Purified Mitochondrial Phosphate Transport Protein. Improved Proteoliposomes and Some Properties of the Transport Protein Sulfhydryl Group(s)†

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ABSTRACT: The incorporation of the beef heart mitochondrial phosphate transport protein, copurified with several minor proteins and the adenine nucleotide translocase, into liposomes has been improved (a) by including the calcium salt of phosphatidic acid in the modified lipid mixture used in the preparation of the proteoliposomes, (b) by freezing the higher lipid/protein ratio mixture during rapid and uniform agitation in liquid nitrogen, and (c) by briefly vortexing the thawed lipid/protein mixture. The transport-active proteoliposomes are kinetically homogeneous, and due to gentle dispersion by vortexing, transport-inactive vesicles can be removed by centrifugation, increasing the V_{max} [22 °C, net (zero trans), pH_i = 8.0, pH_e = 6.8] to 0.22 mmol of phosphate min^{-1} (mg of phosphate transport protein)$^{-1}$ or a turnover number of 7×10^3 min^{-1} for a 34-kilodalton (kDa) monomer or 1.4×10^4 min^{-1} for a 68-kDa dimer. Net (zero-trans) phosphate efflux has a ΔpH dependence and initial transport rate similar to net (zero-trans) phosphate uptake (preferential transport from acidic to basic pH media). The phosphate transport protein is 70–80% inactivated by removal of dithiothreitol from the air-equilibrated suspension medium. The inactivation is readily reversed by dithiothreitol and much less readily by β-mercaptoethanol. Of the reconstituted phosphate transport activity, 50% is inhibited by 100 μM phenylarsine oxide. The autoxidation-inhibited protein can be reactivated by dithiothreitol after exposure to *N*-ethylmaleimide, but the active protein cannot. These results suggest that a dithiol in the phosphate transport protein may form a disulfide during autoxidation. Partial inhibition of transport by *N*-ethylmaleimide or autoxidation decreases the V_{max} and causes a 2–3-fold increase in the K_m. These results suggest either that the *N*-ethylmaleimide-reactive SH group(s) is (are) not essential for transport, that two (or more) different SH groups react with *N*-ethylmaleimide, or that an inhomogeneity in the preparation can be detected under these conditions.

The mitochondrial phosphate transport protein, copurified with several minor proteins and the adenine nucleotide translocase from beef heart, has recently been incorporated as an active transporter into liposomes (Wohlrab, 1980a–c). A modified purification procedure resulted in a transport-active preparation from pig heart that consisted of several major proteins [30–35 kilodaltons (kDa)[1]] with an apparent deficiency in the ADP/ATP carrier (Kolbe et al., 1981; Durand et al., 1981). We have recently been able to prepare a highly active phosphate transport protein without those minor proteins and have demonstrated that contrary to published results (Kadenbach et al., 1982) these proteins do not catalyze phosphate transport (H. Wohlrab et al., unpublished results). We have separated the phosphate transport protein from the adenine nucleotide translocase in the presence of sodium dodecyl sulfate and determined its amino acid composition (Wohlrab et al., 1981; H. V. J. Kolbe et al., unpublished results).

We report now on a modified phosphate transport protein/liposome preparation that catalyzes high net (zero-trans) phosphate transport rates and that as a reconstituted preparation is highly reproducible. We also demonstrate in this paper additional properties that confirm the protein's identity with the mitochondrial phosphate transporter. We have carried out some experiments on the SH group(s) of the phosphate transport protein and report that two SH groups may be located sufficiently close to each other to form a disulfide bond during autoxidation, which results in a dramatically lower V_{max} for the catalyzed transport.

Materials and Methods

Materials. Phospholipids (plant phosphatidylethanolamine, calcium and sodium egg phosphatidic acid, plant phosphatidylcholine, and calcium, magnesium, and sodium cardiolipin) were obtained from Avanti Polar Lipids (Birmingham, AL). Various other lipid samples were obtained from Sigma (St. Louis, MO), Calbiochem (La Jolla, CA), Serdary (London, Ontario), P-L Biochemicals (Milwaukee, WI), and Supelco (Bellefonte, PA); carrier-free [^{32}P]P_i (in dilute HCl) was from Amersham (Arlington Heights, IL), Triton X-100 was from Sigma (St. Louis, MO), asolectin, used without further purification, was from Associated Concentrates (Woodside, L.I., NY), glass-distilled chloroform and methanol were from Burdick and Jackson (Muskegon, MI), and pH paper was from EM Industries (Gibbstown, NJ).

Purification of the Phosphate Transport Protein. Our most recent procedure (Wohlrab & Flowers, 1982) was further modified. Three grams of hydroxylapatite powder (Bio-Rad) was added to 18 mL of medium B [10 mM sodium phosphate, 0.1 mM EDTA, 130 mM sodium sulfate, 5 mM dithiothreitol, and 0.5% (v/v) Triton X-100, pH 7.2]. The slurry was poured into a 1 × 15 cm column, and after the hydroxylapatite had settled, the column was washed with 35 mL of medium B. The column was equilibrated to 4 °C overnight. The solubilized mitochondria, after centrifugation and BioBeads SM2 treatment, were added onto the hydroxylapatite column, and 2-min

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[1] Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; CL, cardiolipin; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane.

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fractions (about 0.80 mL each) were collected. The high OD (275 nm) fractions were pooled, mixed with sonicated lipids, mixed and stirred with BioBeads SM2 (0.5 g/mL suspension, 45 min, 4 °C), frozen in 200-μL aliquots as described before, and stored in a liquid nitrogen freezer (Wohlrab & Flowers, 1982).

Improvements in the method used to preserve the activity of the frozen phosphate transport proteins include the addition of 250 μL of dispersed phosphatidylcholine (4 mg) instead of the original CL/PC/PE lipid mixture per 6.2 mL of pooled hydroxylapatite eluate. Also, 20% (v/v) glycerol was added to the BioBead SM2 treated phosphate transport protein after removal of the BioBeads SM2 and before freezing in liquid nitrogen in experiments were indicated (H. Wohlrab et al., unpublished results).

Preparation of Liposomes. Liposomes for reconstitution were prepared from high-purity commercial lipids as described (Wohlrab & Flowers, 1982). Calcium/sodium egg phosphatidic acid batch 835 (Avanti) was used in most experiments, except where noted. The vacuum-dried lipids (mostly 16 h, but as little as 2 h under high vacuum at room temperature is sufficient) were sonicated with medium C_i [10 mM Tris base and 10 mM 1,4-piperazinediethanesulfonic acid, pH_i (intravesicular pH)] for 6 min at 10 °C to make an optically clearer dispersion which resulted in a somewhat larger amount of phosphate being taken up in 30 s by the reconstituted proteoliposomes.

Incorporation of the Phosphate Transport Protein into Liposomes. The thawed phosphate transport protein (200 μL) was vortexed for 1 s before being transferred to a plastic test tube (Sarstedt, 3.5 mL, no. 55.484). The solution was passed through a Bio-Gel P-6DG (Bio-Rad) column (0.7 × 6cm) that had been equilibrated at 4 °C with solution C_i plus 5 mM dithiothreitol. The phosphate transport protein was diluted less than 2-fold by this medium exchange step. Of this solution (15–20 μg of phosphate transport protein per mL), 60 μL was mixed with 110 μL of liposomes plus 25 μL of medium C_i made, in some experiments as mentioned, to 0.6% (v/v) glycerol. The mixture was vortexed (2 s) under high-purity argon; the test tube was stoppered and mounted in a drill press. The spinning test tube was slowly lowered into liquid nitrogen. The mixture froze evenly along the wall of the test tube with no material frozen at the round bottom; it was thawed and vortexed at low speed for 8 s at room temperature. The vortexing disperses all visually obvious large lipid/protein aggregates. The preparation, stable for at least 24 h at 4 °C, was stored on ice and used immediately for transport experiments or centrifuged first to remove transport-inactive liposomes, proteoliposomes, or protein/lipid aggregates that accumulate in the pellet. All transport-active proteoliposomes remain in the supernatant (see Net Efflux of Phosphate from Proteoliposomes under Results).

Phosphate Transport Assay. The transport experiments were carried out as described (Wohlrab & Flowers, 1982). Mersalyl (624 μM) was used to stop the transport. The anion-exchange resin (AG1-X8, chloride, 50–100 mesh, Bio-Rad) was converted in a big batch to the formate form to increase the amount of $[^{32}P]P_i$ retained. Each anion-exchange column (4 °C) was equilibrated before use with 500 μL of bovine serum albumin (30 mg/mL H_2O) and liposomes (1 mL, 1.4 mg of lipid/mL); i.e., part of the liposomes, used for reconstitution, were diluted to 1.4 mg/mL with water. Up to 30 different samples were applied to a single column. Two external medium samples [each 500 μL, no proteoliposomes], each followed by 3 mL of solution GNN [5% (v/v) glycerol/0.1 mM sodium azide], were applied to each column before the application of samples with proteoliposomes in order to monitor the $[^{32}P]P_i$ binding capacity of the column (99.995% of the $[^{32}P]P_i$ of each of these medium samples is bound by the column). After application of each sample with proteoliposomes, a sample without proteoliposomes was applied to remove a less than 10% $[^{32}P]P_i$ tail that is left by the sample with proteoliposomes and that would, by eluting together with the next sample, contribute to its $[^{32}P]P_i$ counts. This is especially important if a high $[^{32}P]P_i$ uptake sample is followed by a low $[^{32}P]P_i$ uptake sample. The amount of $[^{32}P]P_i$ from a transport medium sample without proteoliposomes not retained by the column at the end of the experiment is as low as at the beginning, demonstrating that the capacity for $[^{32}P]P_i$ binding of the column has not become limiting.

Conversion of Sodium Phosphatidic Acid to Calcium Phosphatidic Acid. The conversion of sodium phosphatidic acid to calcium phosphatidic acid was carried out as follows. Into a conical, ground-glass-stoppered, and graduated 40-mL centrifuge tube was added 2 mL of sodium phosphatidic acid (20 mg/mL chloroform), and the tube was flushed with high-purity argon. Then 3.3 mL of chloroform, 0.7 mL of methanol, and 0.7 mL of water were added, the gas phase was flushed with argon, and the contents were vortexed at maximum speed for 15 s. Four 20-μL aliquots of 1 N HCl were added, each followed by 15 s of vortexing; then a 15-μL 1 N HCl aliquot was added followed by vortexing for 1 min. The pH of the solution should be 2.0–2.5. The solution is then centrifuged for 1 min (all centrifugation steps at room temperature and 570*g*) and the supernatant discarded. H_2O (0.7 mL) is added, the mixture vortexed for 1 min and centrifuged for 1 min, and the supernatant discarded. The small amount of white interphase material is not discarded. A 0.7-mL sample of CaOH (30 mg/20 mL of H_2O) is added and vortexed for 30 s, and again 0.7 mL of CaOH is added, vortexed for 30 s, and centrifuged for 15 min. The clear supernatant is discarded; then 0.7 mL of CaOH is added and vortexed for 30 s, 0.7 mL of CaOH is added and vortexed for 30 s, and 0.5 mL of CaOH is added and vortexed for 30 s. The pH of the solution should now be between 6.0 and 7.0. Centrifuge 15 min. Discard the clear supernatant. Add 2 mL of H_2O, vortex, centrifuge, and discard the clear supernatant. Repeat the water washing step once more. Remove chloroform with high-purity argon and the residual water with high vacuum. Take up dried lipids in 2 mL of chloroform, transfer lipids to an amber glass vial, seal under high-purity argon, and store at −80 °C. The concentration of the calcium phosphatidic acid was determined by the dry weight and by organic phosphate analysis (Ames, 1966).

Other Methods. Protein was determined in the presence of sodium dodecyl sulfate and *N*-ethylmaleimide by using the Lowry method (Lowry et al., 1951) or by scanning the Coomassie Brilliant Blue R250 (Serva) strained protein bands of the phosphate transport protein and the ADP/ATP carrier in sodium dodecyl sulfate–polyacrylamide slab gels. Various concentrations of human carbonic anhydrase were also electrophoresed on the same gel and stained. The integrated absorbance bands were used to generate an OD vs. protein concentration plot. A correlation of amino acid concentration with Coomassie Blue stained phosphate transport protein and human carbonic anhydrase bands shows clearly that the human carbonic anhydrase stained within 10% as intensely per milligram of protein as the nonalkylated beef heart phosphate transport protein (H. V. J. Kolbe et al., unpublished results). Inorganic and lipid phosphorus was determined according to

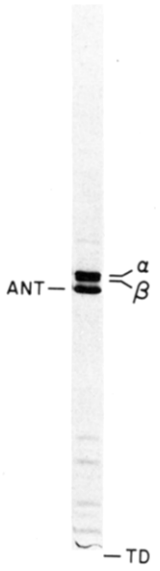

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the phosphate transport protein preparation from beef heart mitochondria. The two phosphate transport protein bands are labeled α and β. ANT is the ADP/ATP carrier, and TD is the position of the tracking dye. The gel was silver stained.

Ames (1966). Phospholipid species were determined by applying microliter quantities of pure phospholipids, proteoliposomes, or fractions directly to Whatman K_6 thin-layer plates equilibrated and developed with chloroform/methanol/28% ammonia (65/35/5) (basic system) or chloroform/acetone/methanol/acetic acid/H_2O (50/20/10/10/5) (acidic system). In the basic system, phosphatidic acid and lysophosphatidic acid have R_f's of about 0.85 and 0.50, respectively. In the acidic system, lysophosphatidic acid remains while phosphatidic acid barely moves away from the origin. The phospholipids were detected with Phospray (Supelco). The concentrations of commercial phospholipids were confirmed by dry weight determinations. Calcium concentrations were determined by atomic absorption. Sodium dodecyl sulfate–polyacrylamide slap gel electrophoresis was carried out essentially according to Laemmli (1970) (16% acrylamide) with an acrylamide/bis(acrylamide) ratio of 150/1, with 750 mM Tris in the separation buffer and 3 mM mercaptoacetic acid in the running buffer (H. V. J. Kolbe et al., unpublished results). The polyacrylamide slab gels were silver stained with minor modifications according to Morrissey (1981).

Results

Reconstitutively Active Phosphate Transport Protein. Our phosphate transport protein preparation consists of the phosphate transport protein (Wohlrab, 1980), which (if not *N*-ethylmaleimide alkylated) runs as two bands (α and β) in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the ADP/ATP carrier (Figure 1). We have purified α and β and find no obvious differences between them (H. V. J. Kolbe et al., unpublished results). The ADP/ATP carrier copurifies, and it is unlikely that other reconstitutively active preparations are deficient in the ADP/ATP carrier with respect to the phosphate transport protein (H. Wohlrab et al., unpublished results). The phosphate transport protein concentration (α and β) was determined from Coomassie Blue stained sodium dodecyl sulfate–polyacrylamide gels (see Other Methods under Materials and Methods).

Incorporation of the Phosphate Transport Protein into Liposomes Using Different Freezing Methods and Lipid/Protein Ratios. We used the freezing method developed by

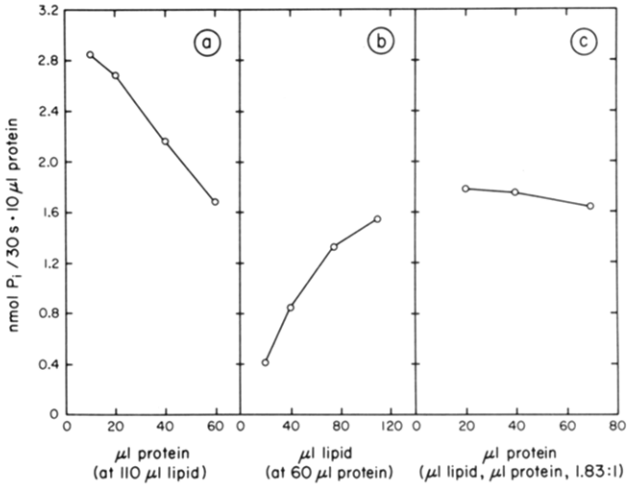

FIGURE 2: Effect of changes in the amount of lipid and protein on reconstituted net (zero-trans) phosphate transport activity. (a) Reconstitution was carried out by keeping the reconstitution mixture at 195 μL. The volume of liposomes was kept at 110 μL, the volume of phosphate transport protein was varied, and the total volume was kept constant by adding various volumes of medium C_i. (b) Similar to (a) except the volume of protein solution was kept constant, the volume of lipid solution was varied, and the total volume was kept constant with medium C_i. (c) The ratio of lipid volume to protein volume was kept constant, the total lipid/protein volume was varied, and the total volume of the reconstitution mixture was made 195 μL with medium C_i. The lipid mixture was kept at PE/PC/PA 53/38.3/8.7 (w/w/w) with pH_i = 8.0, pH_e = 6.8, $[P_i]_i$ = 0 mM, and $[P_i]_e$ = 1 mM.

Kasahara & Hinkle (1977) as well as modifications in order to increase the maximum (net, zero-trans) phosphate uptake within 30 s and the reproducibility of transport-active proteoliposome preparations. The variability and extent of the net phosphate uptake [nanomoles of P_i per 30 s, ±standard deviation of two reconstitutions each with two different protein preparations, pH_i = 8.0, pH_e = 6.8, $[P_i]_i$ = 0 mM, $[P_i]_e$ = 1 mM] catalyzed by proteoliposomes prepared by different methods were the following: (a) water/ethylene glycol bath (−15 °C), 0.50 ± 0.63; (b) freezer compartment (−20 °C), 0.93 ± 0.48; (c) liquid nitrogen, 1.14 ± 0.11; (d) dry ice/ethanol bath, 1.13 ± 0.22; (e) spin/freeze liquid nitrogen (see Materials and Methods), 1.31 ● 0.04. The spin/freeze liquid nitrogen method yielded reproducibly very high phosphate uptake and was used in all experiments.

No phosphate uptake is observed if the thawed reconstitution mixture is not dispersed to some extent. Sonicating it for 30 or 5 s at 4 °C in the sonication bath or vortexing it for 8 s at low speed (room temperature) to disperse large aggregates yields preparations that catalyze about the same amount of phosphate uptake. However, sonication prevents the removal of transport-inactive proteoliposomes or protein/lipid aggregates by centrifugation.

The volume of the reconstitution mixture to be frozen was kept at 195 μL unless noted otherwise. Decreasing the volume of protein solution added to a fixed volume of liposomes (Figure 2a) reduces the phosphate taken up in 30 s, but the amount taken up per protein increases. It is unlikely that this effect is due to residual Triton X-100 since exposing the phosphate transport protein after the hydroxylapatite column step to twice as many BioBeads SM2 yields the same results. Our attempts to assay the detergent concentration directly with ^{3}H-labeled Triton X-100 (New England Nuclear, Boston, MA) were not successful since we found that 5% of the ^{3}H that is not retained by the first passage through a BioBeads SM2 column is also not retained by passage through a second

Table I: Net (Zero-Trans) Phosphate Uptake Catalyzed by Proteoliposomes Reconstituted with Different Kinds and Amounts of Lipids[a]

lipid composition of proteoliposomes	phosphate uptake (nmol of P_i/30 s)
PE/PC/PA (84/62/10)	1.190
PE/PC/PA (60/86/10)	0.680
PE/PC/PA (108/38/10)	0.590
PE/PC (133/97)	0.263
PE/PC/CL (126/92/12)	0.507
PE/PC/CL (119/87/24)	0.099
PE/PC/PA[b] (124/91/15)	0.904
asolectin	−0.102[c]
asolectin/PA (150/10)	−0.101
PE/PC/PA (84/66/10)	1.164

[a] All lipid ratios are dry weight ratios (w/w). Phosphate uptake was assayed with $pH_i = 8.0$, $pH_e = 6.8$, $[P_i]_e = 1$ mM, and $[P_i]_i = 0$ mM. [b] In this experiment, we used PA830 (Avanti). The PA in the other experiments was PA835 (Avanti). [c] In the experiments with asolectin or asolectin with PA, the mersalyl-insensitive phosphate uptake (30 s) was about 3.5 times as high as with the other lipids. Thus, the insensitive uptake in 30 s (mersalyl at 0 s, column after 30 s) was about 0.35 nmol of P_i while the total uptake was about 0.25 nmol of P_i (mersalyl at 30 s, column immediately thereafter).

fresh BioBeads SM2 column. This high concentration of impurities makes tracing of low residual Triton X-100 more difficult. Decreasing the amount of lipid mixed with the protein also decreases the amount of phosphate taken up in 30 s (Figure 2b). When the lipid/protein ratio is kept constant and less lipid and protein are added to a constant volume of reconstitution mixture, the phosphate taken up per protein remains about the same (Figure 2c).

Effect of the Lipid Composition of Proteoliposomes on the Reconstituted Transport Activity. The dependence of lipid composition on transport-active proteoliposomes is of interest for two reasons: (a) to develop an optimum assay system for the phosphate transport protein and (b) to identify lipid(s) that is (are) required for the activity of the protein. We cannot differentiate between these two possibilities at this time.

Table I shows the amount of phosphate taken up by proteoliposomes reconstituted with different ratios and kinds of phospholipids. Phosphatidylethanolamine/phosphatidylcholine/phosphatidic acid yields the highest uptake in 30 s. In some experiments, phosphatidic acid concentration was decreased to less than 0.1% (w/w), and still much more phosphate was taken up in 30 s than in its absence. Replacing PA with cardiolipin lowers dramatically the amount of phosphate taken up. Increasing the CL concentration decreases the amount of P_i taken up further. Asolectin, as had already been observed by Banerjee & Racker (1979) for phosphate/phosphate exchange, is no substitute (even in the presence of PA) for the purified lipids. Maximum uptake occurs at a PE/(PE + PC) ratio of 0.58 with PA (6.4% w/w) present. The amount of phosphate taken up in 30 s dropped by about 50% (Table I) when this ratio was decreased to 0.41 or increased to 0.74.

Different commercial batches of PA resulted in dramatically different amounts of phosphate taken up in 30 s. Thin-layer chromatography demonstrated only minor traces of impurities in the different PA batches, and we found no correlation between the impurities and the resulting transport activities, especially not with pyrophosphatidic acid, the only contaminant in some PA preparations. The UV absorption spectra of different phosphatidic acid batches (after drying, dissolution in cyclohexane, lyophilization, and redissolution in absolute methanol) showed very little autoxidation (Klein, 1970). Small variations in the extent of autoxidation did not correlate with reconstituted transport activity.

Table II: Effect of Different Calcium/Phosphatidic Acid Phosphate Ratios and Calcium Chloride, Magnesium Chloride, and Sodium/Potassium Ethylenediaminetetraacetic Acid on Net (Zero-Trans) Phosphate Uptake by Proteoliposomes[a]

lipid composition	phosphate uptake (nmol of P_i/30 s)	Ca/P[c]
PE/PC/PA835 (84/66/10)	0.997	0.85
plus 2 mM EDTA	0.726	
plus 4 mM EDTA	0.101	
plus 1.5 mM $CaCl_2$	1.086	
PE/PC/PA836 (84/66/10)	0.110	0.01
plus 2 mM EDTA	0.118	
plus 0.5 mM $CaCl_2$	0.080	
plus 1.5 mM $CaCl_2$	0.193	
plus 4 mM $CaCl_2$	0.145	
PE/PC (84/66)[d]	0.268	
PE/PC/PA835 (84/66/10)	1.177	0.85
plus 2 mM $MgCl_2$	0.852	
PE/PC/PA836 (84/66/10)		0.01
plus 200 μM $CaCl_2$	0.211	
plus 4 mM $MgCl_2$	0.228	
PE/PC/PA(CAL)[b] (84/66/10)	0.208	0.01

[a] Phosphate uptake was determined under the conditions of Table I. Lipid compositions are weight ratios. The various reagents were added only to the medium used to sonicate the dried lipids. The PA concentration in that sonication medium was 2.6 mM (average PA M_r 765). PA835 and PA836 were from Avanti. [b] PA(CAL) is the sodium phosphatidic acid (egg) from Calbiochem. [c] Moles of calcium per mole of PA phosphate for the PA used in the preparation of the proteoliposomes. [d] This lipid dispersion contained less than 1% of the calcium of PA835.

Table III: Preparation of Net (Zero-Trans) Phosphate Transport Active Proteoliposomes with CaPA, NaPA, and NaPA Converted to CaPA[a]

	P_i uptake (nmol/30 s)	Ca/P[b]
CaPA (Avanti, 846)	1.54	0.88
NaPA (Calbiochem)	0.26	0.00
CaPA (converted from NaPA)[c]	1.84 ± 0.05	0.99 ± 0.02

[a] Reconstitution was done with glycerol-frozen phosphate transport protein reconstituted in the presence of 0.08% (v/v) glycerol (see Materials and Methods). Results of two experiments with two different converted CaPA preparations with standard deviation, PE/PC/PA (126/99/15), and other conditions as in Table I. [b] Moles of Ca per mole of PA phosphate for the PA used in the preparation of the proteoliposomes. [c] The NaPA was from Calbiochem; see Materials and Methods.

Acidic phospholipids may be associated with different cations. The chloroform from lipid mixtures with PA that yield high transport activity proteoliposomes is much more difficult to remove completely with argon than the chloroform from lipid mixtures with PA that yield low transport activity proteoliposomes. Calcium appears to be the dominating variable. Table II shows the transport activity of proteoliposomes prepared with PE, PC, and different batches of phosphatidic acid. Adding EDTA to medium C_i used to disperse the lipids decreases the amount of phosphate taken up by the proteoliposomes. Adding calcium chloride to the medium used to disperse lipid mixtures containing sodium salts of phosphatidic acid did not result in proteoliposomes with increased phosphate uptake. We have converted the sodium phosphatidic acid to calcium phosphatidic acid (see Materials and Methods) and obtained proteoliposomes with very high phosphate uptake (Table III). It appears then that the calcium salt of PA has

to be present during the mixing of the lipids before the chloroform is removed.

Cardiolipin stimulates phosphate uptake only very little beyond the activity generated with only PE and PC. Preliminary experiments with calcium, magnesium, and sodium cardiolipins show no significant stimulation. Again, increased concentrations of CL decrease the amount of phosphate taken up below that by PE/PC only. Experiments with magnesium phosphatidic acid or the addition of magnesium chloride to the lipid dispersion medium resulted in a decrease in the initial transport rate and the extent of net phosphate uptake.

Kinetic Homogeneity of Proteoliposomes. In preparation for more extensive kinetic studies with the transport-active proteoliposomes, we carried out time-dependent phosphate uptake experiments. We have determined the pH-dependent net phosphate uptake (zero trans; i.e., at $t = 0$, there is phosphate only on one side of the membrane) with a low extravesicular initial phosphate concentration (0.35 mM), which is significantly smaller than the K_m (1.5–2.5 mM) for phosphate transport. Under these conditions, the transport should follow the equation $[P_i]_t = [P_i]_\infty(1 - \alpha e^{-kt})$ with only one α and one k if the vesicles are kinetically homogeneous. With $[P_i]_\infty = 2.149$ mmol, $\alpha = 0.973$, and $k = 0.0185$ s^{-1}, we get a fit with a standard deviation of 0.079. It should be noted that the amount of phosphate taken up at $t = 0$ s (about 2% of the uptake at 150 s) is the actual amount of mersalyl-insensitive phosphate transport that occurs within 150 s; i.e., for the $t = 0$ s point, mersalyl is in the transport medium before the proteoliposomes are added; then after the addition of proteoliposomes, the mixture is kept at the transport assay temperature (22 °C) for 150 s (the longest transport time point) before it is placed on the anion-exchange column (4 °C). This very small amount of mersalyl-insensitive phosphate transport can also now clearly be demonstrated in experiments where the intraproteoliposomal pH is varied independently from the extraproteoliposomal pH. In an earlier experiment (Wohlrab & Flowers, 1982), this mersalyl-insensitive uptake was much higher.

The rate of phosphate uptake catalyzed by proteoliposomes decreases with time. Is this decrease due to the equilibration of phosphate with the pH gradient? Is the pH gradient dissipated faster by unspecific proton leaks in the membrane than by the transport of phosphate? We added [^{32}P]P_i 1 min after mixing the proteoliposomes with the transport medium. Initial rates of phosphate uptake were the same with a 25% decrease in the phosphate taken up at 150 s. Thus, there occurs either a small dissipation of the pH gradient as the proteoliposomes are exposed to the ΔpH in the absence of an initial phosphate gradient or some inactivation of phosphate transport proteins due to autoxidation in the presence of low concentrations (73 μM) of dithiothreitol (see Inhibition of the Phosphate Transport Protein by Autoxidation).

Net Efflux of Phosphate from Proteoliposomes. Net efflux of phosphate must be demonstrated to establish a functional asymmetry of the phosphate transport protein. Since the amount of phosphate [^{32}P]P_i trapped in the proteoliposomes is high, it is difficult to observe phosphate efflux. The proteoliposomes were briefly centrifuged (Table IV). Only transport-inactive lipids and proteins were present in the pellet, since all transport-active (net uptake) proteoliposomes remained in the supernatant. The centrifugation also reduces the larger amount of mersalyl-insensitive net phosphate uptake that occurs in the presence of 5 mM phosphate. Figure 3 shows the net phosphate efflux. The efflux rate is not dramatically different from the uptake rate, and Table V shows

Table IV: Recovery of Protein, Lipids, and Net (Zero-Trans) Phosphate Uptake Activity from Proteoliposomes after Centrifugation[a]

reconstitution mixture	before centrifugation	supernatant after centrifugation[b] (%)
phospholipids (PE/PC/PA, 52/39/9)	0.68 mg	48
adenine nucleotide translocase	0.29 μg	34
phosphate transport protein	0.13 μg	59
phosphate uptake[c]	1.09[c]	1.13[c]

[a] Transport was assayed with $pH_i = 8.0$, $pH_e = 6.8$, $[P_i]_i = 0$ mM, and $[P_i]_e = 1$ mM. The lipid mixture is in weight ratios. The distribution of transport activity in the supernatant and pellet depends on the lipid mixture. With some lipid mixtures, i.e., no PA, active vesicles are also pelleted. Protein concentrations were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie Blue staining. [b] Centrifugation was at 12000g for 15 min at 4 °C. [c] Phosphate uptake values are in the units nanomoles of P_i per 30 s.

Table V: Net (Zero-Trans) Phosphate Efflux from Proteoliposomes[a]

pH_i	pH_e	phosphate efflux (nmol of P_i/30 s)
6.8	6.8	0.376 ± 0.028
8.0	6.8	0.059 ± 0.137
6.8	8.0	0.498 ± 0.147
8.0	8.0	−0.021 ± 0.117

[a] The proteoliposomes (PE/PC/PA 126/99/15, weight ratios) were briefly centrifuged (Table IV) before the mersalyl-sensitive efflux of phosphate ([^{32}P]P_i) was assayed with $[P_i]_i = 1$ mM and $[P_i]_e = 50$ μM. Average with standard deviation of experiments with three different phosphate transport protein preparations.

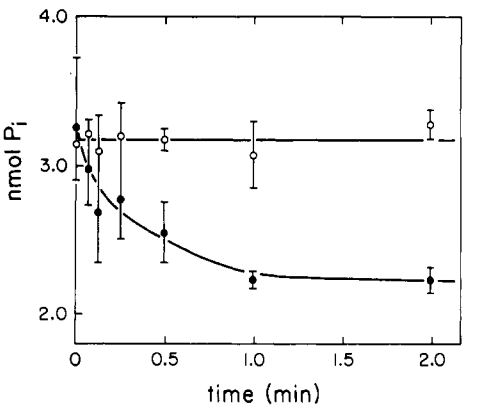

FIGURE 3: Time dependence of net phosphate efflux from proteoliposomes. The transport was determined with $pH_i = 6.8$, $pH_e = 8.0$, $[P_i]_i = 1$ mM, and $[P_i]_e = 50$ μM (from 1 mM reconstitution medium). Experiments were carried out with proteoliposomes prepared with normal phosphate transport protein (●) and phosphate transport protein isolated from mitochondria inhibited with 40 nmol of *N*-ethylmaleimide/nmol of cytochrome *b* (○).

that the net efflux has a pH-gradient dependence similar to the net uptake.

Inhibition of Net (Zero-Trans) Phosphate Uptake by N-Ethylmaleimide and Mersalyl. We demonstrated (Wohlrab, 1980c) that the reconstituted phosphate/phosphate exchange is sensitive to mersalyl and *N*-ethylmaleimide. Mersalyl was used since it was assumed to react more rapidly with the phosphate transport protein. However, *N*-ethylmaleimide is the inhibitor that differentiates phosphate transport catalyzed by the phosphate carrier (phosphate transport protein) from

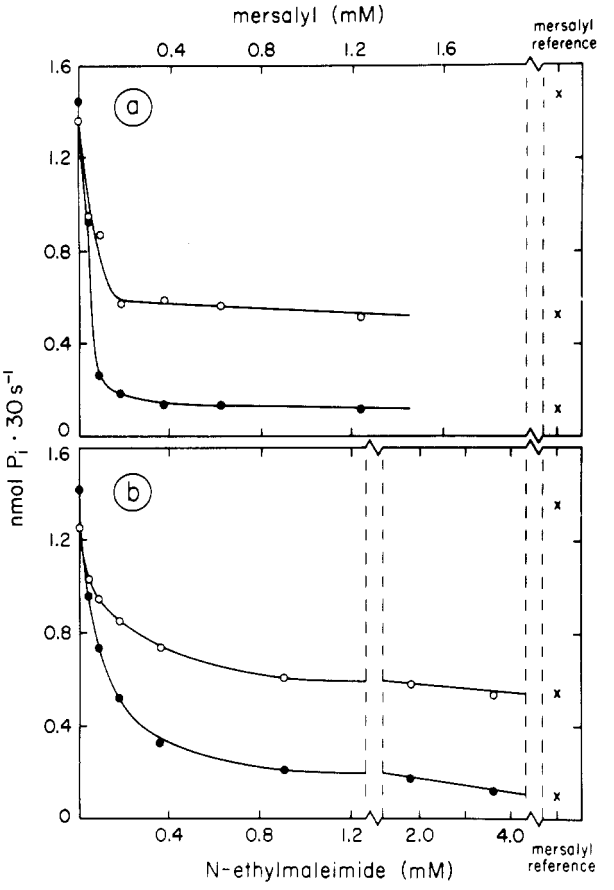

FIGURE 4: Mersalyl and *N*-ethylmaleimide inhibition of net (zero-trans) phosphate uptake catalyzed by proteoliposomes. Different amounts of mersalyl and *N*-ethylmaleimide were present in the transport medium before the addition of the proteoliposomes (●) or were added 7.5 s after the start of the transport (○). Mersalyl (624 μM) was added to all samples at 30 s. The mersalyl reference at the far right (×) had only 624 μM mersalyl added to the transport medium before (bottom) the proteoliposomes, or at 7.5 (middle) or 30 s (top) after the initiation of the transport. The transport conditions were pH_e = 6.8, pH_i = 8.0, $[P_i]_e$ = 1.0 mM, and $[P_i]_i$ = 0 mM.

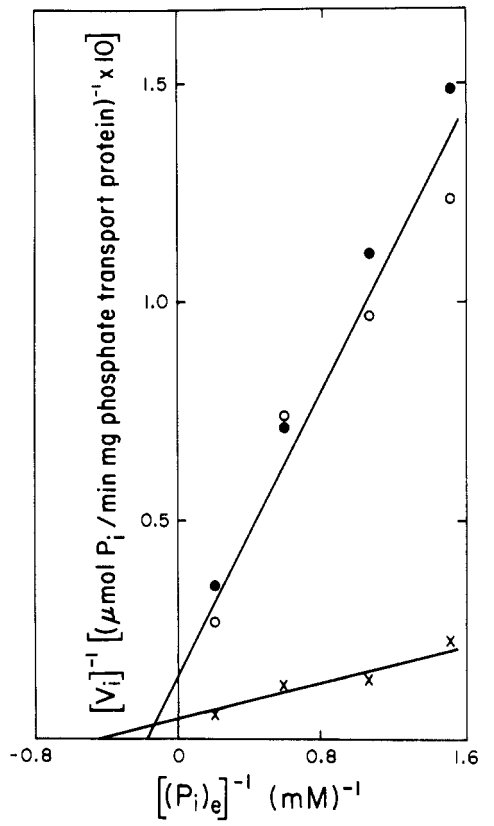

FIGURE 5: Double-reciprocal plot of net (zero-trans) phosphate uptake catalyzed by proteoliposomes. The proteoliposomes were prepared with phosphate transport protein passed through a P-6DG column equilibrated either with (×) or without (●, ○; two different experiments) dithiothreitol. The intraproteoliposomal pH was 8.0 while the extraproteoliposomal pH was 6.8. The initial phosphate uptake rates were determined from time-dependent phosphate uptake experiments with points at 0, 4, 7.5, 15, 22.5, 30, and 45 s.

that catalyzed by the dicarboxylate carrier. Does *N*-ethylmaleimide react sufficiently fast to be useful for inhibitor-stop experiments?

We have added different amounts of mersalyl or *N*-ethylmaleimide to the transport medium before the addition of proteoliposomes or 7.5 s after the start of the transport and have added the normal amount of mersalyl (624 μM) after 30 s of transport to inhibit that fraction of phosphate transport protein that has not been inhibited by the low concentrations of mersalyl or *N*-ethylmaleimide. The resulting plot is thus a function of both the rate of inhibition and the amount of inhibitor added. Figure 4 shows that *N*-ethylmaleimide is adequate for use in inhibitor-stop experiments if used at concentrations higher than mersalyl. The dithiothreitol (73 μM) in the final transport mixture probably has only a small effect on the *N*-ethylmaleimide titer since the thawed phosphate transport protein preparation, which contains 5 mM dithiothreitol, is maximally inhibited by less than 1.5 mM *N*-ethylmaleimide (pH 7.2) (data not shown). Banerjee & Racker (1979) also found that much less mersalyl and $HgCl_2$ were required to inhibit reconstituted phosphate/phosphate exchange activity than the amount required to match the SH's of dithiothreitol.

Inhibition of the Phosphate Transport Protein by Autoxidation. When the purified phosphate transport protein is passed through a P-6DG column, equilibrated without dithiothreitol and then used to prepare proteoliposomes, only 20–30% of the usual phosphate uptake occurs. The full uptake can be completely recovered by adding dithiothreitol to the P-6DG eluate or to the reconstituted proteoliposomes. Figure 5 shows the phosphate uptake kinetics with normal proteoliposomes and with those prepared without dithiothreitol. The initial uptake rates were determined by assuming that only 59% of the phosphate transport protein is incorporated as a transport-active protein (Table IV). The V_{max} is thus 0.22 mmol of P_i min^{-1} (mg of phosphate transport protein)$^{-1}$ with a K_m of 2.3 mM. Assuming a monomeric phosphate transport protein of 34 kDa, a turnover number of 7×10^3 or 1.4×10^4 min^{-1} (for a dimeric transport protein) can be calculated. Proteoliposomes prepared in the absence of dithiothreitol show a V_{max} of only 0.07 mmol of P_i min^{-1} (mg of phosphate transport protein)$^{-1}$ with a K_m of 5.9 mM.

The autoxidation most likely generates a disulfide bond. The fraction of phosphate transport proteins with decreased (or zero) transport activity and the disulfide bond should not react with *N*-ethylmaleimide, and their activity should be reversible even in the presence of *N*-ethylmaleimide, which does not react with disulfides. Table VI shows that proteoliposomes prepared with normally active and with autoxidation-inhibited phosphate transport protein are completely inhibited by *N*-ethylmaleimide. Only the autoxidation-inhibited proteoliposomes, however, recover a substantial amount of transport activity upon addition of dithiothreitol after *N*-ethylmaleimide addition to the proteoliposomes.

Figure 6 shows that partial inhibition of the phosphate transport protein by *N*-ethylmaleimide yields kinetics very

Table VI: Protection of Phosphate Transport Protein (Proteoliposomes) from *N*-Ethylmaleimide Inhibition by Autoxidation[a]

	P_i uptake (nmol/30 s)
phosphate transport protein (P-6DG/dithiothreitol)	1.680
plus *N*-ethylmaleimide	0.012
plus *N*-ethylmaleimide plus dithiothreitol	0.014
phosphate transport protein (P-6DG/no dithiothreitol)	0.529
plus *N*-ethylmaleimide	0.011
plus *N*-ethylmaleimide plus dithiothreitol	0.545

[a] Reconstitutions as in Table III with Avanti CaPA 846. *N*-Ethylmaleimide was added at a final concentration of 3.6 mM to the proteoliposomes (0 °C). Five minutes later, 25 μL of this mixture was assayed for phosphate uptake (see Materials and Methods). Dithiothreitol concentration in the P-6DG column was 5 mM. Dithiothreitol for reactivation (final concentration 1.5 mM) was added 5 min after the *N*-ethylmaleimide, and the transport was assayed 5 min after the addition of dithiothreitol.

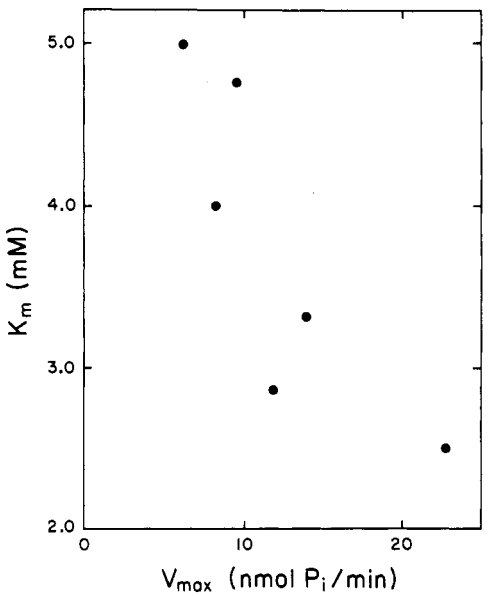

FIGURE 6: Summary of results from double-reciprocal plots. The experiments were carried out as in Figure 5 with dithiothreitol. Different amounts of *N*-ethylmaleimide were added to the phosphate transport protein before passage through the P-6DG column.

similar to those of the autoxidation-inhibited preparation. Thus, as the V_{max} is reduced by *N*-ethylmaleimide, the K_m increases.

Two other experiments suggest that a dithiol may exist in the phosphate transport protein. The autoxidized phosphate transport protein is much more readily reactivated by dithiothreitol than by β-mercaptoethanol. The monothiol β-mercaptoethanol (4 mM), added to the autoxidized phosphate transport protein before reconstitution, recovers 10% of the autoxidation-caused loss of transport activity, while 2 mM dithiothreitol (dithiol) produced a complete reversal of the autoxidation-generated transport inhibition. Proteoliposomes, prepared with phosphate transport protein that was passed through a P-6DG column equilibrated with medium at pH 7.1 with 10 mM EDTA (no dithiothreitol), were inhibited to 50% with 100 μM phenylarsine oxide (dissolved in dimethylformamide) in the presence of 50 μM mercaptoethanol.

Discussion

We have now collected a significant amount of evidence demonstrating that the phosphate transport protein is responsible for net phosphate transport into the mitochondrial matrix, transport that is required for steady-state oxidative phosphorylation to occur in the cell. The primary pieces of evidence are the following: (a) the transport protein can catalyze net phosphate transport; (b) the K_m for phosphate transport is of the magnitude expected from studies with intact mitochondria (Coty & Pedersen, 1974); (c) the extent of phosphate transport depends on the ΔpH across the proteoliposome membrane; (d) the reconstituted transport is more sensitive to mersalyl than *N*-ethylmaleimide as has been shown in mitochondria (Guerin et al., 1970).

The requirement of calcium phosphatidic acid for a large amount of net phosphate uptake is of great interest since the addition of only calcium chloride to the medium used to disperse dried lipids which contain sodium phosphatidic acid is not sufficient. Preliminary experiments carried out with proteoliposomes prepared with NaPA suggest that the addition of calcium to the extraproteoliposomal medium does stimulate net (zero-trans) phosphate uptake. Fonyo & Ligeti (1978a) have demonstrated a correlation between the intramitochondrial phosphate proton release and the amount of strontium (or calcium; Fonyo & Ligeti, 1978b) present in the mitochondrial matrix. The proteoliposomal calcium most likely is bound not free. It is probable that the protons that are cotransported with the phosphate displace the calcium from the phosphatidic acid and make it available for complexation with phosphate. The amount of phosphatidic acid in the mitochondrial membrane is low (Nohl et al., 1981), and our preliminary experiments suggest that calcium cardiolipin cannot stimulate the uptake of phosphate to the extent that calcium phosphatidic acid can. Calcium phosphatidic acid, on the other hand, is not required for phosphate transport protein catalyzed net phosphate uptake (Table I). A ΔpH experiment [Figure 2 in Wohlrab & Flowers (1982)] carried out with proteoliposomes prepared without acidic phospholipids (PA or CL) shows the same ΔpH dependence for net phosphate uptake with, however, less phosphate being taken up under the different conditions (results not shown). Preliminary experiments have shown that CaPA increases the total phosphate taken up within 30 s and also the initial phosphate uptake rate, and thus also the turnover number of the phosphate transport protein.

It is not clear what the calcium binding sites in the mitochondrial matrix are. Acidic phospholipids have been suggested to play such a role. On the other hand, calcium phosphatidic acid could also play a direct catalytic role by binding to the phosphate transport protein. Calcium/phosphatidylserine has been observed to activate a calcium/phospholipid-dependent protein kinase (Takai et al., 1979; Kuo et al., 1980). Phosphatidic acid has also recently been suggested to play an important role in membrane calcium phenomena (Lapetina, 1982). We have observed that as little as 0.07% (w/w) phosphatidic acid in the reconstituted proteoliposomes increases the amount of phosphate taken up dramatically. Phosphatidic acid and cardiolipin have also been suggested from experiments with a Pressman cell to act as calcium ionophores. Cardiolipin has been shown to exchange calcium for proton(s) (Tyson et al., 1976). We are investigating whether these acidic phospholipids can act in such capacity in our proteoliposomes.

Figure 4 shows that *N*-ethylmaleimide can be used in inhibitor-stop experiments, whether stopping the transport from the beginning or at intermediate times. We should be able to use this system in attempts to detect accessibility changes of the phosphate transport protein SH group(s) to *N*-ethyl-

maleimide as the ΔpH, the pHs on either side of the proteoliposomal membrane, or the phosphate concentration on either side of the proteoliposomal membrane is changed.

We have carried out experiments investigating the role of the SH groups of the phosphate transport protein in the mechanism of transport. SH reagents inhibit mitochondrial phosphate transport. Do the phosphate transport inhibitor experiments demonstrate that the *N*-ethylmaleimide-reactive SH group(s) is (are) essential for the catalytic mechanism? Our observation, shown in Figure 6, that increasing amounts of *N*-ethylmaleimide decrease the V_{max} as well as increase the K_m suggests that either a single SH (per dimeric protein?) reacts with the phosphate transport protein and modifies the reaction mechanism without completely blocking it or two SH's react with *N*-ethylmaleimide where one initially modifies the K_m while the second blocks the transport with a reduction in V_{max}. It is also possible that the results are a reflection of SH group heterogeneity in the proteoliposomes. We are determining now the number of sulfhydryl groups in the phosphate transport protein and how many react with *N*-ethylmaleimide to inhibit its activity. Preliminary experiments suggest that *N*-ethylmaleimide has to react with less than one SH group per monomer (one SH group per dimeric protein) to inhibit transport.

Our observation that the purified phosphate transport protein is very sensitive to autoxidation raises the question whether a dithiol/disulfide interchange mechanism is important in phosphate transport. The autoxidation-caused inhibition is reversible, and it is likely that disulfide formation is involved since autoxidation protects the protein from *N*-ethylmaleimide inhibition (Table VI). Autoxidation inhibits the phosphate transport protein in a manner similar to that of *N*-ethylmaleimide, providing further support that autoxidation may affect the same SH group(s) as *N*-ethylmaleimide. The inhibition by phenylarsine oxide also suggests that two SH groups are located close to each other. The observation that mercaptoethanol is a relatively poor reactivating agent compared to dithiothreitol also suggests that two thiols are relatively close to each other. It is most likely that the disulfide bond is intramolecular rather than intermolecular; i.e., it could be between two subunits of a dimeric phosphate transport protein but less likely between subunits of different phosphate transport proteins. Evidence that supports this is that proteoliposomes prepared with autoxidized phosphate transport protein are reactivated by dithiothreitol, and adding various amounts of dispersed phospholipids to the purified phosphate transport protein before passage through the P-6DG column still results in the same amount of inhibition by autoxidation.

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Registry No. Phosphate, 14265-44-2; calcium, 7440-70-2; calcium chloride, 10043-52-4; magnesium chloride, 7786-30-3; ethylenediaminetetraacetic acid, 60-00-4; adenine nucleotide translocase, 9068-80-8; *N*-ethylmaleimide, 128-53-0; dithiothreitol, 3483-12-3.

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