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A Simple and Rapid Method for the Reversible Removal of Lipids from a Membrane-Bound Enzyme

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A simple, rapid and reproducible method for the reversible removal of lipids from a membrane-bound enzyme is described. Essentially, a membrane preparation containing ($Na^+ + K^+$)-dependent adenosine triphosphatase was extracted with the non-ionic detergent Lubrol WX in the presence of glycerol, and partial separation of protein from lipid was achieved with the use of only two centrifugations. About 74% of the endogenous phospholipid and 79% of the cholesterol were removed, concomitant with a virtually complete loss of ouabain-sensitive adenosine triphosphatase activity, but with retention of 60–100% of the K^+-dependent phosphatase activity. The addition of pure phosphatidylserine re-activated the enzyme to more than 80% of the initial activity, and up to 30% of the protein was recovered. Excess of phosphatidylserine could be washed off the enzyme to give a stable 'reconstituted' preparation. The effects of variation in the experimental conditions were examined, and the results are discussed with respect to the possibility of adapting the method to the study of other lipid-dependent enzymes bound to membranes.

The current widespread interest in the structure and functions of biological membranes has generated intensive study of membrane-associated enzymes (Coleman, 1973). Some of these studies have revealed that to function normally, or even at all, many membrane enzymes require the hydrophobic environment provided by membrane lipids. This finding has led to the development of a variety of experimental procedures for the removal of endogenous lipids from such enzyme preparations, to facilitate investigation both of the enzymic reactions themselves and of the protein–lipid interactions involved. The methods that have been used for such studies include the use of organic solvents, phospholipases or detergents, or combinations of these. In addition to reproducibility, an important property of a useful method for lipid depletion is that it must not denature, or otherwise inactivate, the enzyme protein, because recombination of the protein with lipid to give an active complex is essential for these kinds of investigations. Hence it is necessary to control carefully the water content and the temperature when organic solvents are used, and the exclusion of any contaminating proteinase activity is essential when phospholipase treatment is involved. Similarly, both protein denaturation and enzyme inactivation by residual bound detergent are possible complications associated with the use of detergents. In general, however, methods involving the various different detergents have been most widely and successfully applied (Helenius & Simons, 1975; Kimelberg, 1976).

Palatini *et al.* (1972) pioneered a detergent-extraction method using the non-ionic detergent Lubrol WX for partial removal of lipid from the ($Na^+ + K^+$)-dependent ATPase (EC 3.6.1.3), and this procedure was subsequently modified by Wheeler *et al.* (1975). In spite of the excellent reproducibility of that method, however, it is rather complicated, time-consuming, and produces very low yields of lipid-depleted protein. We have therefore examined the method in some detail and found that a simpler and quicker method provides not only an apparently identical lipid-depleted enzyme preparation, but also substantially higher yields of it. Details of this method and its application to the ($Na^+ + K^+$)-dependent ATPase are described below, together with descriptions of what happens when some variations are made in the conditions used. It seems reasonable to assume that this information could enable the method to be adapted to the study of other membrane-associated enzymes.

A preliminary account of part of this work has been presented (Wheeler & Goodman, 1976).

Abbreviation used: ATPase, adenosine triphosphatase.

Experimental

Preparation of the 'normal' ATPase (essentially a microsomal fraction separated from rabbit kidney homogenate and extracted with NaI solution), enzyme-activity measurements, and all other procedures not described below, were carried out exactly as described by Wheeler *et al.* (1975) and Wheeler & Walker (1975).

Lubrol WX solutions

Concentrated solutions of Lubrol WX were prepared with the use of an MSE ultrasonic disintegrator. A mixture of 20g of Lubrol in 95ml of water was subjected to ultrasonic vibrations (settings: medium power, amplitude 2, with a 0.8cm diam. probe) for 30–60min. The mixture was stirred intermittently but not cooled. Concentrated Tris was added to adjust the solution to pH7.4 at room temperature (20°C), the volume was brought up to 100ml and then it was centrifuged at about 60000*g* for 3h at 4°C to sediment titanium particles. The clear supernatant solution remained stable at room temperature.

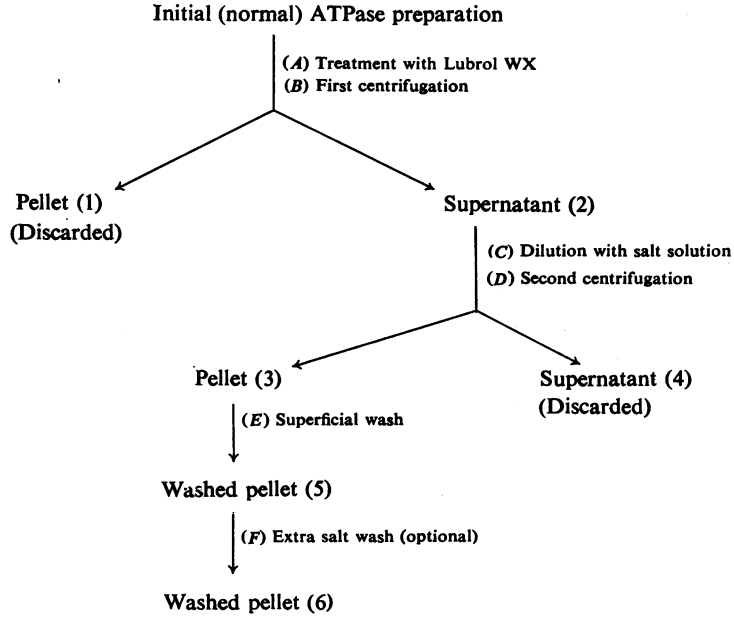
Preparation of lipid-depleted ATPase

For clarity and convenience the procedure is outlined in Scheme 1. The letters and numbers in parentheses refer to the individual steps and fractions (respectively) described below.

(*A*) *Treatment with Lubrol WX.* A sample of the 'normal' ATPase preparation was added to a solution containing Lubrol WX (30mg/ml), glycerol (40%, v/v), 0.45mM-EDTA, 1.25mM-dithiothreitol, 2.5mM-ATP (disodium salt) (adjusted to pH7.4 with Tris at 20°C) and 12mM-Tris (adjusted to pH7.4 with HCl at 20°C). The final concentration of protein was approx. 2mg/ml and the total volume of the mixture about 70ml. The mixture was cooled to 0–4°C in an ice/water bath, and the ATPase preparation dispersed with the use of the ultrasonic disintegrator. The small probe (tip diam. 3.1mm) was used with power set at 'medium' and amplitude at '1'. The power was switched on for 15s at a time, with stirring between each 15s treatment, to give a total of 2min exposure to the ultrasonic vibrations. The mixture was then stirred at 0°C for 1h.

(*B*) *First centrifugation.* The mixture obtained from step (A) was centrifuged at 46000$g_{max.}$ for 30min at 4°C in the 10×10ml rotor of an MSE Superspeed 50 centrifuge. The pale-yellow supernatant solution (2) was carefully decanted and the brown pellet (1) discarded.

(*C*) *Dilution with NaCl solution.* As a routine during development of the method, 1vol. of the supernatant solution (2) was diluted with 0.4vol. of 1M-NaCl, to give a mixture containing 28.5% (v/v) glycerol and 285mM-NaCl. However, it is noteworthy that subsequent examination of this step in more



Scheme 1. *Outline of method for removal of essential lipid from the ATPase*
Details of the method are described in the text, with reference to the letters and numerals in parentheses.

detail has shown not only that some variation is permissible in these values of final glycerol and NaCl concentrations, but also that such variation can significantly improve the yield of final product. Details of these variations and their effects are presented in the Results section.

(*D*) *Second centrifugation.* The mixture obtained from step (C) was centrifuged at 170000$g_{max.}$ for 60min at 4°C, with the use of the same apparatus as for step (B). The very-pale-yellow supernatant solutions (4) were decanted and discarded, and the greenish-brown pellets (3) were retained in the centrifuge tubes.

(*E*) *Superficial wash.* A small amount (2–3ml) of the buffer 'A20' (Towle & Copenhaver, 1970; Wheeler *et al.*, 1975) was used to wash, very thoroughly and carefully, the insides of each centrifuge tube and the surfaces of the pellets. This procedure was repeated until the washing mixture no longer foamed, when one further wash completed the process. Usually three to four washes were sufficient. The pellets (5) were then suspended by gentle homogenization in buffer A20 such that the final concentration of protein was between 5 and 10mg/ml. These suspensions showed no changes in properties for at least 1 month when stored at −20°C.

(*F*) *Extra NaCl wash.* Although the product resulting from step (E) was usually satisfactory in that its lipid content had been decreased until residual ATPase activity was extremely low, if not negligible, we have occasionally obtained a product in which both residual lipid and residual ATPase activity were unacceptably high. When that happened the problem was easily overcome by the addition of an extra wash with NaCl solution. To do this the enzyme was first sedimented by centrifugation, as in step (D), and then resuspended by homogenization in 1M-NaCl. Sedimentation by centrifugation, again as in step (D), gave a pellet (6) that was treated as described in step (E).

Reconstitution with exogenous phospholipid

Samples of lipid-depleted enzyme were 'reconstituted' by the addition of exogenous phospholipid, usually phosphatidylserine. Dispersions of the latter were prepared as described previously (Wheeler *et al.*, 1975; Walker & Wheeler, 1975*a*) and sufficient was mixed with a sample of the lipid-depleted enzyme in 10mM-Tris/HCl (pH7.4 at 37°C) to give a mixture containing at least 3μmol of phospholipid/mg of protein. The concentration of protein was usually about 2mg/ml. The mixture was incubated at 37°C for 10min, cooled to 4°C, and centrifuged at 120000$g_{max.}$ in the SW50.1 rotor of a Spinco centrifuge for 30min. The supernatant dispersion was decanted and the pellet washed by resuspension in 10mM-Tris/HCl (pH7.4 at 20°C) and centrifugation as described above. Usually two washes were sufficient to remove all loosely bound phospholipid. The final pellet was suspended in buffer A20 solution and stored at −20°C, when it was stable for at least 1 month. This product is called the 'reconstituted' enzyme.

Re-activation with exogenous phospholipid

For some purposes, reversal of the lipid-depletion process was monitored by simple addition of the phospholipid dispersion to the incubation medium used to assay the ATPase activity. Details of this method have been given (Wheeler *et al.*, 1975); here we just wish to point out that we have termed this process 're-activation' to distinguish it from the 'reconstitution' described above.

Measurement of Lubrol concentrations

The amount of residual Lubrol associated with the lipid-depleted ATPase was measured with the use of a modification of the method described by Wheeler *et al.* (1975).

Table 1. *Summary of results obtained by application of the method described in Scheme* 1

The terms 'normal', 'depleted', 're-activated' and 'reconstituted' are defined in the text. Lines with (−F) refer to the method excluding step (F) and lines with (+F) to the method including step (F). Ouabain-sensitive ATPase activities and either ouabain-sensitive (−F) or K^+-dependent (+F) phosphatase activities are given; all values are means±S.E.M. for the numbers of preparations indicated in parentheses.

Preparation		Normal	Depleted	Re-activated	Reconstituted
Phospholipid content (μmol/mg of protein)	(−F)	0.70±0.04 (15)	0.18±0.01 (15)	—	0.74±0.12 (6)
	(+F)	0.70±0.04 (27)	0.18±0.01 (11)	—	—
ATPase activity (μmol of P_i/min per mg of protein)	(−F)	1.24±0.07 (15)	0.03±0.01 (15)	1.04±0.09 (14)	0.97±0.15 (8)
	(+F)	1.02±0.08 (26)	0.09±0.02 (11)	0.77±0.11 (11)	—
Phosphatase activity (μmol of *p*-nitrophenol/min per mg of protein)	(−F)	0.19±0.02 (11)	0.17±0.02 (10)	—	0.24 (3)
	(+F)	0.26±0.04 (9)	0.15±0.02 (8)	0.21±0.03 (8)	—
Protein yield (%)	(−F)	100	20.0±1.6 (10)	—	—
	(+F)	100	14.0±1.0 (11)	—	—

Results and Discussion

Effect of extraction with Lubrol WX on ($Na^+ + K^+$)-dependent ATPase

The results of applying the procedure outlined in Scheme 1 to a number of ATPase preparations are summarized in Table 1 (lines –F). The average value for removal of phospholipid was about 75%, and that was associated with a decrease in ouabain-sensitive ATPase activity of more than 97%. (Ouabain-insensitive activity of the initial preparations amounted to only 5% of the ouabain-sensitive value.) However, addition of exogenous phosphatidylserine to the depleted preparations was very effective in reversing this loss of enzymic activity. When the enzyme was 'reconstituted' with phosphatidylserine, the tightly bound phospholipid was restored to the initial value, and this was accompanied by a restoration of ATPase activity to about 80% of the initial value. Re-activation by simple incubation of the depleted enzyme in the presence of excess of phosphatidylserine gave slightly higher activity. The average yield of protein was about 20%, so that the total yield of ouabain-sensitive ATPase activity averaged 16% for the reconstituted enzymes and 18% for the re-activated ones. (Note, however, that subsequent experiments, described below, indicate that these yields can very easily be significantly improved.)

The ouabain-sensitive phosphatase activity associated with the ($Na^+ + K^+$)-dependent ATPase was not significantly decreased by the Lubrol treatment, confirming the earlier conclusion that the phosphatase activity is not dependent on lipid in the same way as the ATPase activity (Wheeler & Walker, 1975).

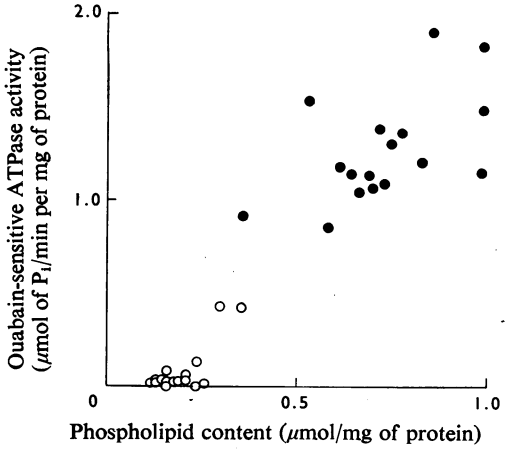


Fig. 1. *Relationship between the phospholipid content and the ATPase activity of different enzyme preparations* Ouabain-sensitive ATPase activities and total phospholipid contents of 17 different enzyme preparations were measured before (●) and after (○) lipid depletion, as described in the text.

Correlation between ATPase activity and phospholipid content

Fig. 1 shows a plot of the ouabain-sensitive ATPase activities and the total phospholipid contents of 17 different enzyme preparations, before and after lipid-depletion according to steps (A)–(E) in Scheme 1. Clearly, enzyme activity and phospholipid content were closely associated (the correlation coefficient was 0.94); but, as found with the longer method (Wheeler *et al.*, 1975), complete loss of ouabain-sensitive ATPase activity was frequently observed, although some lipid always remained. Fig. 1 also shows that the modified procedure for removing lipid failed to do so sufficiently only twice during the 17 experiments. It was the occasional failure such as these that led to the introduction of the arbitrary extra NaCl-wash step (F), which is discussed in more detail below.

Residual Lubrol WX associated with the enzyme

The amount of Lubrol remaining, which was associated with six different lipid-depleted ATPase preparations, averaged 0.27±0.04 (S.E.M.) mg/mg of protein. This mean value is somewhat lower than the values obtained previously (Wheeler *et al.*, 1975) and probably reflects the improved procedure used to assay the Lubrol (S. L. Goodman & K. P. Wheeler, unpublished work). It seems likely that the residual Lubrol had replaced some of the original lipid associated with the enzyme protein, but we have no other direct evidence to support this view.

Effects of variations in the lipid-depletion procedure

The effects of changing some of the possible variables of the method are described below because they not only indicate that further improvement in the final yield of enzyme is possible, but also provide some insight into what happens during the extraction. Such information could be valuable if the method were adapted to other membrane-associated enzymes.

Step (A). (I) Protein concentration. When the concentration of the ATPase was increased, from 2mg of protein/ml to 4.5mg of protein/ml in the initial Lubrol/glycerol mixture, removal of phospholipid was much less efficient and the final product (5) possessed high residual ATPase activity. Thus total phospholipid decreased from 0.76 to only 0.31 μmol/ml of protein, concomitant with a fall in ouabain-sensitive ATPase activity from 0.88 to 0.35 μmol of P_i/min per mg of protein. Moreover, addition of phosphatidylserine to this product re-activated it only to 0.58 μmol of P_i/min per mg of protein, giving a total activity yield of only 11%.

Hence, although we have not excluded the possi-

bility that higher concentrations of ATPase could be used, to do so successfully would seem to entail changing many, if not all, of the other variables.

(II) Time of extractions with Lubrol. It seemed possible that the 60min extraction period with the Lubrol/glycerol mixture was unnecessarily long. We therefore compared the effect of extracting for only 10min instead of 60min, with the following results. Under both conditions there was no significant difference in the extent of removal of phospholipid (about 80%), residual ouabain-sensitive ATPase activities (4–5% of the initial value), or ability to be re-activated by added phosphatidylserine (to 0.90 and 0.98 μmol of P_i/min per mg of protein, compared with the initial value of 1.02). However, after extraction for 10min the yield of protein was 15%, compared with 19% after 60min, so that the total activity yields were 13 and 18% respectively.

The results are therefore somewhat equivocal, because it is not clear if the difference in yields is significant. Clarification of this point would necessitate many repetitions of this, or similar, experiments, so we decided to keep to the 60min period, but it is clearly possible that this step might be modified to save time with little change in the final product.

(III) Effect of temperature on the extraction with Lubrol. It seems reasonable to expect a detergent to interact with the membrane components more efficiently at temperatures above 0°C, although protein denaturation would obviously limit the possible temperature range that could be used. We attempted to investigate this possibility by extracting with Lubrol for 20min at 20°C, followed by 40min at 0°C, instead of the standard 60min at 0°C. There was, however, no change at all in the products or yields obtained by these two procedures.

Step (B). (I) Omission of the first centrifugation. The importance of step (B) was tested first simply by omitting it, so that the procedure involved going directly from step (A) to step (C) in Scheme 1. This resulted in a large increase in the yield of protein in product (5), 65% compared with 29% in the control sample which included step (B). The product (5) also contained more phospholipid per mg of protein than did the control (34% of the initial value, compared with 20%), although residual ouabain-sensitive ATPase activities were both very low, about 3% of the initial value. However, when phosphatidylserine was added to re-activate the depleted enzymes, the specific activity of the experimental product increased only to 46% of the initial value, whereas that of the control increased to 79% of that of the original preparation.

Hence it appears that the first centrifugation (step B) sediments protein that either is not ATPase, or is denatured ATPase. Omission of this step leads to sedimentation of this extraneous protein with the final product, and the extra phospholipid associated with it probably results from the larger pellets finally obtained, because the efficiency of the washing process (E) varies with pellet size (see below).

(II) Centrifugation at higher speeds. When the centrifugal force used in step (B) was increased above about $50000g_{max.}$, the pellets (3) became two-layered. This did not affect the properties of the final product because the extent of lipid depletion, the loss of ATPase activity and the ability to be re-activated by added phospholipid remained constant. However, the final yield of product (5) was lower. For example, in three preparations the speed of the centrifugation step (B) was increased to give $66000g_{max.}$ instead of the usual $46000g_{max.}$, and the final yields of product (5) were 12, 13 and 16%, compared with the mean value of 20% normally obtained.

Hence it seems that the appearance of the second layer in the pellets (3) when the centrifugal force in step (B) was increased signified the sedimentation of some of the required lipid-depleted ATPase. The value of $46000g_{max.}$ was therefore chosen because that produced no visible layering in the pellets (3).

Step (C). (I) Effect of final NaCl concentration. The addition of NaCl obviously increases the final concentration of ions in the mixture and simultaneously decreases the concentrations of all other constituents. Both effects are important, so the effect of increasing the NaCl concentration was examined under conditions of constant dilution. The amount of NaCl added in step (1) was varied to give final concentrations in the range 0–0.8M, whereas the final concentration of glycerol remained constant at 28.5% (v/v). The amount of residual phospholipid, the re-activated ATPase activity and the residual phosphatase activity each remained approximately constant when the final NaCl concentration was varied from 0 to 0.4M. However, there was a marked increase (up to 40%) in the yield of protein at NaCl concentrations in the range 0.1–0.2M, compared with that obtained in the absence of added NaCl. At 0.8M-NaCl, the final yield of protein was 10% less than that obtained without NaCl, and total enzyme activity yield was much lower because the product could not be re-activated to the same extent.

Whether an NaCl concentration greater than about 0.2M actually increases the solubility of the protein again is not clear, because there is also an increase in solution density as NaCl concentration is increased. However, it is clear that for optimum yields of desirable product, a final NaCl concentration in the range 0.1–0.2M is required. (Replacement of NaCl by KCl did not affect these findings.)

(II) Effect of final dilution. Because the high concentration of glycerol in the mixture produced a dense solution, the dilution accompanying the addition of NaCl appeared to be important in as far as it decreased the density, thus making sedimentation of the ATPase protein more likely during subse-

quent centrifugation. The effect of dilution at constant final NaCl concentration was therefore also examined. In the standard procedure the final NaCl concentration was 285 mM, and the dilution such that the final concentration of glycerol was 28.5% (v/v). The effect of variation in the final glycerol concentration relative to that value, with the NaCl concentration constant at 285 mM, was examined. Within the limits tested (10–40% glycerol), the greater the dilution of the glycerol concentration, the greater was the yield of protein finally obtained. (The yield obtained with 10% glycerol was 60% more than that with 40%.) Also, since the ability of the ATPase to be re-activated remained approximately constant, the increased yield of protein obtained at the lower glycerol concentrations resulted in increased total activity yield.

It seems therefore that the 'standard procedure' initially described above can be significantly improved simply by modification of step (C) such that the final concentrations of NaCl and glycerol are about 0.2 M and 10% (v/v) respectively. (Experiments similar to those just described would quickly provide optimum conditions for other enzymes.)

Step (E). The most likely explanation of the occasional variation in the effectiveness of the superficial washing procedure is variation in the size of the pellets (3) obtained after step (D). It is important that these pellets are washed separately and that the conditions are not changed such that the pellet size increases significantly. It appears that most of the phospholipid and Lubrol no longer closely associated with the protein accumulates on the surface of the pellets, thus making their removal by this simple washing procedure effective. The larger the pellet the smaller will be the ratio of the surface/volume, and hence the efficacy of the washing process will decrease.

Step (F). A separate series of experiments was made in which the optional extra NaCl wash (step F) was routinely included. The data obtained from those experiments are collected in Table 1 (lines +F). An obvious and expected result was that the final yield of protein remaining after the extra wash was lower than that obtained from the shorter procedure, the average recovery being decreased from about 20% to about 14%. However, there was no clearly significant difference in the extent to which phospholipid was removed, the relative loss of ATPase activity, or the relative degree of re-activation by added phosphatidylserine. The cholesterol content of the enzyme preparations was also measured (Zak, 1957) in these experiments, and the results showed that the extraction with Lubrol was also effective in depleting the enzyme of this neutral lipid. On average about 79% of the cholesterol was removed [initial value 0.34±0.01 (S.E.M.) (10); final value 0.07± 0.01 (10) μmol/mg of protein], compared with about 74% of the phospholipid, a finding in keeping with the trend described by Wheeler *et al.* (1975).

The only obvious difference in the properties of the lipid-depleted preparations obtained with the use of the extra NaCl wash was the change in their phosphatase activity. The average activity of the depleted enzymes was about 60% of that of the normal preparations (Table 1, line +F), whereas when the extra wash was not used the phosphatase activity remained essentially constant (Table 1, lines −F). However, too much importance cannot be attached to this difference because there was a slight difference in the assay methods used for the two series of experiments summarized in Table 1. The phosphatase activities given in Table 1 (lines −F) were measured as the difference between values obtained in the presence and absence of ouabain, whereas those in Table 1 (line +F) were measured as the difference between values obtained in the presence and absence of K^+. Also, the substrate concentration used in the latter experiments was 6 mM, compared with the 3 mM used for the former series. Both changes in methodology render the results given in Table 1 (line +F) more reliable (see Walker & Wheeler, 1975*b*). Nevertheless, it is possible that the extra NaCl wash was important in this connexion, because the approx. 40% loss of phosphatase activity during lipid extraction (Table 1, line +F) is more in line with the approx. 50% loss observed when several NaCl washes were used (Wheeler & Walker, 1975). Again, however, the noteworthy point is that the phosphatase activity was much more resistant to lipid removal than was the ATPase activity.

General aspects of lipid-depletion

The molecular reactions involved in the interactions of detergents with biological membranes are very complex and difficult to investigate experimentally. For example, the question of whether Lubrol is effective because it replaces essential protein-bound lipid and stabilizes the protein until phospholipid is added again is difficult to answer. We have found that the amount of residual Lubrol associated with the reconstituted enzyme is practically identical with that found in the depleted preparations (S. L. Goodman & K. P. Wheeler, unpublished work), but we do not know if the Lubrol has the same molecular location in the two kinds of preparations. For the time being, at least, the method remains merely a useful experimental tool, with the details of how it works still to be determined.

In a detailed review of the topic, Helenius & Simons (1975) concluded that, at present, non-ionic detergents 'appear to provide the most generally applicable solubilization agents to extract and purify an integrally bound membrane protein lipid-free in its native conformation, or something close to it'. Our aim has been somewhat different from that, but, as

far as the $(Na^+ + K^+)$-dependent ATPase is concerned, it is true that we have found that the use of Lubrol WX for removal of lipid consistently produces an enzyme protein in a state from which it can be reactivated by the addition of exogenous phospholipid. Complete removal of lipid has not been achieved because loss of enzymic activity occurred when about 20–25% of the phospholipid remained, and removal of that fraction of lipid essential for ATPase activity was the objective. Such a preparation permits useful studies to be made both of the interactions between added lipid and the enzyme protein, and of the involvement of that lipid in the complex reaction sequence of the ATPase. In addition to ready reversibility, the important characteristics of the method described above are its simplicity, speed and reproducibility. At present these features compensate for the still relatively low yields (up to 30%) of reactivatable protein obtained, because they do enable extensive investigations of the ATPase system to be made. It seems feasible also that this method could readily be adapted to the study of other integral membrane enzymes.

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