatidylserine content of the preparations and the specific activity range covered were very similar. The apparent correlation noted by Hokin & Hexum (1972) must therefore be interpreted with caution. An alternative view is that those enzyme preparations were still so impure, in the absolute sense, that detection of a specific phospholipid component against the background of extraneous lipid would be extremely unlikely. In fact, we drew that conclusion and abandoned this approach when the work of Kawai *et al.* (1973) appeared. They had analysed some ATPase preparations which were two to three times as active as the best one we had obtained at that time and found that there was still the usual kind of lipid mixture present. [Although they also concluded that the relative content of phosphatidylserine increased during purification, the values quoted were smaller than that of Hokin & Hexum (1972), despite the much higher activity, and without statistical support for their significance that conclusion must be considered unequivocal.]

We could find no evidence for selective removal of any one type of lipid during extraction and inactivation with either deoxycholate or Lubrol. Hence it appears that either the loss of activity was not associated with removal of a particular lipid, or such specific removal was obscured by other contaminating lipids present. The latter interpretation seems to be supported by the demonstration by Roelofsen & van Deenen (1973) that enzymic decarboxylation of the phosphatidylserine in erythrocyte 'ghosts' did not affect the $(Na^+ + K^+)$-dependent ATPase activity until the last 13% of that phospholipid had been converted, after which all activity was abolished. If only such a small fraction of a particular lipid is involved in the enzyme's functioning, detection of its removal during detergent treatment would be extremely difficult and unlikely. Indeed, it is rather remarkable that the decarboxylase selectively removed most of the phosphatidylserine that was not associated with the ATPase before the fraction that apparently was associated with it.

The general pattern of reactivation of the depleted ATPase by exogenous phospholipids that we found is very similar to that reported by Kimelberg & Papahadjopoulos (1972) for deoxycholate-extracted ATPase from rabbit kidney. In addition we have obtained consistent reactivation with phosphatidic acid (J. A. Walker & K. P. Wheeler, unpublished work), as Hokin & Hexum (1972) found with an ATPase depleted by digestion with phospholipase A. In contrast, the consistent reactivation by relatively low concentrations of phosphatidylserine is the only finding in direct agreement with the results of Palatini *et al.* (1972) for their Lubrol-extracted ATPase from bovine heart. Comparison of the relative proportions of the residual phospholipids in their and our depleted enzymes (Table 3) seems to rule out the possibility that the different patterns of reactivation stem from differences in the kind of residual lipids. For example, the proportion of residual diphosphatidylglycerol was very high in the heart enzyme and low in the kidney preparation, yet low concentrations of exogenous samples of diphosphatidylglycerol reactivated the former but not the latter. Again, both kinds of enzyme contained about the same proportions of residual phosphatidylcholine and phosphatidylethanolamine, but only the heart preparation was reactivated by exogenous samples of those lipids. If we may generalize from these findings, it seems very unlikely that the apparent discrepancies or inconsistencies in reactivation reported in the literature arise from differences in the kinds of residual phospholipids in the various lipid-depleted preparations. Different physical states of the protein could be responsible or, alternatively, the nature of the fatty acyl side chains of the phospholipids used. With attention focused mainly on the polar ends of these molecules the hydrophobic portions have been somewhat neglected. However, evidence has been presented showing the importance of fluidity in the phospholipid side chains, both endogenous (Grisham & Barnett, 1973) and exogenous (Kimelberg & Papahadjopoulos, 1974), associated with $(Na^+ + K^+)$-dependent ATPase activity. Consideration of all the variations reported suggests that complete reactivation of lipid-depleted ATPase requires a combination of the 'right' polar head groups (probably one with a net negative charge) with the 'right' kinds of fatty acyl chains (probably unsaturated ones). How far these requirements reflect the position *in situ* is uncertain.

The finding that the reactivated enzymes frequently had higher specific activities than the original unextracted preparations (Fig. 4) is not unreasonable because the recovery of protein after a single Lubrol extraction was only about 10%. In one way, therefore, comparison of a lipid-depleted preparation with the original is rather spurious because the relative content of the ATPase protein was probably higher after the Lubrol treatment. This loss of protein during Lubrol extraction is the only drawback to the method at the moment.

The general correlation between the extent of reactivation and the amount of residual ATPase activity (Fig. 4) raises the question of what the phospholipid is required for. At present we can only speculate that this kind of correlation could be explained by the following sequence of events. Exposure to the detergent strips off phospholipid that is closely associated with the ATPase

protein through hydrophobic binding and replaces it with detergent. This removal and replacement of the phospholipid results in a change in protein conformation and loss of enzymic activity. Sufficient retention of the conformation capable of interacting with exogenous phospholipid, to reverse the process, would then be necessary for reactivation to occur, and residual ATPase activity could be a measure of that retention.

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