Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation

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XXVI. SPECIFICITY OF PHOSPHOLIPIDS REQUIRED FOR ENERGY TRANSFER REACTIONS

YASUO KAGAWA,† ANNE KANDRACH, AND EFRAIM RACKER

From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850

SUMMARY

1. Vesicles catalyzing $^{32}$P-ATP exchange and ATP-driven proton translocation were reconstituted with chemically defined phospholipids and mitochondrial membrane proteins which were virtually free of electron transport carriers.

2. Phosphatidylcholine and phosphatidylethanolamine were both required for the reconstitution of vesicles with high exchange activity. The optimal ratio of the two phospholipids was variable depending on the source and composition of the phospholipids. With highly purified preparations a 3- to 4-fold excess of phosphatidylethanolamine over phosphatidylcholine yielded optimal rates. An equal molar ratio gave low exchange activity, which was markedly accelerated by low amounts of cardiolipin or another acidic phospholipid. Coenzyme Q_{10} was not required.

3. Synthetic preparations substituted for natural phospholipids. The presence of unsaturated fatty acyl groups in the phospholipid appeared to be essential for the reconstitution of active vesicles. Phospholipids with fully saturated acyl groups were actually inhibitory. On the other hand, phospholipids with unnatural side chains were active provided they contained unsaturated groups.

4. The morphology of the reconstituted vesicles as seen in electron micrographs varied with the different phospholipids used in reconstitution.

5. Optimal conditions of reconstitution of the components which were solubilized with cholate were studied with [carboxy-14C]cholate. Rapid removal of cholate by passage of the components through a Sephadex column resulted in inactivation of the phospholipids. With highly purified preparations a 3- to 4-fold excess of phosphatidylethanolamine over phosphatidylcholine yielded optimal rates. An equal molar ratio gave low exchange activity, which was markedly accelerated by low amounts of cardiolipin or another acidic phospholipid. Coenzyme Q_{10} was not required.

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Oxidative phosphorylation is catalyzed by a multienzyme system which is located in the mitochondrial membrane. It consists of an oxidation chain and a coupling device which utilizes the energy of oxidation to generate ATP from ADP and Pi. The coupling device, which has been separated from the oxidation chain, catalyzes an oligomycin- and uncoupler-sensitive $^{32}$P-ATP exchange and contains an ATPase activity which is inhibited by oligomycin and stimulated by uncouplers (1). The coupling device has been reconstituted from a mixture of phospholipids and of hydrophobic and soluble proteins. There are at least four soluble proteins (coupling factors) required for the $^{32}$P-ATP exchange (cf. Reference 2) and at least two hydrophobic proteins (3). The phospholipids which have been used in the past for the reconstitution of the coupling device were crude mixtures of soybean phospholipids.

It is the purpose of this paper to describe experiments which elucidate the specificity of the phospholipids required for the reconstitution of active vesicles. Properties of the reconstituted vesicles and further studies of optimal conditions of reconstitution will also be reported.

EXPERIMENTAL PROCEDURES

Materials—Reagents, mitochondrial preparations, and auxiliary enzymes were obtained as described previously (1). [carboxy-14C]Cholic acid (5.7 Ci per mole) was purchased from Mallinckrodt Nuclear. Filipin was obtained from Upjohn.

Natural Phospholipids—Cardiolipin, phosphatidylserine and phosphatidylinositol were purchased from General Biochemicals, Inc. They gave single spots in thin layer chromatograms. Crude mixtures of soybean phospholipids or methanol-chloroform extracts of bovine heart mitochondria were purified by washing with dry acetone and by extracting with ether as described previously (1) and enriched in phosphatidylethanolamine by fractionation with ethanol (4). The enriched fraction was placed on a column, (2.5 × 16 cm) of Bio-Rad silicic acid HA minus 325 mesh (1 mg of phosphorus per g of silicic acid) and stored in chloroform-methanol (4:1 v/v) under nitrogen at −20°C. Phosphatidylethanolamine obtained by this procedure gave one major spot in thin layer chromatograms but the phosphatidylethanolamine fraction contained several other phospholipids. Phosphatidylethanolamine which gave one major spot was prepared as follows. An ether extract of 4 g of phospholipids (after extraction with acetone) was taken to dryness and suspended in 25 ml of chloroform.
form. A column (2.5 x 30 cm) of 80 g of silice acid was washed with "hexanes" (Fisher, Pesticide grade) and then thoroughly equilibrated with chloroform. The lipids were applied and eluted with chloroform and 10-ml fractions collected. A large peak containing phosphorus and extraneous yellow material was eluted with about 250 ml of chloroform. A second colored peak was eluted with chloroform-methanol (4:1). When there was no more detectable phosphorus eluted, the solvent was changed to chloroform-methanol (3:2) which eluted phosphatidylethanolamine of about 95% purity.

The following synthetic products were obtained: 1,2-di-palmitoyl phosphatidylethanolamine was purchased from General Biochemicals Inc., and 1,2-di-palmitoyl phosphatidylserine from Sigma. We wish to thank Dr. J. Weissbach and Dr. R. Pfeiffer of Smith, Kline and French, Philadelphia, for generous gifts of 1,2-dihexanoyl and 1,2-di-adamantoyl phosphatidyldihydrocholine; of 1-stearoyl-2-10-undecanoyl, diphytanoyl, and di-undecanoyl phosphatidylethanolamine; and of 1,2-dioleoyl, 1,2-dipalmitoleoyl, and 1-palmityl-2-stearoyl phosphatidylglycerol; Dr. L. L. Van Duren, University of Utrecht, The Netherlands, for 1,2-dilinoleoyl phosphatidylethanolamine; Dr. D. Chapman, Sheffield University, England, for 1,2-dioleyl phosphatidylcholine; Dr. E. C. Robles, Unilever Research Laboratory, Vlaardingen, The Netherlands, for 1,2-dioleoyl and 1,2-distearoyl phosphatidylethanolamine, and Dr. R. Kornberg, Stanford University, for 1,2-dihydrosterculoyl phosphatidylcholine.

The purified phospholipids (20 to 50 moles of phosphorus per ml) were suspended in 10 mM Tricine-KOH, pH 8.0, 2% cholate, 20 mM ammonium sulfate, 25 mM sucrose, 0.1 mM EDTA, and 1 mM dithiothreitol. The suspension of the individual phospholipids (0.1 to 5 ml) was placed in a glass test tube, filled with nitrogen, stoppered, and sonicated for 2 to 5 min in a water bath at room temperature with a Sonblaster model G201 (Narda Chemicals Inc., and L-a-dipalmitoyl phosphatidylethanolamine from Sigma. We wish to thank Dr. J. Weissbach and Dr. R. Pfeiffer of Smith, Kline and French, Philadelphia, for generous gifts of 1,2-dihexanoyl and 1,2-di-adamantoyl phosphatidyldihydrocholine; of 1-stearoyl-2-10-undecanoyl, diphytanoyl, and di-undecanoyl phosphatidylethanolamine; and of 1,2-dioleoyl, 1,2-dipalmitoleoyl, and 1-palmityl-2-stearoyl phosphatidylglycerol; Dr. L. L. Van Duren, University of Utrecht, The Netherlands, for 1,2-dilinoleoyl phosphatidylethanolamine; Dr. D. Chapman, Sheffield University, England, for 1,2-dioleyl phosphatidylcholine; Dr. E. C. Robles, Unilever Research Laboratory, Vlaardingen, The Netherlands, for 1,2-dioleyl and 1,2-distearoyl phosphatidylethanolamine, and Dr. R. Kornberg, Stanford University, for 1,2-dihydrosterculoyl phosphatidylcholine.

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Preparation of Hydrophobic Proteins—Submitochondrial particles of bovine heart were extracted with 2% cholate containing 20 mM ammonium sulfate, 25 mM sucrose, 0.1 mM EDTA, 2 mM dithiothreitol, and 5 mM Tricine-KOH, pH 8.2, and fractionated with ammonium sulfate as described previously (1). The fractions which were precipitated between 0 to 35%, between 33 to 50%, between 25 to 40%, and 25 to 50% saturation of ammonium sulfate were designated as 33P, 33 to 50P, 25 to 40P, and 25 to 50P, respectively. The fractions were dissolved in 10 mM Tricine-KOH, pH 8.0 containing 1 mM dithiothreitol to yield protein concentrations between 10 to 30 mg per ml and were stored at −70°C. The fractions could be lyophilized after freezing in liquid nitrogen in the presence of 0.2 M sucrose. These preparations remained active even after being stored at room temperature for several days.

Reconstitution of Active Vesicles—Reconstitution of active vesicles was carried out as described previously (1) except that

1 The abbreviations used are: Tricine, N-tris(hydroxymethyl) glycine; F1, F0, F1, F0, and F, coupling factor 1 (ATPase) 2, 3, 5, and 6, respectively; Q10, ubiquinone or coenzyme Q10; OSCP, oligomycin sensitivity-conferring protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
phospholipid which was recovered in the supernatant fraction. These proteins contained a large proportion of acidic cardiolipin which was difficult to remove. It should be noted that there was almost absolute dependence on added phospholipids for reconstitution of active vesicles (1). It is therefore apparent that the residual phospholipids in the protein fraction were either chemically altered or insufficient in amount to yield active vesicles. On the other hand a possible participation of these phospholipids in conjunction with added phospholipids has not been ruled out.

Gas chromatographic analysis of the fatty acid composition of the protein fractions and of the natural phospholipids used in reconstitution is shown in Table I. Although the highly unsaturated arachidonic and eicosatrienoic acid residues found in mitochondria were not present in soybean phospholipids, the average unsaturation in the latter preparations was actually slightly higher because of high content of linoleic and linolenic residues. The acyl groups of the residual phospholipids in the hydrophobic protein fractions were usually more saturated than those of crude mitochondrial phospholipids which may contribute to the difficulties encountered in removing these residual phospholipids without damage to the protein. The Q_{10} content of the hydrophobic protein fraction was quite low, varying between 0.34 and 0.47 n mole per mg of protein, compared to about 4 n moles per mg of submitochondrial particle protein.

Reconstitution of Active Vesicles with Purified Natural Phospholipids—In view of the different fatty acid composition of bovine and soybean phospholipids, crude bovine phospholipids and partially purified preparations were substituted for the crude soybean lipids. It can be seen from Table II that the crude bovine phospholipids were either equally or slightly less effective in reconstitution than soybean lipids. It was also noted that a mixture of phosphatidylethanolamine and partially purified phosphatidylethanolamine substituted for the crude mixture. However, again relatively large amounts were required and no significant differences were found between phospholipids from bovine heart or soybeans.

A titration with highly purified preparations of phosphatidylethanolamine and phosphatidylethanolamine revealed that an excess of phosphatidylethanolamine over phosphatidylethanolamine was required for optimal activity (Fig. 1). Some variability was noted in the apparent optimal ratio, depending on the origin and composition of the phospholipids. Particularly the exchange activity of vesicles reconstituted with phosphatidylethanolamine alone was quite variable. With some preparations of phosphatidylethanolamine (Fig. 1) considerable activity was noted, while others, as will be shown later, gave low activity. Nevertheless it is apparent that both phosphatidylethanolamine and partially purified phosphatidylethanolamine were required for the reconstitution of vesicles with appreciable \(^{32}\)P-ATP exchange activity.

Reconstitution of Active Vesicles with Synthetic Phospholipids—Since the best phospholipids from natural sources were only about 95% pure, it was desirable to eliminate the possibility that a trace contamination was responsible for activity. Chemically synthesized phospholipids were therefore tested. As can be seen from Table III, either phosphatidylethanolamine or phosphatidylethanolamine from natural sources could be substituted by
corresponding synthetic phospholipid. Dilinolenoyl phosphatidylcholine was as good or better than the natural compound and was completely inactive by itself (Experiment 2). Synthetic dihydrosterculoyl phosphatidylcholine was also an excellent substitute for the natural mixture and had little activity when tested alone (Experiment 3). Considerable exchange activity was observed also when two synthetic phospholipids were used (Experiment 2). It is of interest to note that the phosphatidylethanolamine (1-stearoyl-2-undecenoyl) used in this experiment has an unnatural side chain. Synthetic distearoyl phosphatidylethanolamine was inactive and this saturated phospholipid was in fact inhibitory when included with the two synthetic active preparations (Table III, Experiment 4). Similar observations were made with synthetic dipalmitoyl phosphatidylcholine. The rate of 79 nmoles/10 min per mg of protein with the two synthetic phospholipids is particularly significant in view of the fact that the tests were carried out at arbitrary ratios because of limited availability of linolenoyl phosphatidylcholine.

A titration with two synthetic phospholipids is shown in Fig. 2. Of particular interest is that high exchange rates were observed with diphytanoyl phosphatidylcholine which contains two unphysiological side chains and 1-steinrayl-2-undecenoyl phosphatidylcholine which contains one unphysiological side chain. The 3H-ATP exchange activity of 245 nmoles/10 min per mg of proteins at the optimal ratio of 2 moles of phosphatidylcholine to 3 moles of phosphatidylethanolamine is within the range of values obtained with natural phospholipids. There was virtually no exchange reaction when 5 moles of phosphatidylethanolamine alone and only about 20% of the maximal rate when 5 moles of diphytanoyl phosphatidylcholine alone were used in the reconstitution. It is of interest that the highest rates were obtained when the ratio of the two phospholipids was close to unity, similar to the ratio found in the natural mitochondrial membrane. A synthetic preparation of diphytanoyl

![Fig. 1](http://www.jbc.org/)

**Effect of synthetic phospholipids on reconstitution**

Preparation of phospholipids and reconstitution was carried out as described under "Experimental Procedure." The numbers in parentheses indicate the micromoles of the phospholipid added in the reconstitution with 1 mg of hydrophobic protein. The first three experiments were carried out with 25 to 50P; Experiment 4 with 25 to 40P.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Phospholipids in reconstitution</th>
<th>3H-Pi-ATP exchange (nmoles/10 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soybean PC(2) + soybean PE(2)</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>Soybean PC(1) + diolyl PE(1)</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Soybean PC(2.5) alone</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Diolyl PE(2) alone</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>Soybean PC(2.5) + soybean PE(2.7)</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Soybean PE(5.4)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Diilinolenoyl PC(2.5) + soybean PE(2.7)</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Diilinolenoyl PC(4)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diilinolenoyl PC(2.5) + 1-steinrayl-2-undecenoyl PE(2.8)</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>1-Steinrayl-2-undecenoyl PE(2.6)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Diilinolenoyl PC(2.5) + 1-steinrayl-2-undecenoyl PE(2.6) + distearoyl PE(1.8)</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>Soybean PE(5.4)</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Dihydrosterculoyl PC(5.0)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Soybean PE(2.6) + dihydrosterculoyl PC(5.0)</td>
<td>555</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Soybean PC(2) + soybean PE(2)</td>
<td>308.5</td>
</tr>
<tr>
<td></td>
<td>Dipalmitoyl PC(2) + dipalmitoyl PE(2)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Soybean PC(4)</td>
<td>22.4</td>
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<td></td>
<td>Soybean PE(3.4)</td>
<td>60.8</td>
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<tr>
<td></td>
<td>Dipalmitoyl PC(2) + soybean PE(3)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Dipalmitoyl PE(3) + soybean PC(3)</td>
<td>0.0</td>
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</tbody>
</table>

![Fig. 2](http://www.jbc.org/)

**Table III**

Effect of synthetic phospholipids on reconstitution

Preparation of phospholipids and reconstitution was carried out as described under "Experimental Procedure." The numbers in parentheses indicate the micromoles of the phospholipid added in the reconstitution with 1 mg of hydrophobic protein. The first three experiments were carried out with 25 to 50P; Experiment 4 with 25 to 40P.
phosphatidylethanolamine was active when tested in the presence of soybean phosphatidylethanolamine.

Phosphatidylglycerol with 1,2-divaeeenoyl, 1,2-dipalmitoleyl, and 1-palmitoyl-2-vaccenoyl side chains did not substitute for either phosphatidylethanolamine or phosphatidylinositol. Incorporation of cholesterol during reconstitution had no effect. Moreover, the exchange activity of vesicles containing cholesterol was not inhibited by Filipin.

Effect of Acidic Phospholipids on Reconstitution—In earlier experiments it was observed that acidic phospholipids did not substitute for either phosphatidylethanolamine or for phosphatidylcholine in reconstitution and in fact inhibited when included during the reconstitution of activity phosphorylating vesicles. It was later observed however that inclusion of cardiolipin at low concentrations markedly stimulated the 32Pi-ATP exchange activity of the reconstituted vesicles (Fig. 3) with amounts of phosphatidylethanolamine and phosphatidylinositol which showed low activity by themselves. It is of interest that optimal activity was obtained in the presence of cardiolipin when the ratio of phosphatidylethanolamine per phosphatidylinositol was close to unity as it is in the natural mitochondrial membrane.

As can be seen from Table IV, phosphatidylserine or phosphatidylethanolamine acted similarly to cardiolipin but were not as effective. Higher concentrations of the acidic-phospholipids were less effective or even inhibitory thus explaining the failures of previous attempts.

Reincorporation of Phospholipids by Sonication—In view of the dual phospholipid requirement it was of interest to explore the possibility whether one phospholipid was preferentially required for reconstitution and another for the activity of reconstituted vesicles. Since some incorporation of phospholipids into vesicles can be achieved by sonication (10) the experiment shown in Table V was designed. It can be seen that considerable exchange activity was present when either phosphatidylethanolamine or phosphatidylinositol was incorporated into the vesicles by sonication after formation of the vesicles had been completed.
which was required for the solubilization of the hydrophobic proteins. As shown in Table VI reconstituted vesicles contained reactions it was essential to remove over 95% of the cholate per mg of protein) were already inhibitory for energy transfer of cholate were retained when the critical micelle concentration was exceeded. Since small amounts of the detergent (500 pg per mg of protein) were used in the reconstitution process as been discussed in detail. As shown in Fig. 4, considerable amounts of cholate (containing 0.7 μmole of phospholipid per mg of protein) were used in the reconstitution.

Table VI
Retention of cholate after reconstitution and dialysis

In a final volume of 0.5 ml, 2 mg of 25 to 40P was reconstituted at pH 8.0 with 10 mg of (acetone-washed) soybean phospholipids in the presence of the indicated amounts of sodium [3H]cholate (1750 cpm per mg) as described under "Experimental Procedure." Prior to the addition of cholate, the phospholipids were sonicated in 10 mM Tricine-KOH, pH 8.0, 1 mM dithiothreitol, and 100 mM ammonium sulfate until clarification was achieved. Dialysis and assays were performed as described under "Experimental Procedure." except that 0.2 ml sodium chloride was present in the dialysis fluid. cmc, critical micelle concentration.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Initial cholate</th>
<th>Cholate remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 to 50P + phosphatidylethanolamine (PE)</td>
<td>3.0</td>
<td>0.058</td>
</tr>
<tr>
<td>33 to 50P + phosphatidylycholine (PC)</td>
<td>3.0</td>
<td>0.108</td>
</tr>
<tr>
<td>33 to 50P + PE (2 μmole) + PC (2 μmole)</td>
<td>3.0</td>
<td>0.133</td>
</tr>
<tr>
<td>25 to 50P + crude soybean phospholipid</td>
<td>6.0</td>
<td>0.142</td>
</tr>
<tr>
<td>Submitochondrial particles</td>
<td>0.1</td>
<td>0.007</td>
</tr>
<tr>
<td>Submitochondrial particles</td>
<td>1.0</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Effect of Cholate on Reconstitution of Active Vesicles—The role of detergents on the reconstitution process has been discussed in detail (11). As shown in Fig. 4, considerable amounts of cholate were required for reconstitution. These optimal amounts yield a cholate to phospholipid ratio in the range required to achieve isotropic solution (4). Concentrations above 4 mg of cholate per mg of protein in the presence of 0.1 mM ammonium sulfate yielded less active vesicles. It can be seen that large amounts of cholate were retained when the critical micelle concentration was exceeded. Since small amounts of the detergent (500 μg per mg of protein) were already inhibitory for energy transfer reactions it was essential to remove over 95% of the cholate which was required for the solubilization of the hydrophobic proteins. As shown in Table VI reconstituted vesicles contained less than 200 μg of cholate per mg of protein, an amount which was not inhibitory when added to phosphorylating reconstituted vesicles. More extensive dialysis resulted in further removal of cholate but had little effect on the phosphorylating capacity of the particles. The amount of cholate which remained in the particles after dialysis was a function of their phospholipid composition as indicated by the higher residual values found in vesicles reconstituted with phosphatidylethanolamine. The higher binding capacity of the latter was also ascertained by equilibrium dialysis experiments. Nonpolar lipids such as cholesterol or monoglycerides bound cholate even more avidly (data not shown).

Reconstitution of Active Vesicles by Gel Filtration Chromatography—It was shown previously (1) that rapid removal of cholate from the reconstituted mixture of phospholipids and hydrophobic proteins yielded preparation with no or low activity. It was therefore thought likely that assembly of the membrane takes place when a critical concentration of cholate is reached during the dialysis procedure. Reconstitution of vesicles was therefore attempted by passage of the cholate-containing mixture through Sephadex columns which were equilibrated with various concentrations of cholate and phospholipids. It can be seen from Table VII that passage through columns equilibrated with 0.5% cholate and 0.2% soybean phospholipids yielded vesicles which were no active as those obtained by the dialysis procedure. Particles obtained from columns at 0.2% cholate concentration were about half as active. Submitochondrial particles treated under identical conditions were noted to lose activity after exposure to cholate. At 0.5% cholate, submitochondrial particles were
and phosphatidylcholine accumulated H+ from the medium at pH 6.25 on addition of 10 to 40 nmoles of ATP and MgSO₄. Under these conditions the chemical hydrolysis of ATP does not give rise to a pH change. As can be seen from Fig. 5, A and B, on addition of ATP-Mg⁺⁺ to the reconstituted vesicles caused an uptake of protons which was eliminated by carbonyl cyanide p-trifluoromethoxyphenylhydrazone or rutamycin. When the vesicles were reconstituted with either phosphatidylethanolamine or phosphatidylcholine alone, H⁺ uptake was not observed (Fig. 5, C and D). Similarly, stimulation of ATPase activity by carbonyl cyanide p-trifluoromethoxyphenylhydrazone was only observed when both phospholipids were present in the reconstituted vesicles whereas oligomycin-sensitive ATPase was present in vesicles reconstituted with a single phospholipid. The decay of the pH gradient was much slower in the reconstituted vesicles (t½ = 50 to 100 s) than in submitochondrial particles (t½ = 20 s). The slow leakage of H⁺ may be caused by the high content of phospholipids in the reconstituted vesicles.

Electron Microscopy of Vesicles Reconstituted with Different Phospholipids—Reconstitution with hydrophobic proteins and unsaturated phosphatidylethanolamine usually gave thin walled, extended, and aggregated figures (Fig. 6, a and A). Vesicles reconstituted with unsaturated phosphatidylethanolamine gave thick walled, often multilayered smaller vesicles (Fig. 6, b and B). The vesicles reconstituted with both phospholipids (which were active both in ³²P₁-ATP exchange and H⁺ accumulation) gave single layered round vesicles (Fig. 6, c and C). Similar results were obtained with synthetic unsaturated phospholipids. With dinolenoyl phosphatidylethanolamine and hydrophobic proteins large thin walled vesicles were seen (Fig. 7). However, unless phosphatidylethanolamine was present during reconstitution these vesicles were inactive in ³²P₁-ATP exchange. With synthetic saturated lipids of any combination, the reconstituted preparations appeared polymorphous and sometimes crystalline. As reported earlier (1, 12) phospholipids diazyl without proteins formed multilayered myelin figures which could be converted by sonication into vesicular structures.

DISCUSSION

The experiments with synthetic phospholipid are significant from three points of view. First, we find that highly purified phospholipids from natural sources reveal small amounts of contaminants when examined by two-dimensional thin layer chromatography. Thus, it was necessary to rule out the possibility that one or the other minor component is essential for the exchange activity. Secondly, the experiments with the synthetic phospholipids established that a natural fatty acyl group is not required for the reconstitution. It is noteworthy that whenever active preparations with synthetic phospholipids were obtained, electron micrographs (not shown) revealed the presence of vesicular structures. Thirdly, the requirement for the presence of unsaturated acyl groups in the phospholipids is in line with the dependency of oxidative phosphorylation in yeast on the presence of unsaturated fatty acids (13). With saturated phospholipids the failure to form vesicular structures in the presence of hydrophobic proteins and to obtain active preparations is yet another correlation in this relationship of structure and function.

We conclude from these comparative studies of the structure and function of reconstituted preparations that a vesicular structure appears to be required for the operation of an uncoupler-sensitive ³²P₁-ATP exchange and ATP-driven proton accumulation. It is also apparent from the experiments with single phospholipids that formation of a vesicular structure was not sufficient for the exchange reaction, even when the protein components of a potential phosphorylating apparatus were present. It is not clear as yet why multiple phospholipids are required for the formation of active vesicles, but the striking differences observed

Fig. 5. ATP-driven H⁺ accumulation in reconstituted vesicles. Ten milligrams of 25 to 50P fraction and 50 mg of phospholipids, as indicated below, were mixed with 25 mg of sodium cholate (pH 8.0), 3 mg of F₄, 1 mg of F₁, 1 mM dithiothreitol, 0.1 M ammonium sulfate, 10 mM Tricine-KOH (pH 8.0) in a final volume of 1.5 ml and dialyzed at 4°C against 100 volumes of dialyzing buffer (1) containing 0.2 M NaCl. The buffer was changed every 2 hours during the first 8 hours. Since the coupling factor preparations contain ammonium sulfate their inclusion during reconstitution followed by dialysis was essential to remove the residual ammonium sulfate. After further 12 hours of dialysis the reconstituted vesicles (0.8 to 1.22 mg) were put into a pH-measuring chamber (9) containing 1.1 ml of 150 mm KCl, 2 mm glycyglycine buffer, pH 6.25. After 5 min, the buffering power of the solution was determined by adding 24.2 nmoles of HCl followed by further additions as indicated: valinomycin (0.2 μg), ATP-Mg (40 nmoles, pH 6.25), rutamycin (2 μg), and carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) (2 nmoles). A, vesicles reconstituted with the acetone-washed asolectin; B, vesicles reconstituted with the equal amounts of purified soybean phosphatidylethanolamine and phosphatidylethanolamine; C, vesicles reconstituted with soybean phosphatidylethanolamine; D, vesicles reconstituted with soybean phosphatidylethanolamine.

in fact consistently less active than reconstituted vesicles. Independent tests on the comparative sensitivity of submitochondrial particles and reconstituted vesicles revealed that the latter were more resistant to cholate presumably because of their higher content in phospholipids.

Properties of Reconstituted Vesicles—As mentioned earlier, reconstituted vesicles had a relatively high phospholipid content (1). They were not sedimented by centrifugation at 80,000 × g for 2 hours in a buffered solution containing 0.4 M sucrose. The vesicles had a low buoyant density of 1.05.

The vesicles reconstituted with both phosphatidylethanolamine and phosphatidylcholine accumulated H⁺ from the medium at pH 6.25 on addition of 10 to 40 nmoles of ATP and MgSO₄. Under these conditions the chemical hydrolysis of ATP does not give rise to a pH change. As can be seen from Fig. 5, A and B, on addition of ATP-Mg⁺⁺ to the reconstituted vesicles caused an

² W. S. Thayer, and P. C. Hinkle, unpublished observations.
Fig. 6. Electron micrographs of vesicles reconstituted with purified phospholipids. The vesicles were reconstituted from 25 to 40% with phospholipids purified from soybean phospholipids, as described under "Experimental Procedure." (Phospholipid-cholate-protein = 5:2:1). a and A: vesicles reconstituted with phosphatidylcholine. a, thin sectioning, X 45,000; A, negative staining, X 120,000; b and B: vesicles reconstituted with phosphatidylethanolamine. b, thin sectioning, X 45,000; B, negative staining, X 120,000; c and C: vesicles reconstituted with both phosphatidylethanolamine and phosphatidylcholine (equal weight). c, thin sectioning, X 45,000; C, negative staining (33 to 50% instead of 25 to 40%), X 120,000.
in electron micrographs suggest that the assembly of vesicular structures in the presence of hydrophobic proteins is profoundly influenced by the composition of the phospholipids.

The requirements for multiple phospholipids and for vesicular structures in the energy transfer reactions discussed above are quite different from the phospholipid requirements of the oligomycin-sensitive ATPase (14) and of the electron transport chain.

Our failure to observe an uncoupler-sensitive \( ^{32} \text{P}\) -ATP exchange in the absence of vesicles seems contrary to observations recorded with "soluble" preparations of \( ^{32} \text{P} \) synthetase (16). Through the courtesy of Dr. Sanadi we have examined two samples of such preparations and found that they contained small amounts of vesicles which in our opinion would be sufficient to account for the low rates of uncoupler-sensitive \( ^{32} \text{P}\) -ATP exchange. But it is difficult under the circumstances to make quantitative correlations. Moreover, we cannot conclude from the fact that we observed an absolute dependency on phospholipids that another system may not be capable of catalyzing an uncoupler-sensitive \( ^{32} \text{P}\) -ATP exchange under different experimental conditions.

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