Biochimica et Biophysica Acta, 729 (1983) 62–74
Elsevier Biomedical Press

BBA 71556

LIPID-PROTEIN INTERACTIONS OF RECONSTITUTED MEMBRANE-ASSOCIATED ADENOSINETRIPHOSPHATASES

USE OF A GEL-FILTRATION PROCEDURE TO EXAMINE PHOSPHOLIPID-ACTIVITY RELATIONSHIPS

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(Received August 6th, 1982)
(Revised manuscript received November 15th, 1982)

Key words: Reconstitution; $(Na^+ + K^+)$-ATPase; Mg^{2+}-ATPase; Gel filtration; Membrane fluidity; Liposome; Lipid-protein interaction

A gel-filtration procedure is described for the reconstitution of partially delipidated membrane adenosinetriphosphatases (Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase) into liposomes of defined composition. After detergent solubilization of membrane enzyme preparations, reconstitution of these ATPases was achieved by the rapid removal of deoxycholate by Sephadex G-50 chromatography. Proteoliposomes were separated from unincorporated enzyme by chromatography on Sepharose CL-4B. Sedimentation characteristics in sucrose density gradients and electron microscopy confirmed that both Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase were reconstituted into liposomes of phosphatidylcholine and yielded preparations having high recoveries of enzyme activity by comparison with the control membrane preparations. Reconstitution of $(Na^+ + K^+)$-ATPase into synthetic phosphatidylcholines of defined fatty acid composition reveals an inverse relationship between enzyme activity and the chain length of the saturated fatty acids DMPC, DPPC and DSPC. Higher recoveries were obtained when one or more fatty acid chains was unsaturated. Full reactivation occurred with DOPC (18:1/18:1). There was a positive correlation between the specific activity of reconstituted $(Na^+ + K^+)$-ATPase and the temperature of the thermal phase transition of the synthetic phosphatidyl cholines studied. This was not seen with Mg^{2+}-ATPase. It is suggested that 'membrane fluidity' influences the catalytic activity of $(Na^+ + K^+)$-ATPase but not that of Mg^{2+}-ATPase.

Introduction

Since the original lipid-depletion, enzyme-activation studies of Tanaka and Abood [1] nearly twenty years ago, many workers have confirmed the lipid dependence of membrane-associated $(Na^+ + K^+)$-ATPase by demonstration of the marked loss of enzyme activity which occurs when membrane preparations of this enzyme are treated with appropriate concentrations of detergents [2–4], organic solvents [5,6] or certain phospholipases [7–10].

More recently, reactivation of lipid-depleted $(Na^+ + K^+)$-ATPase by reconstitution into liposomes of defined phospholipid composition has confirmed the role of phospholipid in the restora-

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Abbreviations: DMPC, DPPC, DOPC, and DSPC; dimyristoyl-, dipalmitoyl-, dioleoyl-, and distearoylphosphatidylcholine, respectively; 1-P-2-O-PC, 1-palmitoyl-2-oleoylphosphatidylcholine.

tion of enzyme activity, although these techniques have often resulted in considerable loss of enzyme activity in comparison to the original untreated membrane preparations. Presumably this loss arises from either the denaturant action of detergents at the concentrations necessary to achieve the initial solubilization required for the prolonged cholate-dialysis type procedures [14,15], or the relatively poor incorporation of enzyme protein into preformed liposomes by sonication or other means [16,17,19], or even the direct denaturant effect of sonication on the enzyme protein during the 'freeze-thaw sonication' procedure (originally developed by Kasahara and Hinkle [20] for their studies with the glucose transport protein of erythrocytes) used by Hokin and Dixon [21] in their recent examination of the transport properties of reconstituted ($Na^+ + K^+$)-ATPase. On the other hand, hog kidney aminopeptidase [22] and the hydrophobic peptide of MN-glycoprotein from erythrocyte membranes [23] have both been successfully reconstituted into phosphatidylcholine liposomes by a detergent solubilization procedure which was followed by the rapid and effective removal of detergent by column chromatography during the formation of liposomes. It is of particular relevance to this present study that Hall and Brodbeck have reported high levels of enzyme protein incorporation into egg phosphatidylcholine liposomes and equally high levels of acetylcholinesterase activity in systems reconstituted by this general approach [24]. Because of the demonstrated superiority of the gel-filtration procedure over dialysis for the removal of detergent during membrane protein reconstitution [23], we have developed a detergent-gel filtration technique for the reconstitution of membrane ($Na^+ + K^+$)-ATPase. This procedure has been used to investigate the relationship between enzyme activation and phospholipid 'fluidity' as demonstrated by the known gel to liquid-crystalline phase transition temperatures of the synthetic phosphatidylcholines used for reconstitution. Maximum reactivation was obtained when ($Na^+ + K^+$)-ATPase was incorporated into liposomes prepared with dioleoylphosphatidylcholine.

A preliminary account of this work was presented to the Canadian Federation of Biological Sciences at Vancouver, June, 1979 [28].

Materials and Methods

Enzyme preparation

Membrane preparations enriched in ($Na^+ + K^+$)-ATPase activity were obtained from bovine cerebral cortex. The method of tissue homogenisation and differential centrifugation was similar to method (b) of Charnock et al. [25]. The enzyme enriched membrane fractions were partially delipidated by exposure to 0.1% deoxycholate in the presence of ATP as described previously [25]. The detergent extracted membranes were recovered by centrifugation in an aqueous buffer (20 mM Tris/1 mM EDTA, pH 7.6) at $46\,000 \times g$ for 30 min in a Sorvall RC2-B refrigerated centrifuge fitted with a SS-34 angle rotor. The resultant pellet was washed by resuspension and centrifugation at $46\,000 \times g$ in the same buffer. The final pellet was resuspended in small volume of Tris-EDTA containing 0.25 M sucrose to give about 2–4 mg membrane protein per ml, and could be stored at −20°C if necessary.

Assay of adenosinetriphosphatase activity

All enzyme preparations were assayed for both ouabain-sensitive and ouabain-insensitive adenosinetriphosphatase in a medium containing 80 mM Na^+ and 20 mM K^+ [25]. ($Na^+ + K^+$)-ATPase was taken as the difference in activity with and without 2 mM ouabain at 37 ± 0.2°C. Enzyme activity was determined by the coupled assay procedure of Schoner et al. [26] which we had employed in previous studies of this enzyme [25]. Protein concentrations were determined according to the method of Peterson [27] using fat-free bovine serum albumin as the standard.

Incorporation of ($Na^+ + K^+$)-ATPase into liposomes

The incorporation of adenosinetriphosphatase proteins into phospholipid vesicles was achieved by solubilization of the lipid and protein in deoxycholate, followed by the rapid removal of the detergent by passage down a column of Sephadex G-50 [28]. For the preparation of proteoliposomes containing ATPase protein, a phospholipid : membrane protein ratio of 2.2 : 1 (w/w) was used. In preliminary experiments the elution profiles of detergent was determined by isotopic tracer label-

ling with deoxy[^{14}C]cholic acid. ($Na^+ + K^+$)-ATPase was specifically labelled with [^{3}H]ouabain according to the method of Charnock et al. [25].

An aliquot of lipid (usually 10–15 mg) in $CHCl_3$ was evaporated to dryness under a stream of N_2, and further dried at 20°C for 60 min, in vacuo, by rotary evaporation in a Buchi flash evaporator. The dried lipid was rehydrated in an appropriate volume of 20 mM Tris/1 mM EDTA buffer (pH 7.6) containing 1% sodium deoxycholate, to achieve a detergent : phospholipid molar ratio of 3 : 1, and vortexed until the detergent mixture was clear. To this mixture was added a sample of partially delipidated ATPase-containing membrane preparation (2–4 mg protein/ml, labelled with ([^{3}H]ouabain), to achieve a final protein concentration of 1.0–1.5 mg/ml.

After mixing by vortex and incubation at room temperature for 5 min, the enzyme/lipid/detergent mixture was chromatographed on a column (1.5 × 50 cm) of Sephadex G-50 in 100 mM KCl/20 mM Tris/1 mM EDTA (pH 7.6), containing 0.02% (w/v) NaN_3 as a preservative. The column was eluted with this buffer at 1 ml/min and 2 ml fractions were collected. A 0.5-ml aliquot was withdrawn from each fraction and counted for both ^{14}C and ^{3}H content in 10 ml of 13.5% (v/v) toluene-dioxane fluor containing 0.5% (w/v) PPO and 10% (w/v) naphthalene. These samples were counted to 2% error in a Beckman LS-100 liquid scintillator. The void volume containing phospholipid and enzyme protein was centrifuged at 161 000 × g for 60 min in a Beckman L3-40 refrigerated ultra-centrifuge fitted with a 60Ti rotor. The resultant pellet was taken up in 2–3 ml of Tris-EDTA buffer and rechromatographed on a column (1.5 × 50 cm) of Sepharose CL-4B eluted at a flow rate of 9 ml/h with Tris-EDTA buffer containing KCl. 2-ml fractions were collected and 0.5-ml aliquots again taken for counting of ^{14}C and ^{3}H as described above.

To establish that protein incorporation into liposomes had occurred, small samples (0.2–0.5 ml) of the void volume from the Sepharose CL-4B were layered onto linear density gradients of sucrose (10 ml). These gradients were from 15–40% sucrose (w/v) in 100 mM KCl/20 mM Tris/1 mM EDTA (pH 7.6), which were prepared using a model DGM-15 Chrismac gradient-forming device. Tubes were then centrifuged at 4°C in a SW41 swing-out rotor of a Beckman L3-40 ultracentrifuge at 208 000 × g for at least 6 h. After centrifugation a long needle was introduced through each gradient and twenty 0.5-ml fractions were withdrawn with the aid of a 1 ml syringe and a three-way stopcock. These fractions were examined for [^{3}H]ouabain-labelled ($Na^+ + K^+$)-ATPase and for the presence of liposomal phospholipid by inorganic phosphate analysis by the method of Dittmer and Wells [29]. Absorbance at 230 nm also served as an additional qualitative measurement of protein and lipid.

Small aliquots of reconstituted ATPase were negatively stained with 1% phosphotungstate (pH 7.0) for examination in a JEM-7A electron microscope.

Once the complete elution profiles were established, the [*carboxyl*-^{14}C]deoxycholate and [^{3}H]ouabain tracer labels were omitted from the routine preparative procedures. Therefore, in these latter experiments ATPase-liposomes eluted from the Sepharose CL-4B column at the void volume were concentrated by centrifuging at 161 000 × g for 60 min. The resultant lipid-protein pellet was taken up in a small volume of 20 mM Tris/1 mM EDTA buffer (pH 7.6), and assayed for ouabain-sensitive and ouabain-insensitive ATPase activity [25]. This suspension could be stored at 4°C for 24 h if necessary.

In order to avoid possible thermal denaturation of the enzyme the initial reconstitution experiments were carried out at 4°C in a cold room. However, it was found that the reincorporation of ATPase protein into liposomes could be accomplished successfully at room temperature, without significantly effecting the enzyme activity. Therefore unless otherwise indicated, all of the reconstitution experiments reported here were performed at room temperature.

Lipid extraction and analysis

Enzyme preparations (both native and reconstituted) were extracted with chloroform/methanol (2 : 1, v/v) in the presence of 0.01% butylated hydroxytoluene according to the method of Bligh and Dyer [30]. Phospholipids were separated into their major classes by thin-layer chromatography (TLC) as described by Privett et al. [31]. The

discrete phospholipid spots were detected by exposure of the developed plates to iodine vapour and identified by comparison with purified standards. The spots were scraped off the plates and assayed for phosphorous according to the procedure described by Bowyer and King [32].

Chemicals

L-α-Phosphatidylcholine (Type III from egg yolk), deoxycholic acid (sodium salt), ouabain octahydrate and Sepharose CL-4B were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

The synthetic lecithins, dimyristoyl-, dipalmitoyl-, distearoyl-, and dioleoyl-L-α-phosphatidylcholines were purchased from Supelco Inc. (Bellefonte, PA, U.S.A.). 1-Palmitoyl-2-oleoyl-L-α-phosphatidylcholine was a product of Serdary Research Laboratories (London, Ontario, Canada). Sephadex G-50 was supplied by Pharmacia (Canada) Ltd., [*carboxyl*-^{14}C]deoxycholic acid sodium salt (52 mCi/mmol) was obtained from Amersham/Searle (Oakville, Ontario, Canada) and [^{3}H]ouabain (12 Ci/mmol) was obtained from New England Nuclear (Canada) Ltd. (Lachine, Quebec, Canada).

Tris-ATP was prepared from Na_2-ATP (Sigma) by exchange on a column of Dowex-50W (H^+ form, 200–400 mesh) purchased from Bio-Rad Laboratories (U.S.A.), followed by adjustment of the pH to 7.5–7.7 with Tris (Base).

All other chemicals were analytical grade and purchased from Sigma Chemical Co., or British Drug House Ltd.

Results

Preparation of ATPase-liposomes

Reconstitution of membrane-associated ($Na^+ + K^+$)-ATPase has been achieved previously by detergent solubilization of the enzyme protein in the presence of defined phospholipid. However, whereas the removal of detergent by prolonged dialysis has often taken several days [14,15] the column chromatography procedure described here could be accomplished in a few hours [23,28].

Partially delipidated preparations of membrane associated ($Na^+ + K^+$)-ATPase were tracer-labelled with 10^{-7} M [^{3}H]ouabain by the method described previously [25] and mixed with egg phosphatidylcholine and ^{14}C-labelled deoxycholate in the proportions described under Methods. Passage of this mixture through a column of Sephadex G-50 resulted in the elution profiles shown in Fig. 1. The void volume (fractions 14–19) contained only negligible deoxy[^{14}C]cholate but virtually all of the phospholipid, indicating that almost complete removal of the detergent from the lipid had occurred with one passage through the column, similar to the findings of Hall and Brodbeck [24] and Allen et al. [23]. In addition, a small portion of the [^{3}H]ouabain was found in the void volume in association with the enzyme protein profiles, compared to a large peak of free [^{3}H]ouabain which was eluted later. This profile demonstrates that ATPase protein and lipid were co-eluted from Sephadex G-50 in the void volume and were well separated from free ouabain and detergent.

After concentration by centrifugation at 161 000 × *g* for 60 min, the particulate fraction of the void volume was rechromatographed on a column of Sepharose CL-4B and the resultant fractions analysed for [^{3}H]ouabain, protein and phospholipid. The elution profile is given in Fig. 2. Phospholipid, [^{3}H]ouabain and enzyme protein co-eluted at the void column in a bimodal peak followed by a second smaller peak, presumably of free enzyme containing bound [^{3}H]ouabain.

The relatively broad distribution of protein-lipid in the void volume suggests that the ATPase-containing liposomes were heterogenous in size. This observation was later confirmed by electron microscopy (Fig. 3) which revealed that the proteoliposomes were primarily vesicular, ranging between 50 and 100 nm in diameter. This distribution is similar to that previously described by Wacker et al. [22] and Hall and Brodbeck [24] for other enzyme protein reconstitutions in phosphatidylcholine vesicles.

In order to confirm that incorporation of ATPase protein into liposomes has occurred during these relatively simple chromatographic procedures, rather than, for example, co-elution of aggregated enzyme protein and liposomes (in the void volume) containing little or no incorporated protein, the tracer-labelled proteoliposomes eluted in the void volume of the Sepharose CL-4B column were further fractionated by centrifugation

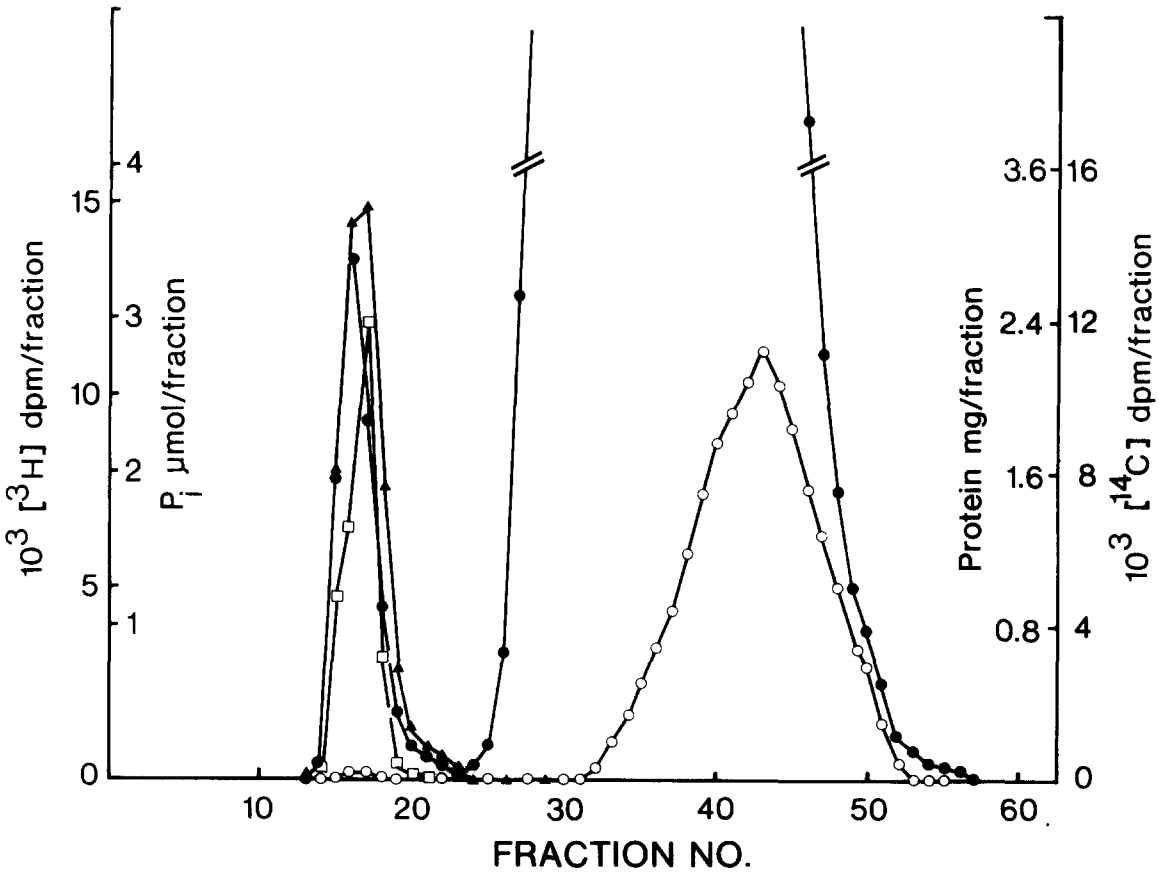

Fig. 1. Elution profile at 4°C from Sephadex G-50 column of [^{3}H]ouabain-labelled bovine brain ($Na^+ + K^+$)-ATPase added to egg phosphatidylcholine dissolved in 1% ^{14}C-labelled deoxycholate as described under Methods. The elution buffer was 100 mM KCl/20 mM Tris/1 mM EDTA/0.02% NaN_3 at pH 7.6. O——O, Deoxy[^{14}C]cholate; ●——●, [^{3}H]ouabain; ▲——▲, inorganic phosphate; □——□, protein.

on a linear gradient of sucrose. After centrifugation at 208 000 × *g* for at least 6 h, the gradients were sampled as described under Methods and the results are given in Fig. 4A. It is clear that enzyme protein eluted in the void volume of the Sepharose column is directly associated with liposomal phospholipid, and is not present coincidentally as protein aggregates. This is confirmed by the sedimentation pattern obtained from control experiments carried out in the absence of exogenous lipid in which the tracer-labelled ATPase protein appeared as protein aggregates having a markedly different sedimentation profile (Fig. 4B) than that seen when phospholipid was present.

The mean specific activity of bovine brain ATPases at various stages of incorporation into egg phosphatidylcholine liposomes is shown in Table I(A). It is clear that there is a major loss of enzyme activity in the enzyme/phospholipid/detergent mixture, presumably due to the demonstrable inhibitory action of detergents [4]. As the detergent is removed by gel-filtration there is a significant increase in enzyme activity (G-50 void volume). In these experiments with egg phosphatidylcholine as the lipid source, the reconstituted enzyme preparation recovered about 86% of the initial total ATPase activity of the membrane-associated starting material. However, during this reconstitution procedure, the specific activity of the ouabain-insensitive Mg^{2+}-ATPase component of the preparation decreased by about 50% (from 14.1 to 6.9 µmol ATP hydrolyzed per

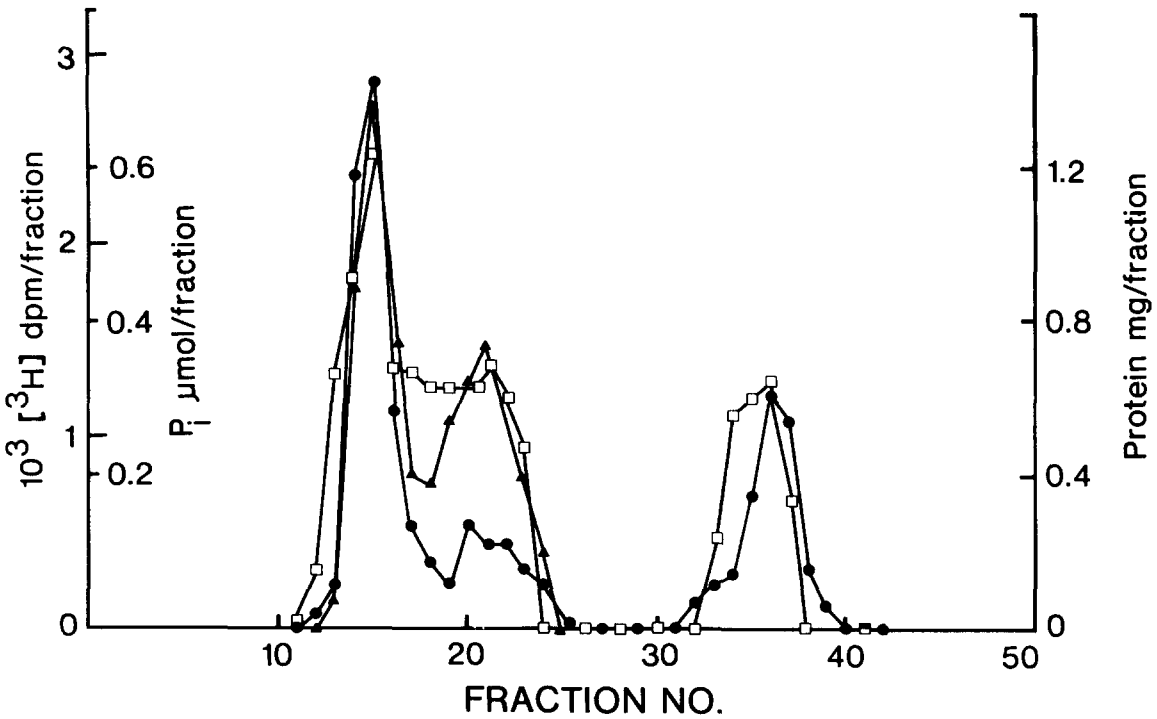

Fig. 2. Elution profile at 4°C from Sepharose CL-4B column of [^{3}H] ouabain-labelled bovine brain ($Na^+ + K^+$)-ATPase incorporated into egg phosphatidylcholine liposomes after removal of detergent by passage down Sephadex G-50. ●———●, [^{3}H]Ouabain; ▲———▲, inorganic phosphate; □———□, protein.

TABLE I

INCORPORATION OF ADENOSINETRIPHOSPHATASES INTO PHOSPHATIDYLCHOLINE LIPOSOMES BY A COLUMN CHROMATOGRAPHY TECHNIQUE

Effect on specific activity in the presence and absence of exogenous lipid. The results are based on four separate experiments all performed at 4°C. Values given are means ± S.E.; µmol hydrolyzed per mg protein per h at 37°C. DOC, deoxycholate.

	Specific activity		
	Total ATPase	Mg^{+2}-ATPase	($Na^+ + K^+$)-ATPase [a]
A. (+) Lipid			
Enzyme	114.2 ± 10.9	14.1 ± 1.5	100.1 ± 9.5
(Enzyme/Lipid/DOC) [b]	44.5 ± 5.7	6.6 ± 0.7	38.3 ± 5.2
G-50 void volume	88.7 ± 9.8	8.2 ± 0.8	80.5 ± 9.9
4-B void volume (reconstituted enzyme)	98.3 ± 5.1	6.9 ± 0.5	91.4 ± 4.6
B. (−) Lipid			
Enzyme	103.9 ± 4.7	11.0 ± 1.4	92.9 ± 3.9
(Enzyme/DOC) [c]	14.3 ± 2.4	5.3 ± 1.0	8.7 ± 1.9
G-50 void volume	7.7 ± 2.1	3.3 ± 0.3	4.4 ± 2.1
4-B void volume	6.6 ± 0.8	3.5 ± 0.7	3.1 ± 0.5

[a] ATPase inhibited by 2 mM ouabain in the presence of Na^+ and K^+.
[b] Enzyme/lipid/detergent mixture, before gel filtration on Sephadex G-50.
[c] Enzyme/detergent mixture before gel filtration on Sephadex G-50.

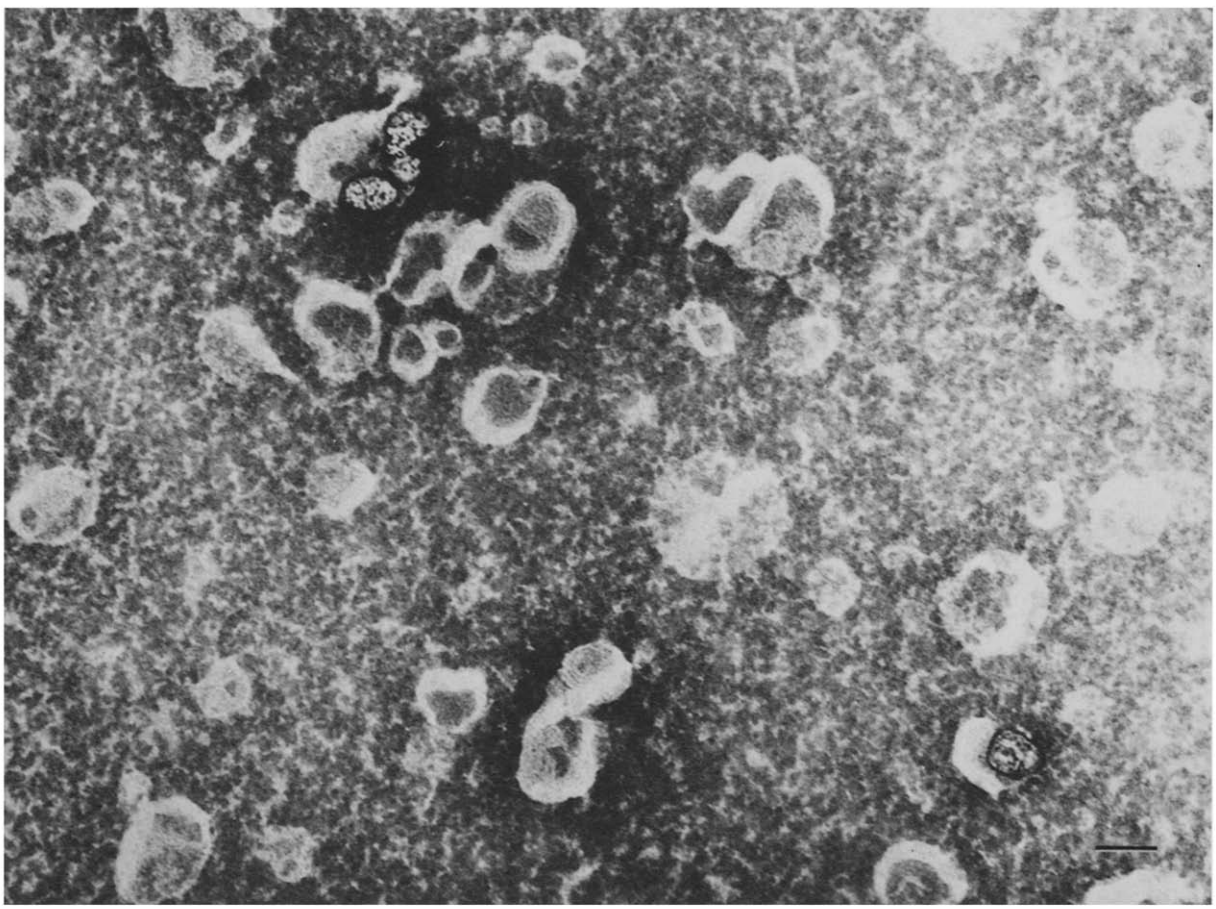

Fig. 3. Electron micrograph of bovine brain ($Na^+ + K^+$)-ATPase reconstituted into egg phosphatidylcholine liposomes of 50–100-nm diameter. Reconstituted enzyme preparation was negatively stained with 1% phosphotungstate. The bar indicates 100 nm.

mg protein per h, at 37°C). On the other hand, the loss of activity in the ouabain sensitive ($Na^+ + K^+$)-ATPase was proportionally much less as this component regained more than 90% of the initial enzyme activity, thus suggesting that some selective reactivation had occurred.

In marked contrast to the activity of the ATPase enzymes reconstituted into egg phosphatidylcholine liposomes, the absence of exogeneous lipid during the chromatographic procedures leads to near total loss of activity for both the ouabain-sensitive and ouabain-insensitive components (Table 1B). Under these latter conditions, activity is not restored by the removal of detergent by column chromatography.

The major lipid classes of the membrane ATPase preparations before and after reconstitution into liposomes of either DSPC or egg phosphatidylcholine are shown in Table II. It is clear from these results that about 70% of the endogenous lipid of the partially purified enzyme preparation was replaced by exogenous lipid after a single exposure of the preparation to the lipid/detergent/chromatography procedure. The level of phospholipid exchange was increased to about 90% by repeated (three cycles) exposure of the enzyme to the lipid/detergent/Sephadex chromatography procedure prior to final chromatography on Sepharose CL-4B. After this repeated (three cycle) procedure, the minor phospholipids (sphingomyelin, phosphatidylserine and phosphatidylinositol) were significantly reduced (to 37% of their initial

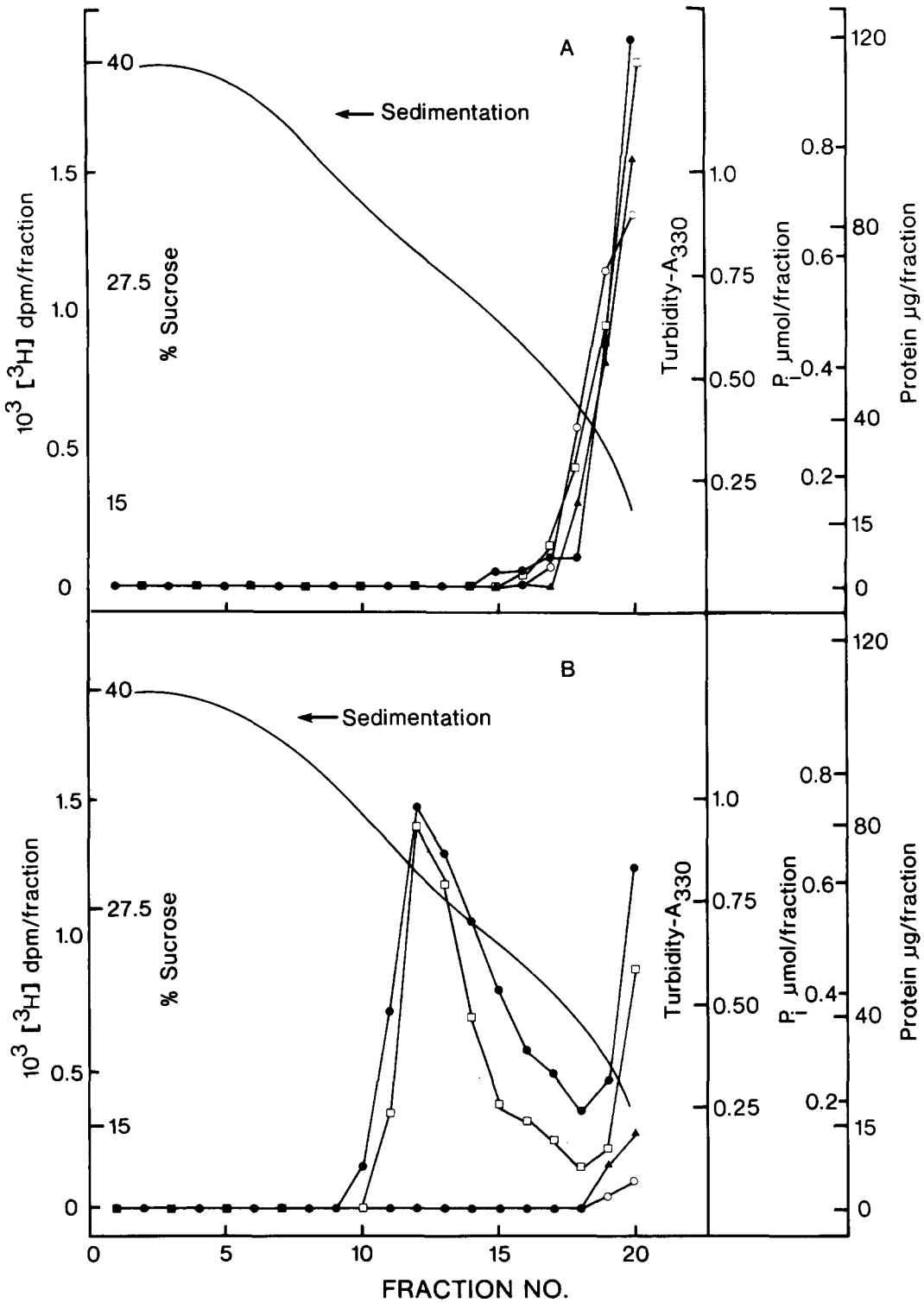

Fig. 4. Sedimentation profile of bovine brain ($Na^+ + K^+$)-ATPase incorporated into egg phosphatidylcholine liposomes after sequential passage down columns of Sephadex G-50 and Sepharose CL-4B. The void volume was layered over linear sucrose gradients as described under Methods. Centrifuged at 298000 × *g* 6 h at 4°C. Panel (A) in the presence of exogenous phospholipid, panel (B) in the absence of exogenous lipid. ———, Sucrose; ○———○, turbidity A_{230}; ●———●, [^{3}H]ouabain; ▲———▲, inorganic phosphate; □———□, protein.

TABLE II

COMPARISON OF THE MEMBRANE LIPID COMPOSITION OF ATPase PREPARATION BEFORE AND AFTER RECONSTITUTION INTO PHOSPHATIDYLCHOLINE LIPOSOMES

Values given are means ± S.E.; $n = 4$. Serial refers to three separate cycles of the lipid, detergent, chromatography procedure. PC, PE, and PI: phosphatidyl-choline, -ethanolamine, and -inositol, respectively. Sph, sphingomyelin.

	Mol%			PE/PC ratio
	PE	PC	Sph + PS + PI	
Enzyme (native)	39.6 ± 1.8	41.9 ± 1.7	18.4 ± 0.8	0.96 ± 0.08
E/DSPC (single)	22.1 ± 1.6	64.4 ± 2.6	13.4 ± 1.1	0.34 ± 0.03
E/egg PC (single)	20.2 ± 0.6	64.6 ± 2.3	15.1 ± 1.8	0.31 ± 0.02
E/egg PC (serial)	10.0 ± 0.8	84.0 ± 1.6	6.9 ± 0.4	0.12 ± 0.01

level) as well as that of phosphatidylethanolamine which was now reduced to 25% of its initial level. Serial reconstitution into egg phosphatidylcholine raised the level of this major phospholipid from 42% to 84% of the total phospholipids. Increased exchange occurs with further cycles of substitution, but appreciable losses of enzyme activity occur [55]. It is thus clear that this reconstitution procedure results in significant exchange of residual (endogenous) membrane lipids by exogenous lipid.

Although several earlier studies has suggested that the charge of the phospholipid headgroup effected the reactivation of $(Na^+ + K^+)$-ATPAse [11–13] recent work has also implicated the phospholipid fatty acid acyl-chain length as a contributing factor in enzyme reactivation. In Table III, we report the results of comparative experiments employing a series of synthetic phosphatidylcholines having defined fatty acid composition. As each experiment was compared to its own control of partially delipidated enzyme prior to reconstitu-

TABLE III

EFFECT OF ACYL CHAIN LENGTH AND SATURATION UPON ADENOSINETRIPHOSPHATASE ACTIVITY RECONSTITUTED INTO PHOSPHATIDYLCHOLINE LIPOSOMES

Conditions	Phospholipid acyl chain composition	Thermal Transition T_c(°C) [a]	Specific activity [b]		
			Total ATPase	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase
Control Membrane	–	–	195 ± 8	27 ± 2	168 ± 8
Enzyme/DMPC	14:0/14:0	+23	143 ± 4	29 ± 2	114 ± 4
Control membrane	–	–	174 ± 7	21 ± 1	153 ± 8
Enzyme/DPPC	16:0/16:0	+41	73 ± 4	19 ± 1	54 ± 3
Control membrane	–	–	181 ± 8	21 ± 2	160 ± 8
Enzyme/DSPC	18:0/18:0	+58	50 ± 1	17 ± 2	33 ± 2
Control membrane	–	–	150 ± 20	21 ± 3	129 ± 17
Enzyme/1-P-2-O-PC	16:0/18:1	−2.6	103 ± 4	18 ± 1	85 ± 4
Control membrane	–	–	145 ± 18	22 ± 2	123 ± 17
Enzyme/DOPC	18:1/18:1	−22	168 ± 14	24 ± 1	140 ± 14

[a] Full literature citations for the thermal transition T_c(°C) of the synthetic phospholipids are given in Refs. 47–53 to this paper.

[b] Results based on at least four separate reconstitution experiments. Values given are means ± S.E.; μmol ATP hydrolysed per mg protein per h at 37°C.

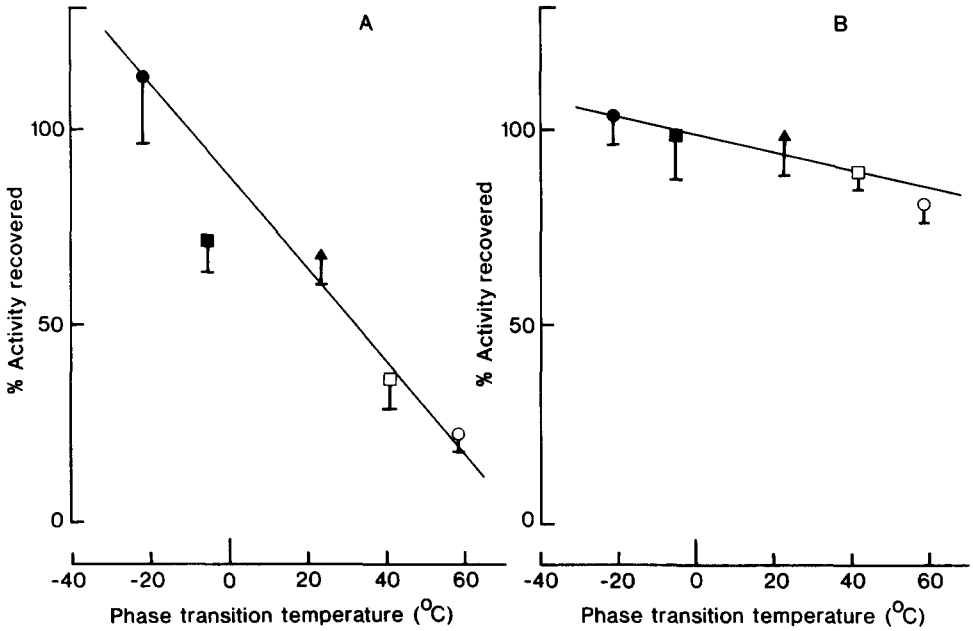

Fig. 5. Relationship between the specific activity of reconstituted bovine brain adenosinetriphosphatases and the thermotropic phase transition temperature of various synthetic phosphatidylcholine liposomes ($r^2 = 0.89$). Panel (A), $(Na^+ + K^+)$-ATPase; panel (B), Mg^{2+}-ATPase. ●, dioleoylphosphatidylcholine; ■, 1-palmitoyl-2-oleoylphosphatidylcholine; ▲, dimyristoylphosphatidylcholine; □, dipalmitoylphosphatidylcholine; ○, distearoylphosphatidylcholine.

tion, it is possible to compare mean activities (as % of control) between the various phospholipids employed. An inverse relationship between enzyme reactivation and fatty acyl chain length can be seen by comparison of the results of reactivation in dimyristoylphosphatidylcholine (70%), dipalmitoylphosphatidylcholine (35%) and distearoylphosphatidylcholine (20%). From this it seems that increasing the fatty acyl chain length of saturated phosphatidylcholines decreased the ability of the lipid to reactivate the $(Na^+ + K^+)$-ATPase, but had little effect upon ouabain-insensitive Mg^{2+}-ATPase which was apparently independent of the lipids involved. By comparison, the presence of a single unsaturated fatty acyl chain, as in 1-palmitoyl-2-oleoyl-phosphatidylcholine (16 : 0/18 : 1), greatly enhanced the degree of reactivation obtained. This effect was even greater when dioleoylphosphatidylcholine (18 : 1/18 : 1) was employed. In the presence of this di-*cis*-unsaturated phosphatidylcholine, complete reactivation of enzyme activity of both ouabain-sensitive $(Na^+ + K^+)$-ATPase and ouabain-insensitive Mg^{2+}-ATPase was achieved.

A further and more definite relationship could be found when the data were considered in terms of the physical properties of the lipid bilayer employed, as expressed by the phase transition temperatures of the pure phospholipids. Values for the transition temperatures with the appropriate literature sources [47–53] are also cited in Table III. Thus, it can be seen that the reactivation of $(Na^+ + K^+)$-ATPase is inversely proportional to the gel to liquid-crystalline transition temperature of the phospholipid, in that the activity declines apparently linearly from a maximum value at a transition temperature of −22°C (DOPC) to a minimum at +58°C (DSPC). A similar but much less marked effect is seen with Mg^{2+}-ATPase where the decrease in reactivation with distearoylphosphatidylcholine is only about 20% less that obtained with dioleoylphosphatidylcholine. These relationships are shown graphically in Figs. 5A and B, respectively.

Discussion

Currently there are a number of different experimental procedures available by which enzyme proteins can be incorporated into lipid vesicles. Preferably, the ideal method for the reconstitution of biological activity should allow the incorporation of enzyme protein into unilamellar vesicles without significant loss of catalytic activity or other damage to the molecular structure of the enzyme. The denaturant effect of the direct sonication process used for this purpose [16,33] severely limits the use of this method for the reconstitution of susceptible membrane-associated enzyme systems. Similarly, the inhibitory action of the relatively high concentrations of detergent that are required for enzyme solubilization, when coupled to the length of time necessary for the subsequent removal of the detergent by prolonged dialysis procedures [14,15], become limiting factors in the application of this latter procedure.

It is of interest to note that whether DSPC or egg phosphatidylcholine was employed after a single exposure to the lipid/detergent mixture used in the reconstituted procedure described in this paper, the exchange of exogenous phospholipid was mainly at the expense of phosphatidylethanolamine, while the quantiatively minor phospholipid classes (sphingomyelin, phosphatidylserine and phosphatidylinositol) were not exchanged to the same extent. Apparently these latter phospholipids are more tightly associated with the enzyme protein. Certainly in the case of the ($Na^+ + K^+$)-ATPase of red blood cell membranes, the earlier studies of Roelofsen and Van Deenen [10] strongly suggest a special relationship between membrane phosphatidylserine and ($Na^+ + K^+$)-ATPase activity. In the present study the phospholipid fraction containing phosphatidylserine only undergoes significant exchange after the repeated exposure of the enzyme preparation to the lipid/detergent mixture, at which time there is a marked decrease in enzyme activity [54,55]; an observation which is consistent with the conclusion of Roelofsen and Van Deenen [10], but which would require quantitative separation of the phosphatidylserine component to establish unequivocal confirmation.

The single cycle gel filtration reconstitution procedure described here for bovine brain ($Na^+ + K^+$)-ATPase appears to be both rapid and efficient in that there was only a 10% loss of specific activity of the ($Na^+ + K^+$)-ATPase when the enzyme preparation was reconstituted into egg phosphatidylcholine liposomes. Even higher enzyme activities were obtained with synthetic dioleoylphosphatidylcholine.

From the results obtained with the series of synthetic phosphatidylcholines of defined fatty acid composition, it seems that the increasing loss of activity observed with dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine can be attributed to the increasing length of the saturated fatty acyl-chains of these phosphatidylcholines which is accompanied by decreasing membrane fluidity [56]. Increasing membrane fluidity through the use of phospholipids in which one or both acyl chains contain unsaturated double bonds, greatly increased the reactivation of the enzyme as shown by our substantially increased reactivation with 1-palmitoyl-2-oleoylphosphatidylcholine (16 : 0/18 : 1) and complete reactivation with dioleoylphosphatidylcholine (18 : 1/18 : 1). Thus it seems that the length and degree of unsaturation of the phospholipid fatty acid acyl chains of the membrane must also play a role in the activity of membrane ($Na^+ + K^+$)-ATPase, in addition to the influence of the phospholipid headgroups demonstrated by other workers [11–13].

Evidence that membrane receptors, enzymes and transport proteins are affected by membrane 'fluidity' has also been reported by many other workers [34–37]. Confirmation that the physical properties of the fatty acyl components of the phospholipids are directly involved in the activation of ($Na^+ + K^+$)-ATPase is suggested by the apparently linear relationship between enzyme activity and the reported gel to liquid-crystalline phase transitions of the phospholipids we employed.

It should be recalled that Chapman [38] has demonstrated that the 'fluidity' of biological membrane is a direct reflection of the transition temperature of the lipids. Thus, at the temperature of these enzyme assays (37°C) there would be a progressive decrease in lipid 'fluidity' from dioleoylphosphatidylcholine to distearoylphosphatidylcholine, according to the ranking of their gel to

liquid-crystalline phase transitions [56].

Dipalmitoyl- and distearoylphosphatidylcholine are both below their phase transition at 37°C, but dipalmitoylphosphatidylcholine shows a greater ability to reactivate the enzyme than does distearoylphosphatidylcholine. The explanation may lie in the presence of residual endogenous phospholipids associated with the enzyme. Because at 37°C, dipalmitoylphosphatidylcholine (T_c, + 41°C) is only slightly below its phase transition mixing of the bulk lipid with residual endogenous lipid may have a small fluidizing effect on the bulk lipid, or allow for some phase separation into regions of greater and less fluidity (e.g. in the immediate vicinity of the enzyme protein). Because at 37°C, distearoylphosphatidylcholine (T_c, + 58°C) is much further below its phase transition, presumably this type of process would be acting to a lesser extent. Support for this suggestion is given by our recent findings that serial (three cycles) detergent solubilization of the enzyme (in the presence of exogenous lipid) resulted in almost total elimination of enzyme activity for both dipalmitoyl- and distearoylphosphatidylcholine [54,55]. Following three cycle serial exchange the level of endogenous lipid was reduced to 10% of the original level; further reduction occurs with further cycles of exchange but serious loss of enzyme activity now occurs [54].

Our results thus confirm the recent findings of Sinensky et al. [39] who used the calculated index of order parameter obtained by electron spin probe studies of cultured cells of hamster ovary, to conclude that fluidity via acyl-chain ordering of the membrane phospholipids has a direct effect upon the rate of $(Na^+ + K^+)$-ATPase catalysis. These authors suggest that this effect arises from the need for a complementary change in the ordering of the surrounding hydrocarbon array of the membrane lipids when the enzyme protein undergoes the required conformational change for catalysis or transport. Silvius and McElhaney [40] have suggested a similar explanation for their observation that the $(Na^+ + Mg^{2+})$-ATPase of *Acholeplasma laidlawii* B is active only in association with liquid-crystalline lipids. The sharp increase in Arrhenius activation energy (E_A) of membrane preparations of $(Na^+ + K^+)$-ATPase at approx. 20°C has been related by several authors to changes in membrane physical parameters at this temperature [11,17,41–44].

In contrast to the $(Na^+ + K^+)$-ATPase system, membrane-associated Mg^{2+}-ATPase appears to be much less sensitive to changes in lipid fluidity; a conclusion in agreement with an earlier study of Bloj et al. [45] who could not find any relationship between Mg^{2+}-ATPase activity and the double bond index/saturation ratio of erythrocyte fatty acids in rats fed different natural fats or oil supplements. Presumably this lack of effect of membrane lipids also accounts for the lack of effect of the local anaesthetic benzyl alcohol upon this enzyme [46].

The lipid depletion-gel filtration method described here for the preparation of reconstituted $(Na^+ + K^+)$-ATPase should provide an additional tool for the study of the effect of lipid-protein interactions upon this enzyme, and be particularly useful for the elucidation of its cardiac-glycoside receptor properties [55].

Acknowledgements

We wish to thank the Medical Research Council of Canada for grants-in-aid (MA-4945 and MA-6489) and a studentship to M.Y.A. The helpful discussion of the manuscript by our many colleagues is gratefully acknowledged.

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