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REACTIVATION OF A PHOSPHOLIPID-DEPLETED SODIUM, POTASSIUM-STIMULATED ATPase

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SUMMARY

1. The effect of phospholipids on (Na^+–K^+)-ATPase was studied in a particulate preparation from bovine heart.

2. After treatment with ultrasonic oscillations the complex became partially soluble in a solution of nonionic detergent lubrol. Fractionation of "solubilized" ATPase with $(NH_4)_2SO_4$, followed by washing in NaCl solutions, resulted in a preparation depleted of phospholipids as well as cholesterol and largely devoid of ATPase activity.

3. Total phospholipids from various sources as well as individual purified phospholipids restored the ATPase activity which was 90 % inhibited by ouabain. Reconstitution was accompanied by reuptake of lost phospholipids. Differences among phospholipids were recorded: phosphatidylserine and diphosphatidylglycerol were the most effective but active preparations were also obtained with phosphatidylcholine and phosphatidylethanolamine. Cholesterol added as aqueous emulsion was ineffective.

4. It is concluded that activation of (Na^+–K^+)-ATPase by phospholipids is a rather aspecific process. Although phosphatidylserine shows remarkable affinity for the enzyme complex and presumably plays an important role in maintaining the activity of the ATPase, it can not be considered as a specific activator. In agreement with recent observations on particulate mitochondrial ATPase from rat liver, acidic phospholipids showed greater effectiveness in the reactivation of transport ATPase and could be bound better.

INTRODUCTION

Ohnishi and Kawamura[1], studying the (Na^+–K^+)-ATPase ("transport ATPase"; ATP phosphohydrolase, EC 3.6.1.3) activity of horse erythrocytes after digestion with phospholipase A, found limited reconstitution of ouabain-sensitive activity with phosphatidylserine. Tanaka and Abood[2] and Tanaka and Strickland[3] using deoxycholate extraction and salt precipitation obtained phospholipid-dependent preparations from rat or bovine brain. This procedure, applied to bovine brain or rabbit kidney, was used in a number of subsequent studies[4–9] in which the activating effect

of phosphatidylserine was confirmed. On these bases it was proposed[8,9] that phosphatidylserine is an essential part of the transport ATPase complex and may participate directly in the transport of Na^+ and K^+.

Hegyvary and Post[10] as well as Roelofsen *et al.*[11] observed that complete depletion of phospholipids by phospholipase A treatment rendered reactivation by the addition of external phospholipids impossible. This raises the question[11] whether the effect of phospholipids on a partially depleted preparation is due to activation of residual ATPase or removal of inhibitory detergent[12] when deoxycholate is used as solubilizing agent.

More recently, Noguchi and Freed[13] found that cholesterol alone could completely reactivate a preparation of transport ATPase rendered inactive by extraction with chloroform–methanol at —75 °C. The extent of phospholipid depletion was not reported.

The present investigation was undertaken with the aim to explore the requirement for phospholipids in a preparation depleted by a procedure different from those generally used (*i.e.* deoxycholate extraction or phospholipase digestion).

MATERIAL AND METHODS

All reagents were commercial grade. Lubrol 'W' (cetyl alcohol–polyoxyethylene condensate) was a gift from ICI Ltd. The purification of a crude extract of bovine brain and rat liver mitochondrial phospholipids was as described[14].

Individual phospholipids were "ultrapure" phospholipids from General Biochemicals (Chagrin Falls, Ohio). They were all from bovine brain with the exception of lysophosphatidylcholine and one sample of phosphatidylcholine (from eggs). In order to exclude contamination of other phospholipids by phosphatidylserine and diphosphatidylglycerol, their purity was carefully checked by thin-layer chromatography using the following systems: (a) chloroform–methanol–water (70:30:5, by vol.); (b) chloroform–methanol–water-acetic acid (25:15:4:2, by vol.); (c) chloroform–methanol–conc. (14.8 M) ammonia (70:20:1.5, by vol.); (d) the two-dimensional system described by Rouser *et al.*[15]. Only phosphatidylethanolamine was contaminated with an unidentified component and further purification was achieved using preparative thin-layer chromatography. All phospholipids were dispersed in a 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.5) solution by ultrasonic oscillation (Biosonik III apparatus). Care was taken to keep the pH at neutral value during and after sonication.

^{32}P-labeled phospholipids were kindly donated by Dr Contessa and Dr Pitotti. They were prepared[16] from rat liver and rat brain after injection of 500 μCi [^{32}P]-phosphate and purified on a column of silicic acid (Bio-Sil HA *minus* 325 mesh, Bio-Rad); the largest part of the radioactivity was in phosphatidylcholine and phosphatidylethanolamine. A stable emulsion of cholesterol in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) was prepared according to Stadtman[17]. Dispersions of cholesterol and egg phosphatidylcholine or L-α-phosphatidylcholine (dipalmitoyl, synthetic) in a molar ratio of 1:1 were prepared as described by Horwitz *et al.*[18].

Solubilization of transport ATPase by lubrol

(Na^+–K^+)-ATPase was the preparation of Matsui and Schwartz[19] obtained as

described previously[20] with the following modifications: (a) treatment with ultrasonic oscillations was for 2 min at 0–5 °C and (b) the final centrifugation (2 h at 40000 rev./min, Spinco No. 40 rotor) was followed by a washing in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4). The preparation was contamined by cytochrome oxidase as revealed by the high content of diphosphatidylglycerol and the presence of a KCN-sensitive oxygen uptake with reduced cytochrome *c*. The inhibition by 0.1 mM ouabain in the presence of 100 mM NaCl and 20 mM KCl was 90–93 %.

In agreement with previous findings on guinea pig brain transport ATPase[21] it was found that, in contrast to various detergents (including the anionic deoxycholate, cholate, sodium dodecylsulfate, the cationic ethylhexadecyldimethylammonium bromide, and the nonionic Triton X-100), lubrol (up to 8 mg/mg protein) did not inhibit the ATPase activity and was selected to attempt solubilization. The enzyme preparation at the stage of purification with NaI was almost insoluble in an aqueous solution of this detergent but became partially soluble after treatment with ultrasonic oscillations.

Removal of phospholipids

To a suspension of the sonicated enzyme preparation (10 mg/ml) were added, in the following order and at the indicated final concentrations, 2.5 mM ATP·Tris (pH 7.3), 10 mg/mg protein lubrol, 35% glycerol, 1 mM β-mercaptoethanol. The final pH was 7.6 and protein concentration 2 mg/ml. After stirring for 60 min at 0 °C the suspension was centrifuged 1 h at 40000 rev./min and the supernatant removed carefully. To the supernatant solid $(NH_4)_2SO_4$ was added up to 35% of saturation at 0 °C and the sample was centrifuged 20 min at 40000 rev./min. The preparation, recovered as a floating layer, was suspended in 0.2 M NaCl and sedimented for 30 min at 50000 rev./min to remove most of the lubrol and β-mercaptoethanol. The sediment was resuspended in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4). After estimation of protein, the treatment with lubrol was repeated. Solid $(NH_4)_2SO_4$ was then added directly and the sample centrifuged for 20 min at 40000 rev./min. The floating layer was recovered, washed once with 0.2 M, once with 0.6 M and finally with 1.0 M NaCl. The final sediment was resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 7.4) and stored in small aliquots at —40 °C where it was stable for several weeks. On the basis of protein content the yield was around 15–25% of the original preparation. Addition of 1 M NaCl during the treatment with lubrol improved the solubilization, but the specific activity after addition of phospholipids was lower.

Analytical procedure

Phosphate was determined according to the method of Fiske and SubbaRow, protein as described by Lowry *et al.*[22] with crystalline serum albumin as standard, after dissolving particulate material with sodium deoxycholate. Phospholipid phosphorus was determined after the ashing procedure of Ames and Dubin[23]: 1 μg of phosphorus was assumed to be contained in 25.3 μg phospholipids[24] except in the case of lysophosphatidylcholine where a mol. wt of 506 was assumed[25].

Phospholipids were extracted overnight at 2 °C with 20 vol. of chloroform–methanol (2:1, v/v), then 30 min at room temperature with the same solution, and

an additional 30 min at room temperature with chloroform–methanol (2:1,v/v) containing 1% (v/v) acetic acid. The pooled extracts were washed with 0.2 vol. of 0.040% $MgCl_2$, taken to dryness and dissolved in chloroform. Qualitative and quantitative estimations of phospholipids were performed as described by Rouser *et al.*[15] using thin-layer chromatography on plates made with Silica Gel HR (Merck) *plus* magnesium acetate as binder. Cholesterol was measured as described by Schwartz *et al.*[26].

ATPase activity was tested at 37 °C in the following incubation medium: 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 3.0 mM ATP·Tris (pH 7.4), 50 mM Tris–HCl (pH 7.4), 50 mM sucrose, 0.2 mM EDTA, 120–150 μg enzyme preparation; final volume, 1.0 ml; incubation time, 20 min. The reaction was stopped by addition of 0.25 ml cold 50% trichloroacetic acid. During this time the reaction was linear. Reconstitution of the phospholipid-depleted preparation was obtained by incubating in 0.2 ml of 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4), the phospholipid and the enzyme (130–160 μg), added in this order, for 30 min at 37 °C. In some instances, the incubation medium was completed and the ATPase activity measured; in others, the samples were centrifuged to remove the unbound phospholipids, washed once and the phospholipid content as well as the ATPase activity determined.

RESULTS

Table I presents the phospholipid and cholesterol values for the preparations. Extraction with lubrol and fractionation with $(NH_4)_2SO_4$ resulted in the removal of about 90% of the phospholipids. The content of cholesterol was reduced to trace amounts (around 0.01 mg/mg protein). Accordingly, the depleted preparation incubated in presence of ATP, Mg^{2+}, Na^+ and K^+, was largely devoid of ATPase activity. The residual activity, when present in measurable extent, was abolished by ouabain.

Substantial recovery of ATPase activity was achieved by addition of a mixture of purified phospholipids which reproduced the composition of unextracted enzyme without diphosphatidylglycerol. The reconstituted ATPase was strongly (91%) inhibited by 0.1 mM ouabain in the presence of Na^+ and K^+. This indicated that reconstitution of (Na^+–K^+)-ATPase activity took place. Simultaneously with reappearance of activity there was a reconstitution of phospholipid complement, which reached 40–60% of the original preparation.

In Table II it is seen that significant enrichment in diphosphatidylglycerol was observed in the residual content of phospholipids of the lubrol-extracted fraction. Most probably this was due to the contamination by cytochrome oxidase since it is known[27] that this phospholipid is tightly bound to this enzyme and difficult to remove. The phospholipid composition of the reconstituted enzyme complex was different from the original preparation because phospholipids with negatively charged head groups showed more affinity and were bound preferentially.

In agreement with these results are the data on the effectiveness of individual phospholipids in the reactivation of ATPase (Table III). Total phospholipids from bovine brain, in the absence of cholesterol, produced complete restoration of activity. The same result was obtained with rat liver mitochondrial phospholipids. Large

TABLE I

LIPID COMPOSITION AND ATPASE ACTIVITY OF LUBROL-EXTRACTED FRACTION

A preparation of (Na^+–K^+)-ATPase from bovine heart was extracted with lubrol as described in Materials and Methods. Reconstitution with phospholipids was performed by incubating 1.7 mg protein (lubrol-extracted fraction) with 10 mg of a mixture of individual, purified bovine brain phospholipids in 2.3 ml 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4). After 30 min at 37 °C the sample was centrifuged, washed once and assayed for ATPase activity and phospholipid content. Individual bovine brain phospholipids were mixed in order to have 46% phosphatidylcholine, 36% phosphatidylethanolamine, 6% sphingomyelin, 7% phosphatidylinositol, 5% phosphatidylserine. The mixture was sonicated three times for 30 s before use. Conditions for ATPase activity: 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 3.0 mM ATP·Tris (pH 7.4), 50 mM sucrose, 0.2 mM EDTA, 50 mM Tris–HCl (pH 7.4), 110–130 μg protein ATPase preparation. Final volume, 1.0 ml; incubation, 20 min at 37 °C. The reaction was terminated with 0.25 ml cold 50% trichloroacetic acid. The data are means of separate experiments with ranges in parentheses.

	Unextracted ATPase	*Lubrol-extracted fraction*	*Reconstituted ATPase*
No. of experiments	4	4	3
Phospholipids (mg/mg protein)	1.67 (1.39–2.18)	0.11 (0.05–0.18)	0.70 (0.62–0.87)
Cholesterol (mg/mg protein)	0.163 (0.145–0.188)	Trace	—
ATPase activity (μmoles ATP split/mg per h)	17.7 (14.0–22.5)	2.2 (1.0–3.4)	14.4 (10.9–16.7)
Per cent inhibition by 0.1 mM ouabain	91.6 (90–93)	100	91 (90–92)

TABLE II

PHOSPHOLIPID COMPOSITION OF LUBROL-EXTRACTED FRACTION

Experimental conditions and number of experiments are reported in Table I.

Phospholipid	% *of total phospholipids*		
	Unextracted ATPase	*Lubrol-extracted fraction*	*Reconstituted ATPase*
Phosphatidylcholine	39.4 (38.0–42.2)	18.5 (0.0–28.0)	18.3 (15.7–21.0)
Phosphatidylethanolamine	31.1 (28.8–33.4)	18.4 (0.0–26.1)	17.5 (14.7–22.1)
Sphingomyelin	6.2 (4.2–10.0)	Trace	9.2 (6.1–10.8)
Phosphatidylinositol	5.8 (4.2–7.0)	Trace	21.1 (17.8–26.9)
Phosphatidylserine	3.4 (2.3–4.8)	Trace	17.7 (16.5–19.0)
Diphosphatidylglycerol	13.7 (11.7–16.7)	63.1 (48.2–100)	12.9 (11.5–14.0)
Others	0.4 (0.0–1.2)	—	3.3 (2.2–4.3)

differences were seen in the effectiveness of individual phospholipids. Moreover, some phospholipids (mainly phosphatidylethanolamine and diphosphatidylglycerol) produced an activation which disappeared on increasing the concentration.

TABLE III

ACTIVATING EFFECT OF PHOSPHOLIPIDS ON LUBROL-EXTRACTED FRACTION

Aliquots of lubrol-extracted fraction corresponding to a mean value of 127 μg protein (range 116–146 μg) were added to 0.2 ml of 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) containing the phospholipids. After 30 min at 37 °C the medium was completed and the ATPase activity measured as outlined in Table I. *V* is the maximum value of ATPase in presence of phospholipids *minus* the activity in their absence which in six preparations was on the average of 2.1 μmoles ATP split/mg protein per h (range 1.0–3.4). *V* values were calculated using Lineweaver–Burk plots. Owing to the non-linearity of Lineweaver–Burk plots, the amount of phospholipids at 50% effect was calculated in plots of activation *versus* phospholipid concentration. The values are mean of separate experiments the number of which is given in parentheses. The inhibition by 0.1 mM ouabain in the absence and presence of phospholipids was 100%.

Phospholipids	*V* (*Δμmoles ATP split/mg per h at 37 °C*)	*Amount of phospholipids at 50% effect* (*mg/mg protein*)
Total bovine brain phospholipids	15.9 (4)	1.06
Bovine brain phosphatidylcholine	5.9 (1)	6.15
Egg phosphatidylcholine	7.4 (2)	2.02
Bovine brain phosphatidylethanolamine	12.3 (2)	3.15
Bovine brain phosphatidylserine	9.7 (5)	0.15
Bovine brain diphosphatidylglycerol	9.3 (4)	0.13
Bovine brain phosphatidylinositol	3.5 (2)	—
Bovine brain sphingomyelin	2.5 (1)	—
Egg lysophosphatidylcholine	0.8 (1)	—

Three patterns can be described: (a) Phospholipids acting at high concentrations. These include phosphatidylcholine and phosphatidylethanolamine, the latter being more active. The extent of reactivation induced by phosphatidylethanolamine, which is weakly acidic at neutral pH, is the highest recorded and comparable to that induced by total phospholipids from bovine brain. (b) Phospholipids with high affinity, acting at low concentration. These are the highly acidic phosphatidylserine and diphosphatidylglycerol. The extent of reactivation afforded by these phospholipids, although remarkable, was limited. It can be added that the stimulation by a low amount of external diphosphatidylglycerol is a further indication that the diphosphatidylglycerol present in the lubrol extracted fraction is not associated with transport ATPase. (c) Phospholipids with slight or nonreproducible activity. These are sphingomyelin, phosphatidylinositol and lysophosphatidylcholine.

In every instance the ATPase activity reconstituted by addition of individual phospholipids was completely inhibited by 0.1 mM ouabain.

These results are in contrast with other reports on transport ATPase. Tanaka and Strickland[3], using a preparation extracted with deoxycholate, found no activation with diphosphatidylglycerol (used, however, at a very high and thus ineffective concentration). Moreover, in similar preparations no activation was observed with phosphatidylethanolamine[4,6,9] and phosphatidylcholine[6,9]; the latter, however, was found to be active by Tanaka and Abood[2] and Tanaka[4].

The disagreement is not difficult to explain since several conditions are expected to influence the effect of external phospholipids: (a) the conditions and the detergent

used to deplete the preparation of transport ATPase, (b) the extent of depletion and (c) the way to add the phospholipid. Dispersion by ultrasonic oscillations is more effective than the simple homogenization sometimes used. In addition, it must be emphasized that the activity of a phospholipid can not be judged by testing a single, often high, concentration because a significant activation produced at low concentration can be absent at a higher amount.

In Table IV the influence of other phospholipids on the reactivation induced by saturating amounts of phosphatidylserine is shown. It can be seen that both phosphatidylcholine and phosphatidylethanolamine could further stimulate the ATPase activity. This is taken as evidence that phosphatidylserine alone can not satisfy the requirement for phospholipids by transport ATPase.

TABLE IV

ADDITIVE EFFECT OF PHOSPHATIDYLSERINE AND OTHER PHOSPHOLIPIDS

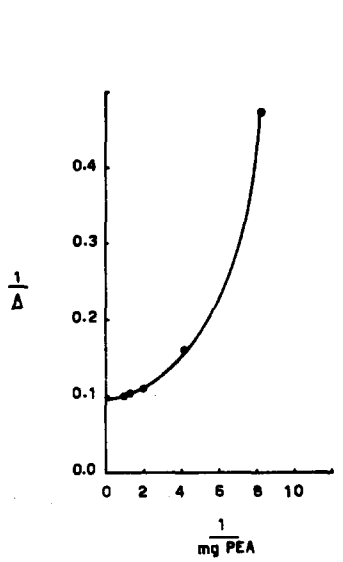
Experimental conditions as in Table II. 125 μg protein (lubrol-extracted fraction). The data are the increase in the activity due to phospholipid addition. The activity without phospholipids was 3.4 μmoles ATP split/mg per h.

Additions	*Stimulation of ATPase activity (Δμmoles ATP split/mg per h)*
30 μg bovine brain phosphatidylserine	7.7
60 μg bovine brain phosphatidylserine	8.2
800 μg bovine brain phosphatidylethanolamine	10.6
520 μg egg phosphatidylcholine	3.4
60 μg phosphatidylserine + 800 μg phosphatidylethanolamine	13.6
60 μg phosphatidylserine + 520 μg phosphatidylcholine	10.2

Noguchi and Freed[13] observed reactivation of transport ATPase by cholesterol after extraction with chloroform–methanol at —75 °C. Cholesterol was effective only when added in organic solvents at —75 °, and not when added at 37 °C as an aqueous emulsion. In agreement with those results, it was found that emulsion of cholesterol alone added at 37 °C was unable to produce reactivation of the lubrol-extracted fraction. When cholesterol was included in micelles of egg or synthetic phosphatidylcholine, this did not improve, but actually decreased the reactivation elicited by the phospholipids. Since complete reactivation of transport ATPase was produced by phospholipids in the absence of cholesterol, these data do not indicate a role of cholesterol in the transport ATPase.

The kinetics of activation of transport ATPase by phosphatidylethanolamine are reported in Fig. 1. A double reciprocal plot of the rate of activation *versus* phospholipid concentration showed a clear deviation from normal Michaelis–Menten kinetics. In the presence of 0.1 mM ouabain the stimulation by phosphatidylethanolamine was completely abolished.

The deviation from normal kinetics was strongly evident with all individual or pooled phospholipids. To further explore the reason for this behavior the activation induced by phospholipids was studied as a function of their binding to the preparation



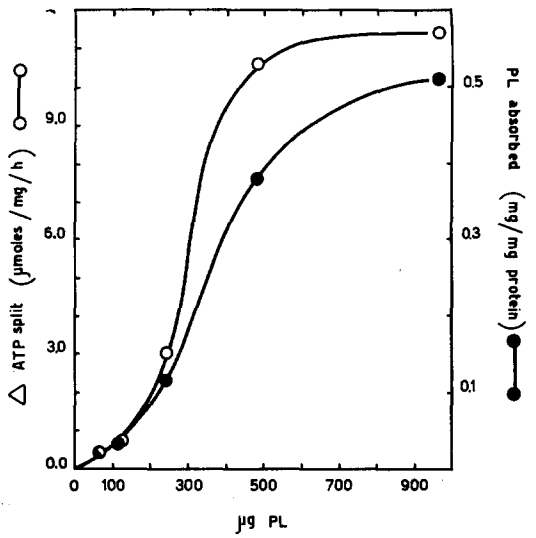


Fig. 1. Activating effect of bovine brain phosphatidylethanolamine (PEA). Experimental conditions as described in Table II. 116 μg protein (lubrol-extracted fraction). Δ is the activity (μmoles ATP split/mg per h) elicited by the varying amounts of phosphatidylethanolamine *minus* the activity in the absence of phospholipid which was 2.0 μmoles ATP split/mg per h.

Fig. 2. Binding of phospholipids (PL) and activation of transport ATPase. Reconstitution of ATPase was performed as described in Table II using ^{32}P-labeled phospholipids from rat liver and brain (20600 cpm/mg phospholipid). After incubation the samples were centrifuged. The supernatant was used for measurement of inorganic phosphate liberated from ATP, the sediment, washed once, was dissolved in 10% sodium deoxycholate and counted after determination of protein content. Activity is expressed as increase due to addition of phospholipids.

(Fig. 2). It can be seen that the sigmoidal relationship between the concentration of added phospholipid and the induced activation is paralleled by a similar relationship between the added phospholipid and the amount which was bound. Clearly, the extent of activation was dependent on the binding of phospholipid to the preparation and this exhibited the kinetics of an apparent cooperative effect.

DISCUSSION

An ouabain-sensitive, (Na^+–K^+)-stimulated activity was elicited by phospholipids in a lipid-depleted preparation of transport ATPase.

Two possibilities can explain the effect of phospholipids (*cf* ref. 11): (a) activation of residual ATPase activity and (b) reconstitution of the ATPase complex. The removal of inhibitory detergent, as suggested[12] for explaining a similar phospholipid effect in deoxycholate-extracted preparations, does not seem to be applicable here since lubrol, unlike deoxycholate, does not inhibit the bovine heart transport ATPase. Attention to the first possibility comes from the observation that extensive cleavage of phospholipid by phospholipase digestion was not followed by activation after addition of external phospholipids[10,11]. Moreover, phosphatidylserine[11] (as well as some detergents[21,28]) activated transport ATPase many-fold also in the absence of

phospholipid depletion. Removal of phospholipids by phospholipase is different from removal by a detergent. Inhibitory products can be left in the preparation; moreover, detergent, replacing phospholipids, may produce protection of essential groups in the protein. Jorgensen and Skou[28] showed that in the absence of phospholipid depletion the activation of kidney transport ATPase by deoxycholate is not accompanied by binding of detergent to the preparation. Their conclusion was that removal of proteins and lipids from the membranous complex is presumably responsible for activation. This could be the case also in the activation induced by phosphatidylserine since phospholipids have some detergent-like properties.

In contrast, the activation by phospholipids of the lubrol-extracted fraction is dependent on their binding to the preparation. This fact speaks in favor of reconstitution of a lipoprotein complex responsible for ouabain-sensitive ATPase activity.

In agreement with the results on the stimulation by phospholipids of particulate mitochondrial ATPase from rat-liver[29], it was observed that the saturation curves for phospholipid effect showed divergences from Michaelis–Menten kinetics. At variance with mitochondrial ATPase, in the transport ATPase the divergence was more pronounced and clearly evident with all phospholipids, including those (phosphatidylcholine and phosphatidylethanolamine) existing in micellar state at extreme dilution[30]. Possibly, this is correlated with the greater extent of phospholipid depletion afforded by extraction with lubrol or with a greater effectiveness of bound lubrol, in comparison to cholate, in inhibiting the binding of active micelles of phospholipids. However, the possibility that this behavior reflects induced conformational changes in the enzyme protein or structural organization of the complex as suggested earlier[29] can not be excluded.

In comparison to other preparations[6,9] a different picture of phospholipid requirement emerged in the lubrol-extracted fraction. Neutral as well as acidic phospho- were found to be active in the reconstitution of ATPase activity. The acidic phospholipids showed higher affinity for the depleted preparation and could be bound better. This is in agreement with recent findings on particulate mitochondrial ATPase from rat liver[29] and confirms the importance of these phospholipids in the structural organization and function of cellular membranes. The high effectiveness of phosphatidylserine is consistent with the possibility that an essential role is ascribed to this phospholipid in the activation and regulation of transport ATPase activity. However, the requirement for phospholipids by this system can not be fulfilled by phosphatidylserine alone, as suggested[6,8,9]. Full activation of transport ATPase has to be regarded as an effect in which more than one phospholipid cooperates.

In comparison to particulate mitochondrial ATPase from rat liver, the requirement for phospholipids by (Na^+–K^+)-ATPase from bovine-heart is more specific. In this case phosphatidylinositol and lysophosphatidylcholine were not active. Of interest is the observation that phosphatidylserine and diphosphatidylglycerol, which are distributed differently in the cellular membranes, were the only phospholipids sharing high activity and affinity in both ATPases.

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REFERENCES

1 T. Ohnishi and H. Kawamura, *J. Biochem. Tokyo*, 56 (1964) 377.
2 R. Tanaka and L. G. Abood, *Arch. Biochem. Biophys.*, 108 (1964) 47.
3 R. Tanaka and K. P. Strickland, *Arch. Biochem. Biophys.*, 111 (1965) 583.
4 R. Tanaka, *J. Neurochem.*, 16 (1969) 1301.
5 R. Tanaka and T. Sakamoto, *Biochim. Biophys. Acta*, 193 (1969) 384.
6 L. J. Fenster and J. H. Copenhaver Jr, *Biochim. Biophys. Acta*, 137 (1967) 406.
7 D. W. Towle and J. H. Copenhaver Jr, *Biochim. Biophys. Acta*, 203 (1970) 203.
8 K. P. Wheeler and R. Whittam, *Nature*, 225 (1970) 449.
9 K. P. Wheeler and R. Whittam, *J. Physiol.*, 207 (1970) 303.
10 G. Hegyvary and R. L. Post, in D. C. Tosteson, *The Molecular Basis of Membrane Function*, Prentice Hall, New Jersey, 1969, p. 519.
11 B. Roelofsen, R. F. A. Zwaal and L. M. van Deenen, in G. Porcellati and F. di Jeso, *Membrane-Bounp Enzymes*, Plenum Press, New York, 1971, p. 209.
12 P. Emmelot and C. J. Bos, *Biochim. Biophys. Acta*, 150 (1968) 341.
13 T. Noguchi and S. Freed, *Nature New Biol.*, 230 (1971) 148.
14 A. Bruni and E. Racker, *J. Biol. Chem.*, 243 (1968) 962.
15 G. Rouser, A. Yamamoto and S. Fleischer, *Lipids*, 5 (1970) 494.
16 W. C. McMurray and R. M. C. Dawson, *Biochem. J.*, 112 (1969) 91.
17 T. C. Stadtman, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 392.
18 C. Horwitz, L. Krut and L. Kaminsky, *Biochim. Biophys. Acta*, 239 (1971) 329.
19 H. Matsui and A. Schwartz, *Biochim. Biophys. Acta*, 128 (1966) 380.
20 A. R. Contessa and A. Bruni, *Biochim. Biophys. Acta*, 241 (1971) 334.
21 P. D. Swanson, H. F. Bradford and H. McIlwain, *Biochem. J.*, 92 (1964) 235.
22 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
23 B. N. Ames and D. T. Dubin, *J. Biol. Chem.*, 235 (1960) 769.
24 E. Bachmann, D. W. Allmann and D. E. Green, *Arch. Biochem. Biophys.*, 115 (1966) 153.
25 H. Hamori and A. M. Michaels, *Biochim. Biophys. Acta*, 231 (1971) 496.
26 A. Schwartz, H. S. Bachelard and H. McIlwain, *Biochem. J.*, 84 (1962) 626.
27 Y. C. Awasthi, T. F. Chuang, T. W. Keeman and F. L. Crane, *Biochim. Biophys. Acta*, 226 (1971) 42.
28 P. L. Jorgensen and J. C. Skou, *Biochim. Biophys. Acta*, 233 (1971) 366.
29 A. Pitotti, A. R. Contessa, F. Dabbeni-Sala and A. Bruni, *Biochim. Biophys. Acta*, 274 (1972) 528.
30 D. Chapman, *Introduction to Lipids*, McGraw-Hill, London, 1969, p. 14.