A CHOLATE-DILUTION PROCEDURE FOR THE RECONSTITUTION OF THE Ca^{++} PUMP, $^{32}P_i$–ATP EXCHANGE, AND OXIDATIVE PHOSPHORYLATION

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1. Introduction

We have described a method for the reconstitution of the mitochondrial proton pump [1,2] which we refer to as the cholate-dialysis procedure. Phospholipids are sonicated in the presence of 2% cholate, then are mixed with the isolated membrane proteins and dialyzed to remove cholate. A second method of reconstitution referred to as the sonication procedure [3] avoids the use of detergents, but requires exposure of the proteins to sonication. Some of the disadvantages of these methods which will be discussed later, prompted us to develop alternative procedures of reconstitution in which both sonication and dialysis are avoided. One of these, which we refer to as a cholate-dilution procedure, is suitable for the reconstitution of several membrane systems as described in this communication.

2. Materials and methods

2.1. *Preparations*

Hydrophobic protein fractions from mitochondria and phospholipids were prepared as described previously [1,2]. Complex III was prepared according to Rieske [4], Complex V according to Hatfi et al. [5], Ca^{++} ATPase from sarcoplasmic reticulum according to MacLennan [6], and cytochrome oxidase according to Kuboyama et al. [7] as modified by Eytan et al. [8]. All other materials were as described in previous references [1,2]. We wish to thank Dr Hosley of Eli Lilly for a gift of A-23187.

2.2. *Methods*

$^{32}P_i$–ATP exchange [2], Ca^{++} translocation [9], respiratory control [10] and oxidative phosphorylation [11] were measured as described in the references.

2.3. *Reconstitution by the cholate-dilution procedure*

The general procedure used was as follows. Phospholipids were sonicated either in the presence or absence of cholate and mixed with the membrane proteins at a final concentration of about 0.7% cholate. After an optimal time of incubation (usually 20 min at 4°C) a sample was diluted at least 25-fold into the appropriate assay mixture. In each case the optimal phospholipid composition and phospholipid/protein ratio was determined. Usually a 20 to 1 or 40 to 1 ratio was used. The optimal cholate concentrations varied with the phospholipid concentration between 0.5 to 0.8%. Specific descriptions of individual reconstitution conditions are in the legends of the tables and figures.

3. Results

3.1. *Reconstitution of the $^{32}P_i$–ATP exchange*

The effect of varying amounts of cholate on the reconstitution of the $^{32}P_i$–ATP exchange with a preparation of the mitochondrial hydrophobic protein fraction is shown in table 1. With crude soybean phospholipids or with a mixture of pure phosphatidylcholine and phosphatidylethanolamine, the optimal cholate concentration varied with the concentration and composition of phospholipids between 0.5 to 0.8%. In our hands the preparation of complex V gave lower specific activities of $^{32}P_i$–ATP exchange than the

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Table 1
Effect of detergent concentration in the cholate-dilution reconstitution of the $^{32}P_i$–ATP exchange reaction

Concentration of added cholate %	Expt. 1	Expt. 2	Expt. 3
	$^{32}P_i$–ATP exchange		
	Purified P-lipids	Crude lipids	Crude lipids
	(nmoles/min/mg protein)		(+ complex V)
None	12	15	6
0.25	17	23	8
0.5	38	73	21
0.75	148	65	28
1.0	100	9	26

The phospholipids were dried, washed with ether and then suspended in 50 mM Tricine-KOH, pH 8.0 to give a final concentration between 20 to 50 mM. This suspension was sonicated to clarity as described previously [2]. Between 1 to 2.5 μmol of the phospholipids were incubated at 4°C in a final volume of 0.08 ml of 50 mM Tricine-KOH, pH 8.0 in the presence of varying amounts of sodium cholate, 0.1 μmol of dithiothreitol and 250 μg of hydrophobic protein fraction. After 20 min 25 μl were transferred to another test tube at room temperature and 20 μg of F_1 were added. After 10 min $^{32}P_i$–ATP exchange was measured as described previously [2]. In experiment 1, purified mitochondrial phosphatidylethanolamine 1.25 μmol, phosphatidylcholine 1.25 μmol and 42 nmol of cardiolipin were used. In experiments 2 and 3 crude soybean phospholipid (1 μmol) was used with hydrophobic protein fraction or complex V were used respectively.

hydrophobic protein fraction with either mitochondrial or soybean phospholipids and at low or high phospholipid concentrations. It can be seen from table 1 that also in the case of complex V additional cholate (there is some cholate present in the fraction) was required for optimal activity.

As shown in table 2 both phosphatidylethanolamine and phosphatidylcholine were required for the $^{32}P_i$–ATP exchange in vesicles obtained by the cholate-dilution procedure. However, the highest activities were observed with a 1:1 mixture rather than with the 4:1 ratio of phosphatidylethanolamine:phosphatidylcholine opti-

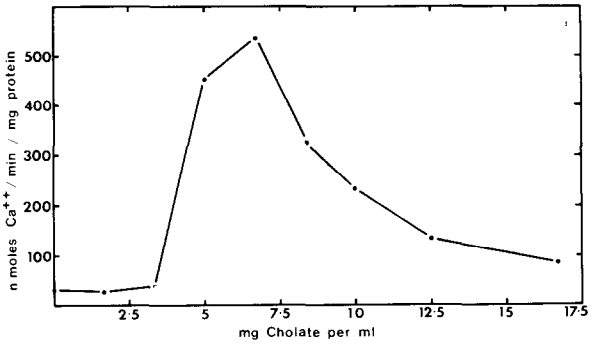

Fig.1. A suspension of 4% soybean phospholipids in 0.2 M oxalate was sonicated to clarity. An aliquot of 0.1 ml was placed into a test tube and incubated at 4°C in a final vol of 0.12 ml together with 80 μg of Ca^{++}-ATPase and various concentrations of sodium cholate. After 30 min a sample (20λ) was assayed for $^{45}Ca^{++}$-transport activity at 24°C.

Table 2
Effect of phospholipid composition on $^{32}P_i$–ATP exchange

	$^{32}P_i$–ATP exchange (nmoles/min/mg protein)
Phosphatidylethanolamine (PE)	45
Phosphatidylcholine (PC)	2
PE:PC (1:1)	136
PE:PC (4:1)	93
PE:PC:Cardiolipin (1.25:1.25:0.02)	143

The procedure of reconstitution was as described in the legend of table 1 except that the mixtures of mitochondrial phospholipids (2.5 μmol total) were sonicated in small aliquots together with cholate and dithiothreitol and the final cholate concentration was adjusted to 0.7%, then the hydrophobic protein fraction was added and finally F_1.

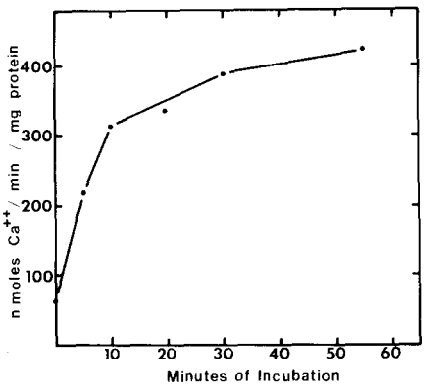

Fig.2. In the presence of 0.68% sodium cholate the Ca^{++}-ATPase was incubated with phospholipids as described in the legend of fig.1 expect that the incubation time was varied as indicated in the figure.

mal in the cholate-dialysis procedure. Moreover, cardiolipin which considerably increased the $^{32}P_i$–ATP exchange in 1:1 vesicles obtained by the dialysis procedure, had little effect in vesicles prepared by the cholate-dilution method. The reconstituted vesicles retained between 60 to 100% of activity when stored in the cholate mixture for 24 hr at 4°C, but F_1 had to be added prior to assay. The rates of $^{32}P_i$–ATP exchange obtained with this procedure were as high or higher than with the cholate-dialysis procedure and about twice as high as with the sonication procedure.

3.2. *Reconstitution of the Ca^{++} pump*

The effect of cholate concentration on the rate of $^{45}Ca^{++}$ translocation is shown in fig.1. The optimal concentration was again about 0.8%. In the absence of the ATPase, or with the complete system in the presence of the ionophore A-23187, virtually no Ca^{++} translocation was observed. The effect of the time of incubation before dilution is shown in fig.2. Each system had to be tested for optimal cholate concentration and time of incubation, since considerable variations were encountered with different phospholipid compositions. Even when cholate was added

Table 3
Reconstitution of vesicles catalyzing phosphorylation coupled to the oxidation of succinate

Reconstitution procedure	Additions	Oxidative phosphorylation natoms O	nmoles glucose-6-P	P:O
Expt. 1				
Cholate-dilution	Succinate	140	43	0.31
Cholate-dilution	PMS-ascorbate	145	30	0.21
Cholate-dialysis	Succinate	64	40	0.63
	PMS-ascorbate	184	64	0.35
Expt. 2				
Cholate-dilution	Succinate	170	47	0.28
	Succinate + rutamycin (4 μg)	160	0	0
	Succinate + 1799 (0.2 mM)	142	7	0.05
	Succinate + antimycin (10 μg)	26	0	0

For the cholate dilution procedure mitochondrial phosphatidylethanolamine (20 μmol) and phosphatidylcholine (5 μmol) were sonicated to clarity in 0.3 ml of 50 mM Tricine-KOH (pH 8.0) which contained 3.5 mg Na-cholate. To the chilled phospholipids 1 μl of 1 M dithiothreitol, 1.5 mg of hydrophobic proteins, 300 μg of complex III, 225 μg of cytochrome oxidase and 1.8 mg cytochrome *c* were added to a final volume of 0.5 ml. After 30 min at 4°C external cytochrome *c* was removed by centrifugation and assays performed as described previously [11]. For the cholate dialysis procedure the same phospholipid mixture was clarified by sonication and mixed in a final volume of 0.7 ml with 1 μl of dithiothreitol, 1.5 mg of hydrophobic protein, 300 μg of complex III, 450 μg of cytochrome oxidase and 1.5 mg of cytochrome *c*. The mixture was dialyzed in 1/4 inch, 0.002 inch thick dialysis tubing against 300 vol of 50 mM Tricine-KOH, pH 7.5, containing 1 mM dithiothreitol for about 20 hr. Removal of cytochrome *c* and assays were performed as described previously [11].

before rather than after the sonication of the phospholipids the results were affected. The reconstituted vesicles were quite stable at 4°C in the presence of cholate (fig.2). The rates of Ca^{++} transport obtained with this procedure were twice as high as with the sonication procedure and about the same as the rates obtained with the cholate-dialysis procedure.

3.3. *Reconstitution of a coupled succinoxidase*

As shown in table 3 vesicles catalyzing oxidative phosphorylation from succinate to oxygen were reconstituted by the cholate-dilution and cholate-dialysis procedure. It can be seen from experiment I that reconstitution of site II plus III can be achieved by either the cholate-dialysis or dilution procedure. The former usually yielded higher P:O ratios and greater differences between Site II plus III and site III phosphorylation. Phosphorylation was sensitive to rutamycin or uncouplers and oxidation of succinate was inhibited by antimycin A (experiment 2).

4. Discussion

There are advantages ond disadvantages associated with each of the reconstitution methods described thus far. The major advantage of the cholate-dialysis procedure is its high degree of reproducibility and it is therefore particularly suited for comparative studies, e.g. effect of phospholipid compositions, etc. Its major disadvantage is that some membrane proteins are inactivated by the prolonged exposure to the detergent. Moreover, the removal of cholate is time consuming so that the method is not convenient when, e.g. fractions obtained during purification of a protein have to be tested. This disadvantage becomes even greater when several days of dialysis are required as in the case of the Na^+ pump of dog kidney [12].

The major advantage of the sonication procedure is that it can be performed in the absence of detergent, it is rapid and the vesicles obtained in some cases have been stable for weeks. Another advantage is that the phospholipid vesicles can be formed at high temperatures (e.g. with dipalmitoyl phosphatidylcholine at 48°C) and then the protein can be incorporated by sonication at somewhat lower temperatures [13]. Finally the activity of vesicles obtained by sonication may be considerably higher than those obtained by the cholate-dialysis procedure as in the case of the proton pump reconstituted with bacteriorhodopsin [3].

The major disadvantage of the sonication procedure is the variability caused by difficulties in controlling sonication. Duplicate samples prepared under seemingly identical conditions of temperature and power output, may vary considerably. Moreover, optimal times of sonication depend on the phospholipid composition so that comparative studies, e.g. on the effect of phospholipids, have limited value. Finally, there are some proteins (e.g. cytochrome oxidase) which are quite sensitive to exposure to sonic oscillation which limits the time of exposure and precludes exploration of optimal conditions for other protein components used in the same reconstitution mixture.

The major advantage of the cholate-dilution described in this communication is rapidity and reproducibility. Activities in several systems tested compare well with those obtained by other reconstitution procedures. However, the cholate-dilution procedure is not suitable for the reconstitution of the proton pump of bacteriorhodopsin, at least under the conditions described in this paper. Probably, other systems requiring more drastic conditions of dissociation may not be responsive to this reconstitution procedure.

Finally, with the demonstration of the reconstitution of succinoxidase catalyzing oxidative phosphorylation all segments of oxidative phosphorylation have now been reconstituted.

Acknowledgements

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