Biochimica et Biophysica Acta, 323 (1973) 547–559

BBA 76479

A NEW METHOD FOR MAKING PHOSPHOLIPID VESICLES AND THE PARTIAL RECONSTITUTION OF THE (Na^+,K^+)-ACTIVATED ATPase†

J. R. SLACK*, B. H. ANDERTON** and W. A. DAY

Medical Research Council Neurobiology Unit, Department of Biophysics, King's College London, 26/29 Drury Lane, London (Great Britain)

(Received June 4th, 1973)

SUMMARY

It has been found that vesicles of phospholipid (96% (w/w) phosphatidylcholine: 4% (w/w) phosphatidylserine) can be formed by dialysis of a solution of the phospholipid in the detergent, sodium deoxycholate. Depending upon the composition of the dialysis medium, small closed vesicles apparently bounded by one or two membranes or large multi-walled structures are produced. The former are predominant if only univalent ions are present in the dialysis buffer. As the Mg^{2+} concentration is raised above about 0.1 mM multiwalled structures are found.

The (Na^+,K^+)-ATPase (EC 3.6.1.3) from cattle brain microsomes has been solubilized with deoxycholate. Dialysis of this material after the addition of the above phospholipid mixture in detergent also produces membrane-bound vesicles. Sucrose density gradient centrifugation has been used to demonstrate that the phospholipid, (Na^+,K^+)-ATPase and protein reaggregate together only if the phospholipid and solubilized protein are mixed before dialysis. This method of forming artificial membranes may be a useful way of studying transport proteins in isolation as the vesicles appear to be small and closed.

INTRODUCTION

An important problem in the study of cell membranes is that of developing methods for the isolation, purification and reconstitution of the membrane components. If this can be achieved, then the particular molecules responsible for specific membrane functions may be studied in simple, chemically well-defined lipid membranes. Such preparations would clearly be useful in the elucidation of molecular mechanisms of the membrane functions. This paper describes an attempt to develop a method of reconstitution, applicable to membrane proteins.

The (Na^+,K^+)-ATPase was chosen for this work because of the fairly well-established association of the enzyme with active Na^+ and K^+ transport across cell

* Present address: Institute of Physiology, University of Glasgow, Glasgow G12 8QQ, Great Britain.

** Present address: Department of Life Sciences, The Polytechnic of Central London, London, W.1, Great Britain.

† A preliminary account of this work was presented at the 8th meeting of the Federation of European Biochemical Societies[10].

membranes[1]. In addition, the enzymatic activity provides an important monitor for the enzyme in free solution, whereas the transport activity can only be assayed using intact membranes.

In order to isolate the (Na^+,K^+)-ATPase from the membrane matrix we used the detergent sodium deoxycholate as a solubilizing agent. A detergent was chosen in preference to an organic solvent[2,3], because of the large number of reports[4–9], describing the preservation of binding or enzymic activity in membrane proteins after solubilization in detergent. Reaggregation of the protein with phospholipid was achieved by removing the detergent by dialysis against detergent-free buffer. Depending upon the composition of the dialysis buffer used, the resulting material could be made to contain what appeared to be small membrane-bound vesicles.

MATERIALS

Disodium ATP, phosphoenolpyruvate tricyclohexylammonium salt, pig muscle lactate dehydrogenase (EC 1.1.1.27) and rabbit muscle pyruvate kinase (EC 2.7.1.40) were purchased from Boehringer, Mannheim. Deoxycholic acid, maleic acid, ouabain and Trizma base were from Sigma. Egg lecithin grade 1 and bovine brain monosodium phosphatidylserine grade 1 were from Lipid Products. Human serum albumin grade B was from Calbiochem. All other chemicals were analytical grade.

Water was distilled twice in an all-glass apparatus.

Tris–maleate buffers were prepared with calculated weight of Trizma base to give the stated concentration and then titrated to the desired pH with maleic acid.

Dialysis tubing was always boiled twice in 1 mM EDTA, pH 7.0, and then washed with distilled water.

Purification of deoxycholic acid

100 g of deoxycholic acid were dissolved in 500 ml of boiling redistilled methanol. 5 g of activated charcoal were added and mixed for 5 min. The charcoal was then removed by filtration. 100 mM EDTA, pH 7.0, was added to give a final concentration of 0.1 mM and the deoxychloic acid precipitated by the addition of 500 ml of distilled water containing 0.1 mM EDTA, pH 7.0. After standing at 6 °C overnight, the white precipitate was filtered off and dried in a desiccator.

Preparation of sodium deoxycholate solutions

Deoxycholic acid was dissolved in distilled water by titration with concentrated NaOH solution until all the solid was dissolved and the pH 7.6. The solution was then made up to volume to give a final concentration of deoxycholate of 50 mg/ml. Other deoxycholate solutions were prepared by the appropriate dilution with distilled water.

METHODS

ATPase assay

ATPase activity was measured at 37 °C in approx. 100 mM Tris–maleate buffer, pH 7.40, (at 37 °C) by coupling the production of ADP to NADH reduction with pyruvate kinase and lactate dehydrogenase. Initial rate determinations of the hydro-

lysis of ATP could then be followed by the decrease in absorption at 340 nm in the Pye–Unicam SP 800 recording spectrophotometer. The assay volume was 1.00 ml containing 2 mM ATP, 1.5 mM phosphoenolpyruvate, 0.23 mM NADH, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 4.5 units* of lactate dehydrogenase, 3.75 units* of pyruvate kinase. This gave approx. 6.5 mM $(NH_4)_2SO_4$ from the stock $(NH_4)_2SO_4$ suspensions of pyruvate kinase and lactate dehydrogenase. All assays were allowed to proceed until a sufficiently long linear trace was obtained and then 25 μl of ouabain were added to give a final concentration of 0.1 mM, and the assay allowed to proceed. Ouabain-sensitive (Na^+, K^+)-ATPase was then taken as the difference between the slopes of the two traces, the dilution being virtually insignificant.

Preparation of phospholipid vesicles

All phospholipid consisted of a mixture of 96% (w/w) phosphatidylcholine and 4% (w/w) phosphatidylserine. Appropriate volumes of these were pipetted into a round-bottom flask and dried down by rotary evaporation. The dry phospholipid mixture (usually a minimum of 50 mg total phospholipid) was then dissolved under a N_2 atmosphere in 50 mg/ml deoxycholate solution which had been filtered through an 0.45 μm Millipore filter just prior to use. A ratio of deoxycholate to total phospholipid of 2.5:1 (w/w) was used routinely. The phospholipid solution was then diluted with distilled water to a final deoxycholate concentration of 1.14 mg/ml. Centrifugation of this phospholipid solution at $250000 \times g$ for 3 h failed to sediment any material and after some initial experiments centrifugation was omitted. This solution was then dialysed against five batches, each of 5 l of the desired dialysis buffer. All dialysis buffers contained 5 mM Tris–maleate, pH 7.0. Dialysis was usually carried out for a total of about 70 h at 6 °C. N_2 was bubbled through the dialysis solution continuously. After the first change of dialysis buffer a slight turbidity appeared inside the dialysis bag.

The precipitate was centrifuged down at $250000 \times g$ in the Beckman 60Ti rotor for varying times between 50 min and 3 h. In order to obtain a well-packed pellet it was necessary to centrifuge for 3 h with preparations of the smallest and single-walled vesicles. These small vesicles gave pellets which were completely translucent and this proved to be a useful criterion for a successful preparation of small vesicles. White opaque pellets were obtained with preparations from some dialysis conditions. When examined by electron microscopy these opaque pellets appeared to consist of large multi-walled structures.

Preparation of microsomes

The cortex from 8–12 cattle brains was removed and homogenized cold in a Waring blender as a 20% (w/v) homogenate in 0.32 M sucrose, 1 mM EDTA, pH 7.0. The homogenate was centrifuged for 15 min at $10000 \times g$ in the 6 × 250-ml rotor of the MSE angle 18 centrifuge. The supernatant was retained and the pellets resuspended to their original volume in fresh sucrose solution. The suspension was recentrifuged for 15 min at $10000 \times g$ and the supernatant was combined with the first. The total supernatant was then centrifuged for 3 h at $22000 \times g$ and the resultant pellets resuspended to a final protein concentration of about 25 mg/ml in 1 mM

* 1 unit of lactate dehydrogenase or pyruvate kinase is that amount of enzyme which catalyzes the conversion of 1 μmole of substrate in 1 min at 25 °C.

EDTA, pH 7.0. This microsomal fraction was divided into 5-ml aliquots and stored at −20 °C for subsequent use as the source of (Na^+, K^+)-ATPase.

Solubilization of (Na^+, K^+)-ATPase

A curve showing the amount of ATPase activity solubilized at increasing deoxycholate concentrations was determined for each fresh preparation of microsomes, *e.g.* Fig. 1. It was thus possible to ascertain the optimum conditions for solubilization. With the microsome preparation used for the profile shown in Fig. 1, a final protein concentration of 5 mg/ml and deoxycholate concentration of 3.5 mg/ml were used routinely.

Frozen microsomes were thawed as required, diluted with distilled water to a protein concentration of 10 mg/ml and homogenized. An equal volume of 7.0 mg/ml deoxycholate solution was slowly added with stirring. The mixture was stirred in an ice bath for 25 min and then centrifuged for 1 h at 250000 × *g* in either the Beckman 60Ti or Type 65 rotors. The supernatant was poured off, taking care to prevent any of the loose particulate matter from leaving the centrifuge tube. This supernatant fraction will be referred to as solubilized ATPase.

Preparation of phospholipid vesicles containing (Na^+,K^+)-ATPase

Solubilized (Na^+,K^+)-ATPase was diluted with distilled water to a final deoxycholate concentration of 1.5 mg/ml and mixed with the appropriate amount of 96% (w/w) phosphatidylcholine: 4% phosphatidylserine also in solution in deoxycholate. Prior to mixing, the phospholipid had been dissolved in 50 mg/ml deoxycholate and subsequently diluted to 1.5 mg/ml deoxycholate with distilled water. The mixture was then dialysed in the usual manner. After dialysis, centrifugation at 250000 × *g* for 50 min was sufficient to give well-packed pellets of membranes. The material will be referred to as ATPase reaggregated after the addition of phospholipid. ATPase without the addition of phospholipid was prepared in the same manner,

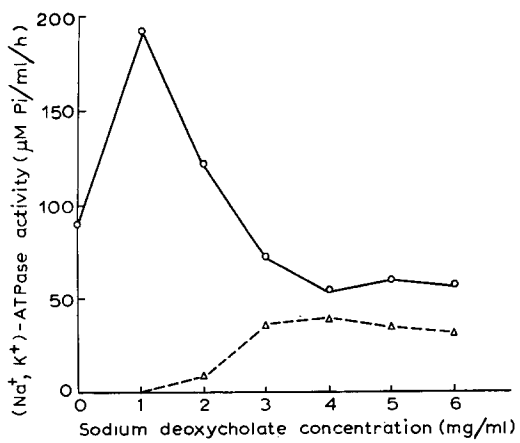

Fig. 1. Deoxycholate solubilization profile. Thawed microsomal suspension was mixed with the appropriate sodium deoxycholate solution to give a final protein concentration of 5 mg/ml. The final pH was about 7.4, no buffer was present. ○—○, total (Na^+, K^+)-ATPase; △—△, solubilized (Na^+, K^+)-ATPase.

omitting the addition of phospholipid. The solubilized ATPase was diluted to a deoxycholate concentration of 1.5 mg/ml before dialysis.

Sucrose density centrifugation

Sucrose density gradients were preformed in tubes for the Beckman SW27.2 rotor and after loading, samples were centrifuged for 65 h at 25000 rev./min. Gradients of 16 ml ran from zero sucrose to either 0.5 or 1.5 M sucrose, underlaid with a cushion of 1.8 M sucrose. Sucrose solutions were made up either in dialysis buffer or 0.5 M NaCl, 5 mM Tris–maleate, pH 7.0.

After centrifugation, fractions were collected by pumping from the bottom of each tube and analysed for ATPase activity, protein, total phosphorus and sucrose.

Protein was assayed by the method of Lowry *et al.*[11] with human serum albumin as the standard. Total phosphorus was assayed by the method of McClare[12] assuming that all phosphorus present was from phospholipid.

Electron microscopy

Phospholipid vesicles were viewed under negative stain. They were diluted in dialysis buffer and incubated at room temperature for 15 min in 0.5% $(NH_4)_2MoO_4$, made isotonic with ammonium acetate and adjusted to pH 7.0. Collodion-coated grids were floated on to the surface of a drop of the vesicle suspension for 3–4 min, excess fluid was removed, and the grids allowed to dry. Grids were viewed in an AEI EM6B electron microscope.

ATPase-containing vesicles were either viewed under negative stain or fixed in 2% glutaraldehyde, (buffered with isotonic cacodylate, pH 7.0) pelleted at $250000 \times g$ as described, resuspended in 2% agar, post-fixed in buffered 1% OsO_4, dehydrated in acetone series and embedded in araldite. Thin sections cut on a Cambridge ultramicrotome were mounted on collodion-coated grids and stained with uranyl acetate and lead citrate.

RESULTS

Formation of phospholipid membranes

Electron microscopic examination revealed that phospholipid vesicles formed by dialysis against buffers containing no divalent metal ions appear to be small, of uniform size distribution (500–1000 Å diameter) and bounded by either one or two membranes each about 50 Å across (Fig. 2). Johnson *et al.*[13] have reasoned that the double-walled appearance of negatively-stained vesicles prepared by sonication of phospholipid is, in fact, an artifact of electron microscopy. They suggest that the vesicles are single-walled and collapse on drying down on the electron microscope grid, giving rise to the apparently double-walled structure. As the morphology of our preparations is similar to that described by Johnson *et al.*[13] we suggest that vesicles prepared by dialysis in the absence of divalent metal ions are predominantly single walled. We found similar vesicles in all preparations (over 50), in which the salt (NaCl or KCl) concentration was in the range of 25–200 mM. There was often a small proportion of the material present as large multi-walled structures. Inclusion into the dialysis buffers of either Mg^{2+} or Ca^{2+} at concentrations greater than approx. 0.1 mM caused the formation of larger multi-walled structures with a wide

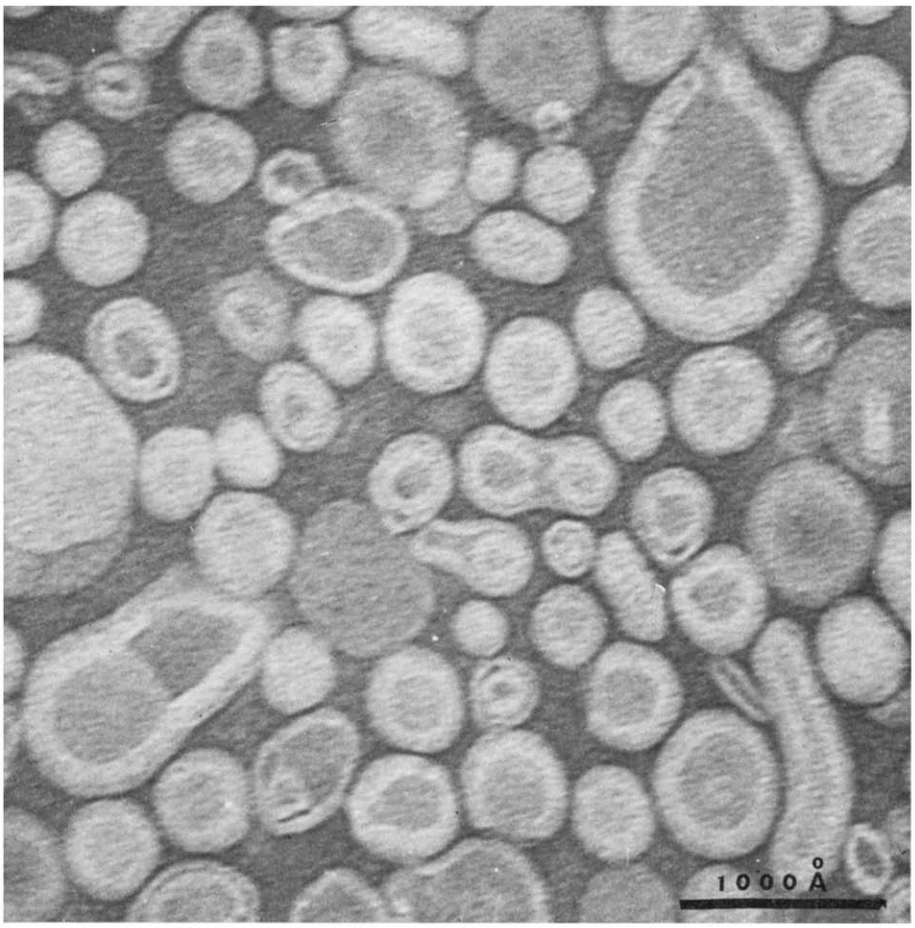

Fig. 2. Electron micrograph of negatively-stained pure phospholipid vesicles formed by dialysis in the absence of Mg^{2+}. Prepared by dialysis against 50 mM NaCl, 50 mM KCl, 5 mM Tris–maleate buffer, pH 7.0.

size distribution (Fig. 3). A concentration of less than 0.1 mM did not seem to produce this effect. Such large multi-walled vesicles were also found after dialysis against buffer containing 500 mM NaCl.

Reincorporation of (Na^+,K^+)*-ATPase into phospholipid vesicles*

Table I presents the recoveries at different stages of (Na^+,K^+)-ATPase, protein and phospholipid from a representative preparation of ATPase-containing vesicles. Comparable results were found in all our experiments (more than 30). About 40% of the initial (Na^+,K^+)-ATPase activity present in the microsomes was solubilized under the conditions described. This is borne out by Fig. 1. It can be seen that major losses of enzymic activity occurred on addition of the phospholipid solution to the solubilized (Na^+,K^+)-ATPase and also upon concentration of the dialysed material by centrifugation. The loss of activity when the phospholipid was

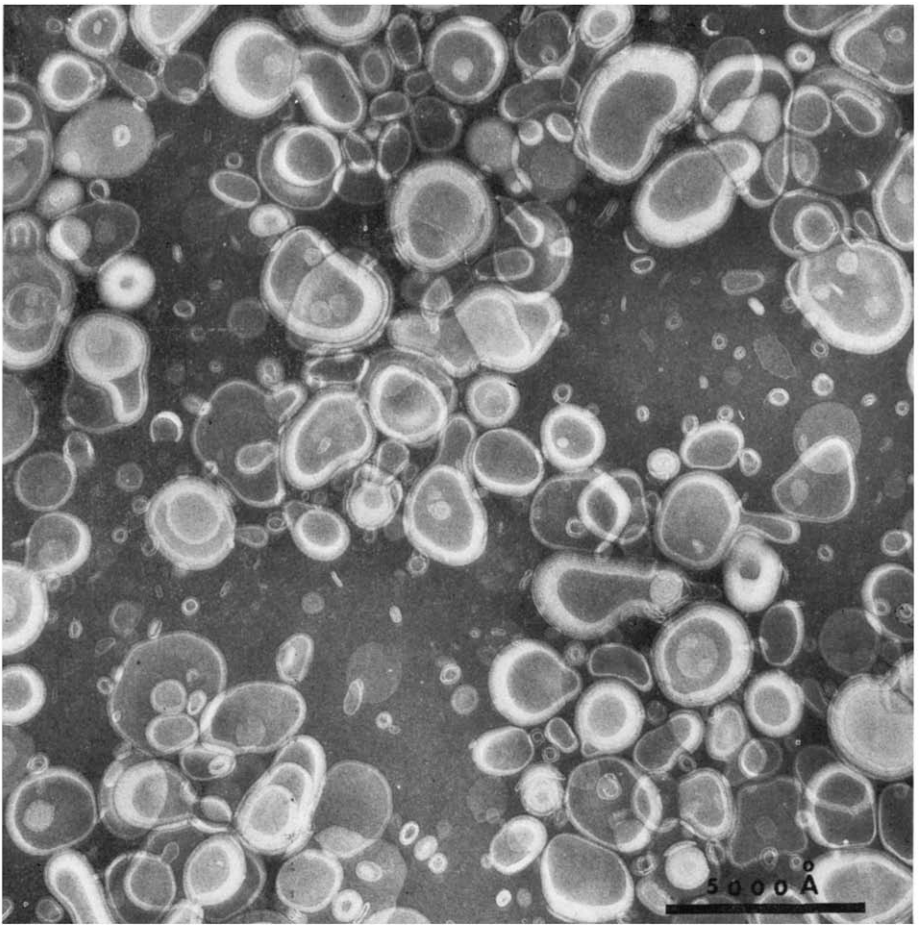

Fig. 3. Electron micrograph of negatively-stained pure phospholipid vesicles formed by dialysis in the presence of Mg^{2+}. Prepared by dialysis against 100 mM NaCl, 100 mM KCl, 2 mM $MgCl_2$, 5 mM Tris–maleate buffer, pH 7.0.

added may be due to the increase in the deoxycholate to ATPase concentration ratio. We cannot yet account for the observed loss of activity on centrifuging the reconstituted membranes. ATPase activity was not detected in the supernatant after centrifugation, nor was there any recovery if the pellet was resuspended and diluted in supernatant fluid before assaying. Increasing the centrifugation time did not improve the yield of (Na^+,K^+)-ATPase. The difficulty of quantitatively resuspending 6 or 7 pellets in a final volume of 1 ml (last column of Table I) resulted in some losses of material, and will account for some of the variation between Columns 3 and 4. This was unavoidable as it was necessary to have very concentrated preparations for subsequent experiments with the material. We have found that the recovery of solubilized (Na^+,K^+)-ATPase in the final resuspended pellet varied from about 1 to 8%. It should be pointed out that the protein concentration in the diluted material before and after dialysis was too low to be assayed with any confidence.

TABLE I

TABLE SHOWING THE RECOVERY OF LIPID PHOSPHORUS, PROTEIN AND (Na^+, K^+)-ATPase ACTIVITY DURING THE PREPARATION OF SOLUBILIZED AND DIALYSED MATERIAL

Dialysis buffer was 60 mM NaCl, 100 mM KCl and 5 mM Tris–maleate buffer, pH 7.0.

	Deoxycholate-solubilized ATPase	*Solubilized ATPase plus added phospholipid before dialysis*	*Reformed membranes before concentration by centrifugation*	*Resuspended pellet of concentrated reformed membranes*
Volume (ml)	8	180	175	1
ATPase activity (*minus* ouabain)	26.5	0.2	0.325	12
(μmoles P_i formed h/ml) (*plus* ouabain)	4.0	0.125	0.175	5
ouabain sensitive	22.5	0.075	0.15	7
Total units ouabain sensitive μmoles P_i formed/h	180	13.5	26	7
Recovery of ouabain-sensitive ATPase (%)	100	7.5	14	4
Protein concentration (mg/ml)	2.8	0.1	0.2	7.7
Total protein (mg)	22.4	25	30	7.7
Recovery of protein (%)	100	100	100	35
ATPase specific activity (μmoles P_i formed/h per mg)	8	0.5	0.9	0.9
Phospholipid concentration (mg/ml)	1.6	0.64	0.54	47
Total phospholipid (mg)	13	115	94	47
Recovery of phospholipid (%)	—	100	82	41
Percent (w/w) of phospholipid in fraction	37	82	76	86

Dialysis of solubilized ATPase without prior addition of extra phospholipid resulted in a precipitate which in thin sections of fixed and embedded material had a membrane-like structure (Fig. 4) but many of these membranes are fused and not in the form of pinched-off vesicles of uniform size distribution. There is also a considerable amount of amorphous non-membrane-like material present.

When ATPase was reaggregated with extra phospholipid then the negatively-stained material showed much less non-membrane-like aggregates than the negatively-stained ATPase reaggregated in the absence of extra phospholipid (Figs 5 and 6). Usually solubilized protein was mixed with phospholipid solution in the ratio of protein to phospholipid approximately 1:5 (w/w) and this mixture produced membranes which were 80–95% (w/w) phospholipid. Membranes prepared by dialysis of solubilized ATPase without prior addition of extra phospholipid contained only 35% (w/w) phospholipid.

The presence of Mg^{2+} in the dialysis medium produced effects on these preparations similar to the effects observed on preparations of pure phospholipid membranes.

Sucrose density gradient centrifugation

A convenient method of investigating reaggregation of the ATPase and

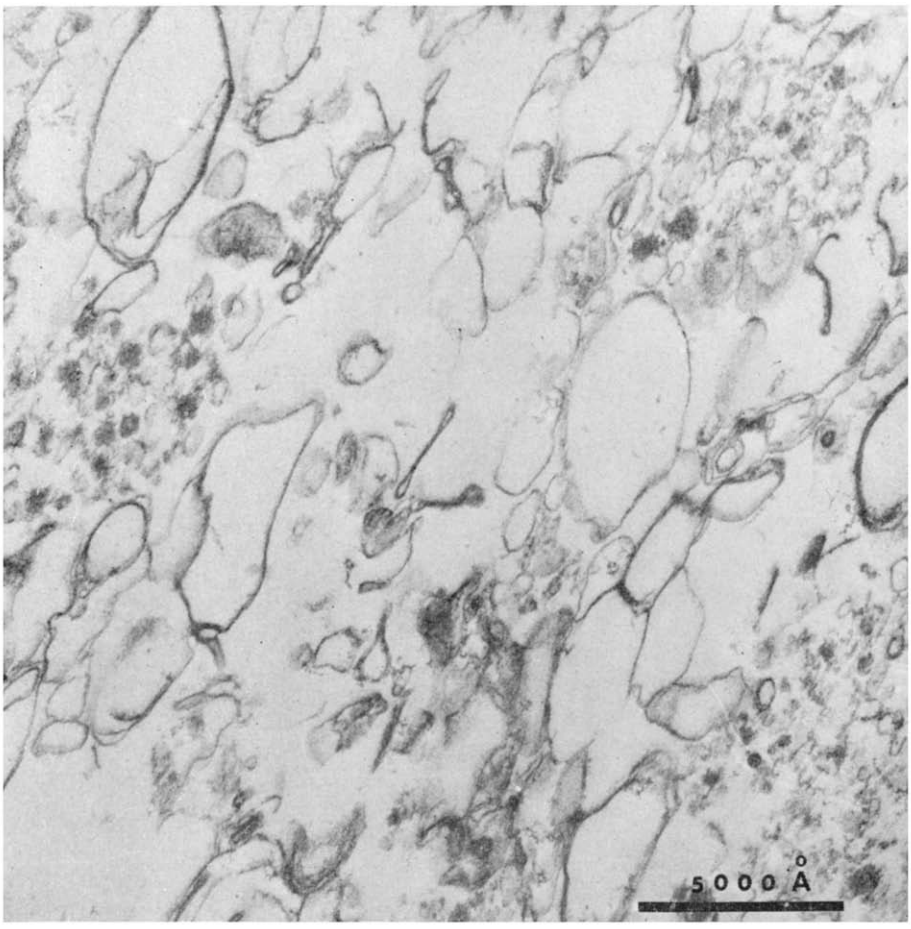

Fig. 4. Electron micrograph of thin-sectioned preparation of solubilized and dialysed membrane lipid and protein-containing (Na^+, K^+)-ATPase. Dialysed without prior addition of extra lipid, against 75 mM NaCl, 75 mM KCl, 20 mM Tris–maleate buffer, pH 7.0.

phospholipid is by centrifuging the reaggregated material into a sucrose gradient. At equilibrium any subfractions are distributed throughout the gradient according to their buoyant density. Fig. 7 shows the results of one experiment from a series of four. All four experiments gave consistent results. The bands were approximately the same, irrespective of whether the gradients were made up in 60 mM NaCl and 100 mM KCl or 500 mM NaCl. Three of the experiments were identical to the one illustrated in Fig. 7 and consisted of centrifuging four different samples in the same run. The samples were as follows: (a) ATPase reaggregated after the addition of phospholipid. (b) ATPase reaggregated without the addition of extra phospholipid. (c) Reaggregated phospholipid alone. (d) Reaggregated ATPase mixed just prior to centrifugation with reaggregated phospholipid.

Fig. 7a shows that the ATPase mixed before dialysis with phospholipid formed a single band on the gradient. Reaggregated ATPase, however, without added

phospholipid formed a different band at higher density, Fig. 7b. ATPase and phospholipid mixed after separate reaggregation formed two separate bands (Fig. 7d) of densities corresponding to those of each material run separately. This implies that the ATPase and phospholipid mixed before dialysis formed a single phase. Moreover, once the phospholipid and ATPase had been reaggregated from solution, they no longer formed a single phase, when mixed. The high salt concentration present in the sucrose gradient was introduced in order to reduce any ionic interactions between phospholipid and protein.

The reasons for the material in the Figs. 7a and 7c remaining at such low densities are uncertain. One possibility is that the high phospholipid content of the material made the vesicles very impermeable to sucrose and consequently prevented sedimentation to the true equilibrium density within the duration of the experiment.

Mg^{2+} in the dialysis buffer was not necessary for ATPase and phospholipid to reaggregate together. This is at variance to the findings of Engelman and Morowitz[14], who have suggested that divalent metal ions may be required for reassembly

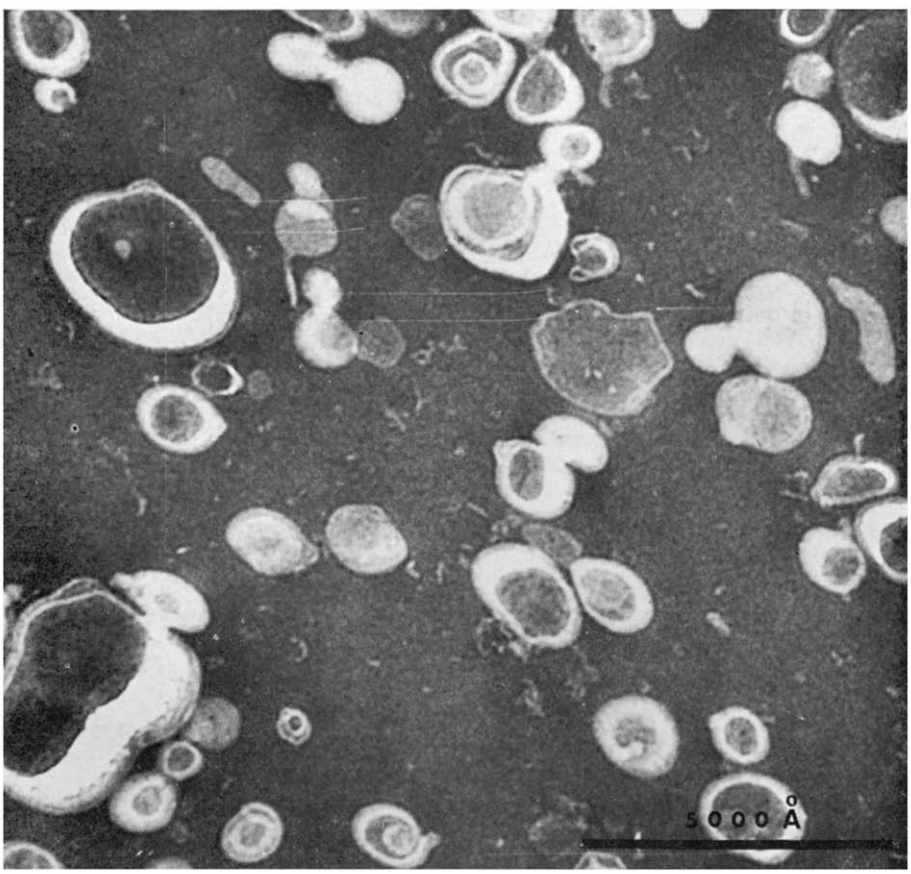

Fig. 5. Electron micrograph of negatively-stained preparation of solubilized and dialysed lipid and protein-containing (Na^+, K^+)-ATPase. Dialysed after the addition of extra phospholipid. Dialysed against 60 mM NaCl, 100 mM KCl, 5 mM Tris–maleate buffer, pH 7.0.

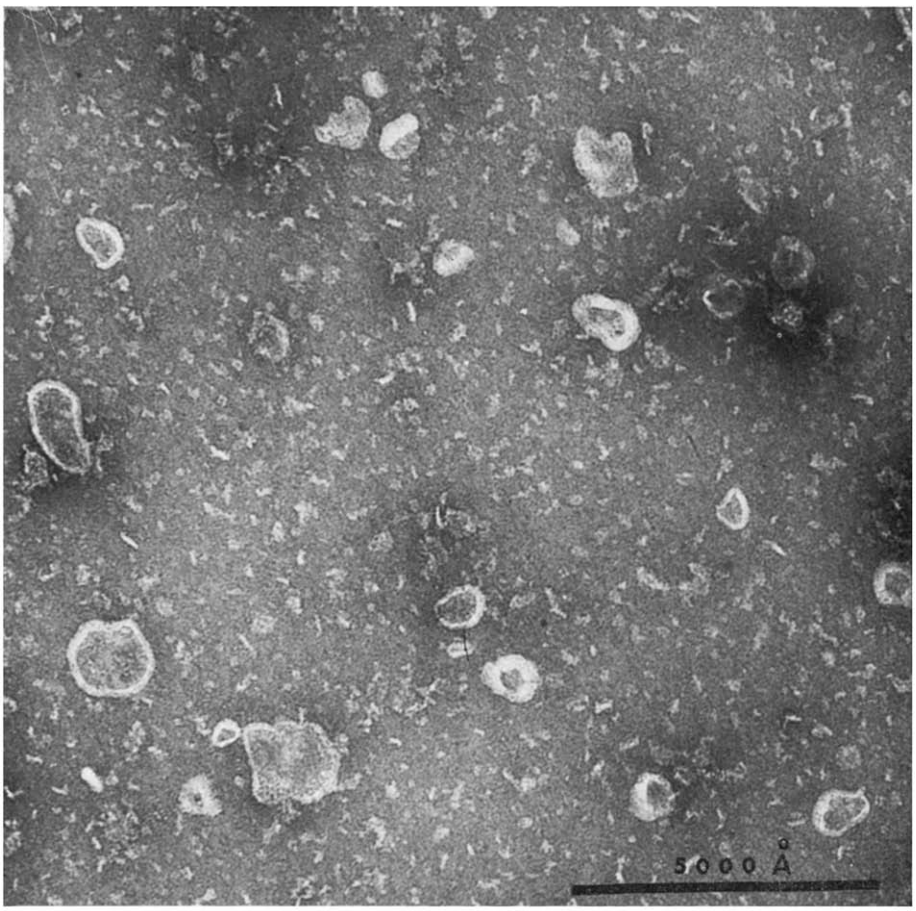

Fig. 6. Electron micrograph of negatively-stained preparation of solubilized and dialysed lipid and protein-containing (Na^+, K^+)-ATPase. Dialysed without the prior addition of extra phospholipid. Dialysed against 60 mM NaCl, 100 mM KCl, 5 mM Tris–maleate buffer, pH 7.0.

of membrane structure. We attribute this discrepancy to the rather high centrifugation forces necessary to form pellets of very small membrane vesicles.

DISCUSSION

It has been shown that the (Na^+,K^+)-ATPase can be reaggregated from detergent solution with predetermined amounts of phospholipid. On removal of the detergent by dialysis, the ATPase remains associated with the phospholipid. The ATPase and phospholipid cannot, however, be reassociated after reaggregation. Electron microscopic examination of the reaggrated material showed it to be membrane-like and in the form of small vesicles probably bounded by a single-membrane thick wall. The association between the protein and the phospholipid appears not merely to be due to ionic bonding but probably involves hydrophobic interactions; this is implicated by the behaviour of the material on the sucrose density gradients containing 0.5 M NaCl.

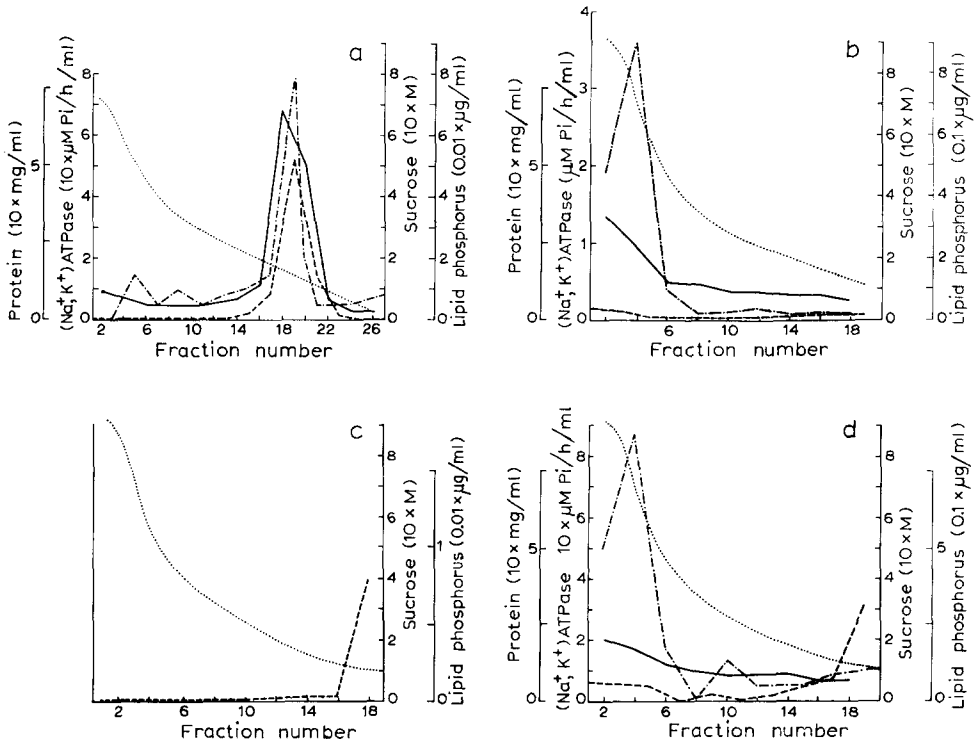

Fig. 7. Sucrose density gradient profiles of various solubilized and dialysed material. (a) Protein-containing ATPase mixed with extra phospholipid before dialysis. (b) Protein-containing ATPase dialysed without additional phospholipid. (c) Pure phospholipid dialysed alone. (d) Protein-containing ATPase and pure phospholipid dialysed separately and combined on the sucrose gradient. All material was dialysed against 60 mM NaCl, 100 mM KCl, 5 mM Tris–maleate buffer, pH 7.0. All gradients made up in 0.5 M NaCl, 5 mM Tris–maleate buffer, pH 7.0. Note, smaller fractions were collected from tube (a). All other experimental details given in Methods. ·····, sucrose concentration; –·—·–, (Na^+, K^+)-ATPase activity; ———, protein concentration; – – – –, lipid phosphorus content.

Following the ideas of Dervichian[15] and Shankland[16], we picture the formation of these membranes from deoxycholate solutions as the slow association of phospholipid molecules around the protein molecules. This is essentially different from the addition of the solubilized protein to preformed phospholipid vesicles or black films. Some membrane proteins, which can be obtained in solution by means other than the use of detergent may be re-incorporated into preformed lipid membranes. An example of such a protein is the ATP asefrom *Streptococcus faecalis* cell membranes[17]. The (Na^+,K^+)-ATPase, however, does not appear to be of this kind (our own observations using black films). Recently[3], the reconstitution into a black film of a sucrose-mediated sugar transport protein was reported. The protein was mixed with the lipid in chloroform–methanol before forming the membrane. Again this method is not suitable for the (Na^+,K^+)-ATPase since the protein is denatured by organic solvents, as probably are many other membrane proteins.

Although it cannot be ruled out that membrane proteins in detergent may be

re-incorporated into preformed membranes, we feel that the most promising approach to reconstitution is that outlined here. The technique of formation of membrane-like structures from detergent solution is not new but so far has been little exploited and then mostly with sodium dodecyl sulphate as the detergent. This detergent denatures many proteins. Since the initiation of this work Hinkle *et al.*[18] have reported some success with the reconstitution of membrane vesicles containing cytochrome oxidase using a dialysis technique similar to that described here. Hong and Hubbell[19] have also recently reported that rhodopsin can be reaggregated with phospholipid using similar methods.

We are at present investigating the permeability properties of the reconstituted (Na^+,K^+)-ATPase membrane preparations.

ACKNOWLEDGEMENTS

We should like to thank Professor M. H. F. Wilkins for support and encouragement; others, especially Dr W. Lieb, for helpful discussion and Mrs Jennie Town for technical assistance. Two of us, B. H. Anderton and J. R. Slack, held Medical Research Council Junior Research Fellowships.

REFERENCES

1 Skou, J. C. (1960) *Biochim. Biophys. Acta* 42, 6–23
2 Sanderman, H. and Strominger, J. L. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 2441–2443
3 Storelli, C., Vogeli, H. and Semenza, G. (1972) *FEBS Lett.* 24, 287–292
4 Wheeler, K. P. and Whittam, R. (1970) *J. Physiol. London* 207, 303–328
5 Changeux, J.-P., Kasai, M., Huchet, M. and Meunier, J.-C. (1970) *C.R. Acad. Sci.* 270, 2864–2867
6 MacLennan, D. H. (1970) *J. Biol. Chem.* 245, 4508–4518
7 Sutherland, E. W., Rall, T. W. and Menon, T. (1962) *J. Biol. Chem.* 237, 1220–1227
8 Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 1277–1281
9 Allen, D., Auger, J. and Crumpton, M. J. (1972) *Nat. New Biol.* 236, 23–25
10 Anderton, B. H. and Slack, J. R. (1972) *Abstr. 8th FEBS Meeting Amsterdam*, Abstr. No. 108
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
12 McClare (1971) *Anal. Biochem.* 39, 527–530
13 Johnson, S. M., Bangham, A. D., Hill, M. W. and Korn, E. D. (1971) *Biochim. Biophys. Acta* 233, 820–826
14 Engelman, D. M. and Morowitz, H. J. (1968) *Biochim. Biophys. Acta* 150, 385–396
15 Dervichian, D. G. (1968) *Adv. Chem.* 84, 78–99
16 Shankland, W. (1970) *Chem. Phys. Lipids* 4, 109–113
17 Redwood, W. R., Muldner, H. and Thompson, T. E. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 989–996
18 Hinkle, P. C., Kim, J. J. and Racker, E. (1972) *J. Biol. Chem.* 247, 1338–1339
19 Hong, K. and Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 2617–2621