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CALCIUM TRANSPORT IN VESICLES ENERGIZED BY CYTOCHROME OXIDASE

VOLUME I

By Randy N. Rosier

Submitted in Partial Fulfillment
of the
Requirement for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: Thomas E. Gunter

Department of Radiation Biology and Biophysics
The University of Rochester
Rochester, New York
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DISCLAIMER

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Vitae

The author was born in [redacted]

He was graduated from Dover Area High School in 1969 and began his undergraduate work at Dickinson College in Carlisle, Pennsylvania that year. He majored in physics and was graduated summa cum laude in 1972, and was elected to Phi Beta Kappa and Sigma Xi that year. In September of 1972 the author began his studies at the University of Rochester in the M.D.-Ph.D. program . He received support from the Medical Scientist Training Grant. Dr. Thomas Gunter supervised his training in the Department of Radiation Biology and Biophysics of the University of Rochester. The author received a Master of Science Degree from the Department of Radiation Biology and Biophysics in May of 1977. During his years at the University of Rochester the author pursued an integrated course of medical and graduate studies. He received his Medical Degree in May of 1978 and began his training at the University of Iowa as a resident in Orthopaedic Surgery in July of 1978.

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List of Abbreviations

ADP - adenosine diphosphate

ATP - adenosine triphosphate

BSA - bovine serum albumin

CCCP - carbonyl cyanide m-chlorophenylhydrazone

CL - cardiolipin

complex V-COV - cytochrome ocidase vesicles containing complex V

COV - cytochrome oxidase vesicles

cyt. ox. - cytochrome oxidase

DCCD - dicyclohexylcarbodiimide

Di-O-C_3-(5) - 3,3'-dipropyloxadicarbocyanine iodide

Di-S-C_3-(5) - 3,3'-dipropylthiadicarbocyanine iodide

DTT - dithiothreitol

EDTA -- ethylenediamine N,N'-tetracetic acid

ETC - electron transfer complex

FCCP - carbonyl cyanide p-trifloromethylphenylhydrazone

F_1 - mitochondrial ATPase

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HP - hydrophobic protein

ISO -- inside out (with respect to orientation or energization of membrane when compared to intact mitochondria)

ITC - ionophore transfer complex

MSH - mannitol-sucrose-HEPES solution

NE - nonenergized

O.D. - optical density

OSCP - oligomycin sensitivity-conferring protein

PC - phosphatidylcholine

PCOV - phosphorylating cytochrome oxidase vesicles

PE - phosphatidylethanolamine

P_i - inorganic phosphate

PLV - phospholipid vesicles

PMS - phenazine methylsulfate

PS - phosphatidylserine

RCR - respiratory control ratio

RR - ruthenium red

RSO - right side out (with respect to orientation or energization of membrane compared to intact mitochondria)

SDS - sodium dodecyl sulfate

SMP - submitochondrial particles

TEMED - N,N,N'N'-tetramethylethylenediamine

TMPD - N,N,N',N'-tetramethylphenylenediamine

TPB - tetraphenylboron

Tricine - N-tris(hydroxymethyl)methylglycine

Tris -hydroxymethylaminomethane

Δ pH - pH gradient

$\Delta \psi$ - membrane potential

ABSTRACT

Experiments on the reconstitution of cytochrome oxidase into phospholipid vesicles were carried out using techniques of selectively energizing the suspensions with ascorbate and cytochrome c or ascorbate, PMS, and internally trapped cytochrome c. It was found that the K^+ selective ionophore valinomycin stimulated the rate of respiration of cytochrome oxidase vesicles regardless of the direction of the K^+ flux across the vesicle membranes. The stimulation occurred in the presence of protonophoric uncouplers and in the complete absence of potassium or in detergent-lysed suspensions. Gramicidin had similar effects and it was determined that the ionophores acted by specific interaction with cytochrome oxidase rather than by the previously assumed collapse of membrane potentials.

When hydrophobic proteins and appropriate coupling factors were incorporated into the cytochrome oxidase vesicles phosphorylation of ADP could be coupled to the oxidation reaction of cytochrome oxidase. Relatively low P:O, representing poor coupling of the system, were problematical and precluded measurements of protonmotive force. However the system was used to study ion translocation.

The primary problem in the study of ion transport in proteoliposomes is the difficulty of separating the small vesicles from the suspending medium by conventional techniques of filtration or centrifugation. A system of vesicle aggregration was developed which involved the addition of polylysine or protamine to vesicle suspensions to cause clumping so that conventional filtrations could readily be used to quantitate ion

transport. The system was found to be applicable to a wide range of types of artificial and natural vesicles.

Using this separatory system, cation uptake in submitochondrial particles and cytochrome oxidase vesicles was studied. A number of tests of the sidedness of the vesicles demonstrated the cytochrome oxidase to be in bidirectional orientation in both types of vesicles, and techniques of selective energization were developed to allow generation of either internally positive or negative membrane potentials in the vesicles. Under these conditions of selective energization it was found that submitochondrial particles accumulated calcium when either internally negative or positive membrane potentials were generated. The characteristics of the transport were studied and uptake against the electrochemical gradient (in internally positive submitochondrial particles) of both calcium and manganese was found to be inhibited by levels of protonophoric uncoupler too low to dissipate the protonmotive force. Other experiments indicated that the mechanism of this type of transport was either an electrogenic pump coupled to oxidation or an antiport type of carrier, while the mechanism in the internally negative particles was consistent with a uniport.

Cytochrome oxidase vesicles were found to take up calcium and manganese when energized to generate inside negative membrane potentials. Uptake in inside positive vesicles or enhancement of uptake in inside negative vesicles could be obtained by co-reconstitution with complex V. This uptake was also sensitive to low levels of uncouplers and further experiments indicated similarity to the submitochondrial particle experiments, in that uptake in internally negative vesicles was mediated

most likely by a uniport type of carrier while uptake against the electrochemical gradient in the internally positive vesicles was more consistent with an antiport or electrogenic pump coupled to oxidation. Uptake of divalent cation in both submitochondrial particles and the reconstituted system was sensitive to uncouplers or inhibitors of oxidation.

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Preface

The nature of this work required the use of numerous techniques, procedures and systems. The experimental methodology was quite diverse and variations of the same methods were frequently used in many phases of this research. Because of this the MATERIALS and METHODS sections are contained in a single chapter to minimize redundancy in the subsequent material. The chapters on the different experimental areas each include a pertinent Introduction, Results, and Discussion section.

The experimental work was done in several phases and covers a number of areas such as vesicle reconstitution, mitochondrial calcium transport, and oxidative phosphorylation. These areas are so broad that it was necessary to present a somewhat condensed introductory section on each focussed on the more relevant aspects of the topic and its relation to the present work.

MATERIALS

^{54}Mn, ^{45}Ca, ^{32}P, and ^{14}C-sucrose were purchased both from Amersham Searle and New England Nuclear. ^{32}P was used without any further purification procedures. ^{14}C-ADP and ^{14}C-ATP were purchased from Amersham Searle, and ^{86}Rb and ^{22}Na were purchased from New England Nuclear.

All ammonium sulfate used was Schwarz-Mann ultra pure enzyme grade. Deoxycholic acid and cholic acid were purchased from Sigma and purified as described later in this section. Lysolecithin, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, hexokinase (type C-300), pyruvate kinase (type II), protamine, polylysine, cytochrome c (type III, horse heart), PMS, CCCP, and valinomycin were purchased from Sigma Chemical Co. Soy asolectin was obtained from Associated Concentrates and from Sigma (type II-S). The Ca^{++} ligand was kindly supplied by Dr. W. Simon of the Swiss Federal Institute of Technology, Zurich. FCCP was a gift from Dr. P. G. Heytler of Du Pont, A23187 was generously provided by Dr. R. J. Hosley of Eli Lilly Co., and the dicarbocyanine dyes Di-S-C_3-(5), Di-O-C_3(5), and Di-O-C_5-(3) were a gift from Dr. Alan Waggoner of Amherst. Dr. E. Racker of Cornell kindly provided samples of hydrophobic protein, mitochondrial phosphatidyl choline and phosphatidyl ethanolamine, and antibodies to F_1. Samples of cytochrome oxidase were supplied by Dr. B. Love and highly purified F_1 by Dr. A. Senior. Dr. R. Mavis provided a sample of lysolecithin.

All distilled water used in these experiments was passed through an ion exchange column to remove residual heavy metal ions.

In the protein extraction procedures which will be detailed below, modifications of the original procedures were liberally employed with respect to centrifugation. All centrifugations were designed to allow an amount of g force x minutes equivalent to the original published procedures, but frequently because of lack of availability of specific rotors or centrifuges, or differences in the amount of tissue processed, substitutions had to be made.

METHODS

Purification of phospholipids

(A) Removal of neutral lipids from asolectin (Kagawa and Racker, 1971)

5 grams of crude asolectin was placed in a flask with 100 ml of acetone and .5 mg of antioxidant (N,N'-diphenyl-p-phenylenediamine). The mixture was stirred with a magnetic stir bar at room temperature and under nitrogen for 24-48 hours. The acetone was carefully removed with a Pasteur pipet and the residue dried under nitrogen and then dissolved in 20 ml of diethyl ether containing approximately 20 μg of antioxidant (usually a small speck was added). This solution was centrifuged in a JA-20 rotor at 0°C. for 10 minutes at 10,000 rpm. The supernatant was decanted and dried under nitrogen. The yellow residue was dissolved in a 4:1 chloroform:methanol solution at a concentration of about 100 mg/ml (as determined by weighing an aliquot of known volume after drying it on a pre-weighed piece of paper). The phospholipid solution was stored in the dark under nitrogen at -20° C.

(B) Ethanol extraction of asolectin (Small, Bourgès, Dervichian, 1966)

Acetone-treated asolectin was extracted with ethanol to produce a phospholipid fraction enriched in phosphatidyl choline and suitable for use in the reconstitution of oxidative phosphorylation (personal communication, A. Kandrach). An aliquot of the chloroform-methanol asolectin solution was dried under nitrogen and redissolved in a small

volume of diethyl ether (2 ml/g of phospholipid). 12.5 volumes of 100% ethanol was added and the mixture stirred at room temperature under nitrogen for 2 hours. The solution became quite turbid upon addition of the ethanol. After the 2 hour incubation the suspension was centrifuged in a JA-20 rotor at 10,000 rpm for 10 minutes and the supernatant was dried under nitrogen and redissolved in 4:1 chloroform:methanol at a concentration of 30-40 mg/ml. The ethanol extracted phospholipid solution was stored under nitrogen at -20°C.

Purification of cholic acid and deoxycholic acid
(Kagawa and Racker, 1971)

100 g. of cholic acid or deoxycholic acid was dissolved in 800-900 ml of 95% ethanol by heating on a magnetic stir plate. While boiling, a 250 ml beaker of activated charcoal powder was gradually added and the mixture allowed to gently boil for about 30 minutes. A large Buchner funnel was fitted with Whatman #1 filter paper, and the filter paper then washed with hot ethanol to remove any residual detergents. The charcoal mixture was poured into the Buchner funnel, to which vacuum was applied from a water aspirator. The filtrate was then refiltered through two layers of fresh filter paper which had been washed with hot ethanol. The clear filtrate was concentrated by boiling until the cholic acid or deoxycholic acid just started to come out of solution as a milky suspension. .367 volumes of distilled water was added to give a final ethanol concentration of 70%, and the mixture heated until complete dissolution of the solute occurred. The solution was then cooled to 4°C. and some seed crystals of either

cholic acid or deoxycholic acid were added. The crystallization process was allowed to proceed for about 48 hours at 4°C. The precipitate was removed by filtration through Whatman #1 filter paper in a Buchner funnel. Cholic acid crystals were washed with diethyl ether and dried in a drying oven overnight. Deoxycholic acid crystals were not washed with ether (since it dissolves them) but were simply dried in an oven overnight. All cholate and deoxycholate solutions were made from this purified material by gradually adding KOH or NaOH while stirring and continuously monitoring pH until all the cholic acid or deoxycholic acid was in solution and the appropriate pH was reached.

Protein measurement

Protein concentrations were generally measured by a modification of the biuret technique (Jacobs, Jacob, Sanadi, Bradley, 1956). The modification simply involved subtraction of the intrinsic protein absorbance at 560 nm from the absorbance of the sample to be measured by using a control sample containing no copper. When the protein concentration was expected to be less than 10 mg/ml the Lowry method was used (Lowry, Rosebrough, Farr, Randall, 1951).

Preparation of beef heart mitochondria

For protein extractions and other experiments beef heart mitochondria were prepared as follows (Crane, Glenn, Green, 1956):

All operations were carried out at 0-4°C. Two beef hearts were trimmed to remove fat and connective tissue, cut into small pieces, and ground in a meat grinder. The ground heart tissue was homogenized

by placing 100 ml of mince, 300 ml of .25 M sucrose, 10 mM Tris-Cl (pH 7.4), .4 ml of 1 M EDTA (final concentration of 1 mM), and 1.0 ml of 6 N KOH in a 1-liter Waring blender and homogenizing for 40 seconds at the maximum speed. The pH of the homogenate was checked after the first aliquot was blended and the amount of KOH added to subsequent aliquots adjusted so as to give a final pH of 7.8. The homogenate was filtered first through 2 layers of cheesecloth and then through 4 layers of cheesecloth, and the filtrate centrifuged at 3000 rpm in a JA-14 rotor or at 2700 rpm in a JA-10 rotor for 5 minutes. The centrifugation times were clocked from the time the rotor reached about 2/3 of its final speed. The supernatant was carefully decanted through 4 layers of cheesecloth and recentrifuged in a JA-10 or JA-14 rotor at 2000 rpm for 3 minutes. The supernatant was carefully decanted and centrifuged in a JA-14 for 20 minutes at 14,000 rpm or in a JA-10 at 10,000 rpm for 40 minutes. These times and speeds apply to all subsequent centrifugations. The brown pellet was homogenized using a glass-tube/teflon pestle in 1/8 of the volume of the original homogenate of .25 M sucrose, 10 mM Tris-Cl, pH 7.4. After centrifugation the pellet was homogenized and centrifuged again. The final pellet was dark brown in the center (heavy layer mitochondria) and the rest of the pellet is a lighter brown in color and more fluffy in consistency (light layer mitochondria). The pellet was resuspended in about 25 ml of sucrose-Tris per liter of original homogenate and stored at 40 mg/ml (protein concentration determined by biuret method) in liquid nitrogen.

Preparation of rat liver mitochondria

Rat liver mitochondria were made according to a modification of the method of Schnaitman and Greenawalt (1968). Sprague-Dawley rats were killed by decapitation after being anesthetized with diethyl ether, and the livers were removed, blotted, and placed in ice cold mannitol prep solution (.22 M mannitol, 70 mM sucrose, 2 mM HEPES, and 1 mM EDTA, pH 7.4-7.5). All subsequent operations were carried out at 0-4°C. The maximum amount of liver which could be conveniently processed was 60-65 g. The livers were finely chopped with scissors and washed with the mannitol prep solution until the wash solution was clear. The mince was then homogenized in mannitol prep solution using a 40 ml glass tube with a motor-driven teflon pestle. The final volume of the homogenate was approximately 320 ml. The homogenate was centrifuged in a JA-20 rotor at 1800 rpm for 12 minutes. The supernatant was decanted into cold centrifuge tubes and centrifuged at 1800 rpm for 2 minutes. This supernatant was then centrifuged at 7000 rpm for 20 minutes. The supernatant was discarded and the loosely packed pellet retained. The white lipid layer which is adherent to the sides of the centrifuge tubes was removed by wiping with tissue paper. The pellets were then resuspended in mannitol prep solution as before and the suspension centrifuged at 8000 rpm for 11 minutes. The supernatant and loose upper portion of the pellet were decanted and the brown pellet was resuspended in mannitol-sucrose-HEPES (MSH; 135 mM mannitol, 45 mM sucrose, 24 mM HEPES, .5 mM sodium succinate, pH 7.2 - after Lowenstein, Scholte, Wit-Peeters, 1970)

The homogenate was adjusted to a final volume of 40 ml and cen-

trifuged at 12,500 rpm for 5.5 minutes. The brown pellet was resuspended in MSH to a final protein concentration of 75 mg/ml as determined by the biuret method.

Preparation of submitochondrial particles

Method 1 (digitonin method)

SMP for sidedness and cation uptake studies were generally made by sonication of mitoplasts made by digitonin treatment of Schnaitman and Greenawalt (1968). An aliquot of mitochondrial suspension (75 mg/ml) was treated with digitonin as follows: .12 mg digitonin/mg protein was weighed out and dissolved in a volume of suspending medium (MSH for rat liver mitochondria or .25 M sucrose, 10 mM Tris-Cl, pH 7.4 for beef heart mitochondria) equal to 1/2 the volume of the mitochondrial suspension to be treated. The solution was heated while stirring to dissolve the digitonin, and was then cooled to 4°C. This digitonin solution was slowly added to the mitochondrial suspension, which was magnetically stirred with two small stir bars in a 40 ml centrifuge tube at 0°C. The suspension was stirred slowly for 15 minutes and then rapidly diluted to a total volume of 80 ml with the buffer solution and centrifuged in a JA-20 rotor at 12,500 rpm for 5.5 minutes. The pellet was resuspended in 80 ml of buffer with a glass/teflon homogenizer and recentrifuged. The pellets were resuspended in a small volume (about 5 ml) of buffer and powdered cytochrome c was then added to a final concentration of 12 mg/ml. This step was omitted when beef heart SMP' were being made for protein extractions. The suspension was sonicated in an L&R type 210

(or equivalent) bath sonicator for 15-20 minutes at 4°C. The sonicator was tuned to maximum sonic activity by observing the agitation of the suspension. The suspension was then diluted to approximately 40 ml with .125 M KCl, 10 mM HEPES, .5 mM sodium succinate, pH 7.2. For SMP made without cytochrome c the suspension was diluted with the previous suspending medium. The diluted suspension was then centrifuged for 1 hour at 40,000 rpm in a Sorvall type A-841 rotor and Sorvall type OTD-65 ultracentrifuge at 4°C. The pellet was washed with suspending medium and then resuspended with a glass/teflon homogenizer in either MSH or sucrose-Tris at a protein concentration of 30-40 mg/ml as determined by biuret. The SMP were either kept on ice until used or frozen as described below.

Method 2 (osmotic shock method; Loyter, Christiansen, Steensland, Saltzgaber, Racker, 1969)

This method was used only to produce SMP for protein extractions. After the last centrifugation of the beef heart mitochondrial preparation, the fluffy light brown layer of the pellet (light layer mitochondria) was removed by gentle agitation with a small amount of .25 M sucrose, 10 mM Tris-Cl, pH 7.4 added to the centrifuge tube. The firm dark brown pellet remaining in the tube after decanting the liquid was separately resuspended in sucrose-Tris, and represented the heavy layer mitochondria. Both fractions of mitochondria were homogenized with a glass/teflon homogenizer to a final protein concentration of approximately 40 mg/ml, and were then stored at -20°C until needed. SMP were made as follows:

(A) Light layer SMP

Light layer mitochondria were diluted to 20 mg/ml using 30 mM Tris-SO_4, pH 7.4. The suspension was sonicated in 75 ml batches at the maximum setting of a Branson type W 140 probe sonicator for 2 minutes. The sucrose concentration of the suspension was increased to .65 M by the addition of an appropriate amount of 2 M sucrose. The suspension was then homogenized using a glass/teflon homogenizer and stirred at 0°C. for 10 minutes. 9 volumes of cold 10 mM Tris-SO_4 (pH 7.4) was added and the diluted suspension centrifuged in a JA-10 rotor at 4000 rpm (2000 xg) for 5 minutes. The pellet was discarded and the supernatant centrifuged at 10,000 rpm for 45 minutes in a JA-10 rotor or at 14,000 rpm for 20 minutes in a JA-14 rotor. The pellet, representing the SMP, was resuspended in .25 M sucrose, 10 mM Tris-Cl (pH 7.4) and the protein concentration determined by the biuret technique.

(B) Heavy layer SMP

Heavy layer mitochondria were diluted to 20 mg/ml by addition of 30 mM Tris-SO_4 (pH 7.4) and then sonicated at the maximum power setting of the Brancon probe sonicator in 60 ml batches for 2 minutes. The sucrose concentration was increased to .65 M by addition of 2 M sucrose and the suspension was then homogenized and stirred for 10 minutes. 9 volumes of cold 10 mM Tris-SO_4 (pH 7.4) were added and the suspension was centrifuged in a JA-10 rotor at 4000 rpm (2000 xg) for 5 minutes. The pellet was resuspended in .25 M sucrose, 10 mM Tris-Cl (pH 7.4) and protein concentration determined by biuret.

Freezing of mitochondria, submitochondrial particles, and mitoplasts

Mitochondria, SMP , and mitoplasts were stored after freezing according to the method of Walton, Kervina, Fleischer, and Dow (1970). Mitoplasts were obtained by stopping the SMP preparation (digitonin method) just before addition of cytochrome c and sonication. This was done because the yield of SMP made from mitoplasts which had been frozen was greater than the yield of SMP which was obtained after freezing them. Dimethyl sulfoxide was added to a suspension of mitochondria, mitoplasts or SMP to be frozen to a final concentration of 10% (v/v). Crystalline bovine serum albumin was added to a final concentration of 10 mg/ml and the suspension stirred until the BSA was entirely dissolved. The suspension was then stored frozen in liquid nitrogen for up to 2-3 weeks. To remove the dimethyl sulfoxide and BSA from a suspension before use, the suspension was thawed at room temperature and then diluted by a factor of approximately 20-40 with cold suspending medium (usually MSH). The diluted suspension was then centrifuged either in a JA-20 rotor at 12,500 rpm for 6 minutes (for mitochondria and mitoplasts) or in a Sorvall A-841 rotor at 40,000 rpm for 1 hour (for SMP). The pelleted particles were resuspended in suspending medium using homogenization and then used or processed further.

Preparation of cytochrome oxidase

Cytochrome oxidase was prepared by both a modification of the method of Kuboyama, Yong, and King (1972) and by the method of Fowler Richardson, and Hatefi (1962). The Kuboyama method (Method 1) was

more time-consuming and difficult, but produced oxidase which gave good respiratory control ratios in COV and was used for all reconstitution experiments. The Fowler oxidase (Method 2) was used only for the experiments on subfractionation of the oxidase into component parts and was not found to be suitable for reconstitution experiments.

Method 1

The mince was made by the method of Yonetani (1966) as follows: Fat and connective tissue were cut into small pieces and ground in a meat grinder in a cold room at 4°C. The mince was washed 2 times with 10 volumes of crushed ice and distilled water for 30 minutes with occasional stirring, then with 10 volumes of 20 mM sodium phosphate (NaPi) buffer pH 7.4 and finally 2 more times with crushed ice and distilled water. The mince was collected after each wash by squeezing through 2 layers of cheesecloth. The washed mince was either processed further or stored overnight at 0-4°C.

The mince was subsequently processed according to the method of Kuboyama et al (1972), and all further operations were carried out at 0-4°C. 300 gram aliquots of the mince were homogenized at the maximum speed setting of a 1 liter Waring blender with 150 ml of .2 M sodium phosphate (pH 7.4) and 150 grams of crushed ice for 8 minutes. 2/3 volume of distilled water was added to the homogenate and the mixture was centrifuged in a Beckman JA-10 rotor at 2700 rpm (800 xg) for 20 minutes. The supernatant was saved and the loose precipitate rehomogenized for 3 minutes with 1/5 volume (of the diluted homogenate) of 20 mM sodium phosphate (NaPi), pH 7.4. This second homogenate was recentrifuged as before and the precipitate was discarded. The pooled

supernatant was acidified to pH 5.6 by gradual addition of 1 M acetic acid while stirring in centrifugable batches (3 liters for the JA-10 rotor). Approximately 13 ml of acetic acid was required per liter of supernatant. The suspension was immediately centrifuged in the JA-10 rotor at 2700 rpm for 10 minutes. The precipitate was washed with 10 volumes of distilled water and recentrifuged. The washed precipitate was resuspended in approximately 40-60 ml (per liter of original diluted homogenate) of .2 M NaPi, pH 7.4, using a 40 ml glass homogenizer tube with a motor driven teflon pestle. The pH of the homogenate was adjusted to 7.4 with 3M ammonium hydroxide if necessary, and the homogenate then diluted by addition of an equal volume of distilled water. Ordinarily this particulate preparation was then stored overnight at 0-4°C. before further processing.

While stirring, a 10% (w/v) solution of sodium cholate (pH 7.4) was added to give a final cholate concentration of 1%. Solid ammonium sulfate was ground to a fine powder with a mortar and pestle and then slowly added (over a period of about 30 minutes) to the suspension with continuous stirring to a final ammonium sulfate saturation of .25, as calculated from the formula

$$x = \frac{.506\ (S_2 - S_1)}{1 - (.286\ S_2)}$$

where x is the amount of ammonium sulfate added in g/ml, S_2 is the desired final ammonium sulfate saturation, and S_1 is the initial ammonium sulfate saturation of the suspension. Saturation here is used as a fractional value, with 1.00 representing a completely saturated solution of ammonium sulfate. At 0°C., this is about 4.1 M in ammonium

sulfate. Therefore at .25 saturation the solution contains 25% of the concentration of ammonium sulfate at full saturation, or 1.025 M ammonium sulfate. The pH was adjusted to 8.0 with 1 N sodium hydroxide (usually required about 1.2 ml/100 ml of suspension), and the suspension was then incubated for 1 hour with occasional stirring. Additional solid ammonium sulfate was then added to bring the saturation to .35. After 10 minutes, the suspension was centrifuged at 14,000 rpm in a JA-14 rotor for 90 minutes (or at 20,000 rpm in a JA-20 rotor for 60 minutes, depending on the size of the batch). The clear and faintly orange supernatant was discarded and the precipitate was resuspended in .1 M NaPi (pH 7.4) to a final volume equal to 3/4 of the previous particulate preparation volume using a glass/teflon homogenizer. While stirring the suspension, 10% sodium cholate was added to give a final cholate concentration of 2%. Solid powdered ammonium sulfate was added as before to a saturation of .25 and the pH was adjusted to 7.6 with 1 N sodium hydroxide. The mixture was then incubated at 0°C. for 10-12 hours.

The suspension was centrifuged at 19,000 rpm in a JA-20 rotor for 30 minutes. The pellet was discarded, and the supernatant filtered through Whatman #1 filter paper to remove any small particles of the pellet. The supernatant was then brought to .40 saturation of ammonium sulfate by addition of powdered ammonium sulfate as before. After 10 minutes of incubation with occasional stirring the mixture was centrifuged at 19,000 rpm in the JA-20 rotor for 15 minutes. All subsequent centrifugations in this preparation were done at 30,000 rpm in a Sorvall A-841 rotor and a Sorvall OTD-65 ultracentrifuge for 20

minutes, or at 19,000 rpm in the JA-20 rotor for 30 minutes, depending on the batch. The supernatant was discarded and the dark green-brown precipitate was dissolved in 1/4 volume (of the previous particulate preparation) of .1 M NaPi (pH 7.4) containing 1.5% sodium cholate (w/v). While this clear green solution was stirred, a saturated (at 0°C.), neutralized (to pH 7.0 by addition of a small amount of 1 N ammonium hydroxide) solution of ammonium sulfate was added slowly until a slight but persistant turbidity became apparent. This was usually judged by observing a decrease in the visibility of the graduations on the side of the beaker when viewed through the solution from above. The turbidity usually occurred at an ammonium sulfate saturation of approximately .25. The amount of saturated ammonium sulfate solution to be added to a solution to achieve a desired saturation was calculated from the formula

$$y = \frac{S_2 - S_1}{1 - S_2}$$

where y is the amount of ammonium sulfate solution to be added in ml per ml of cytochrome oxidase solution, S_1 is the initial ammonium sulfate saturation, and S_2 is the desired final saturation. The mixture was incubated for 30 minutes and then centrifuged. The brownish opaque pellet was discarded and saturated ammonium sulfate solution added to the supernatant to give a saturation of .37. Since the oxidase should not be exposed to ammonium sulfate concentrations higher than necessary to minimize denaturation of the enzyme, whenever large aggregates (visible clumping) of the precipitate occurred during ammonium sulfate addition, further addition of the saturated ammonium sulfate solution

was discontinued. This would usually occur at about .36 saturation at this stage of the preparation. The turbidity and aggregation were used as criteria for determining the cutoff points for ammonium sulfate addition and the subsequent saturations suggested here are taken from the Kuboyama procedure (1972) and were only used as guidelines, since it was found that usually adding slightly less ammonium sulfate was necessary. Any floating oily precipitates noticed after centrifugation were carefully removed by filtration through glass wool.

After a 10 minute incubation, the suspension was centrifuged and the clear or slightly green supernatant was discarded. The dark green-brown pellet was dissolved in approximately 20 ml of .1 M NaPi (pH 7.4), 1.5% cholate per 100 ml of original particulate preparation. Saturated ammonium sulfate was again added to the solution until a slight but persistant turbidity appeared. After centrifugation, the supernatant was brought to about .36 saturation by addition of saturated ammonium sulfate solution. The precipitate obtained by centrifugation was dissolved in 15 ml per 100 ml of original particulate preparation of .1 M NaPi (pH 7.4) containing 1% Tween 20. The solution was brought to slight turbidity by addition of saturated ammonium sulfate solution and incubated for 30 minutes with occasional agitation. After centrifugation the ammonium sulfate fractionation procedure was repeated twice, but each time the ammonium sulfate saturation used to precipitate out the oxidase was decreased by about .01. The final pellet was dissolved in .25 M sucrose and then dialyzed against about 25 volumes of .25 M sucrose for 8 hours. The dialysis solution was changed and dialysis then continued for an additional 8 hours to

remove residual detergents and ammonium sulfate.

The oxidase protein concentration was determined by a modified biuret assay and the protein concentration was adjusted to 25 mg/ml by addition of .25 M sucrose. The cytochrome oxidase was then stored in .5-1.5 ml aliquots in liquid nitrogen.

Method 2 (Fowler et al, 1962)

Beef heart mitochondria prepared as previously described were thawed and diluted with 3 volumes of .25 M sucrose, 10 mM Tris-Cl, pH 7.4. The suspension was centrifuged in a JA-20 rotor at 14,000 rpm for 15 minutes. The mitochondria were resuspended using glass tube/teflon pestle homogenizer in .66 M sucrose, 1 mM histidine, 50 mM Tris-Cl (pH 8.0). Protein concentration was determined by the biuret method and adjusted to 23 mg/ml. 10% (w/v) potassium deoxycholate solution (pH 9.0) was slowly added with stirring to give a final concentration of .3 mg/mg protein. Crystalline KCL was then slowly added (72 g/l of suspension) while stirring over a period of about 15 minutes. After the KCL was completely dissolved the suspension was centrifuged in a Sorvall A-841 rotor and Sorvall Model OTD-65 ultracentrifuge at 30,000 rpm for 30 minutes.

The reddish supernatant was discarded and the dark green pellets were resuspended in an equal volume of .66 M sucrose, 50 mM Tris-Cl, 1 mM histidine (pH 8.0), homogenized, and recentrifuged as before. The supernatant was discarded, and the pellet, which has 3 layers (a light brown fluffy upper layer, a dark green middle layer representing the bulk of the pellet, and a dark brown bottom layer) was selectively removed by aspiration of the top layer with a pasteur

pipet connected by plastic tubing to a water aspirator, with subsequent removal of the middle layer with a small spatula (avoiding any of the bottom layer). This separation is critical to the purity of the preparation, and must be done very carefully. The middle layer of the pellet was then resuspended and homogenized in sucrose-Tris-histidine to a final protein concentration of 19 mg/ml. 10% potassium deoxycholate was added to a final concentration of .5 mg/mg protein and then 74.5 g/l of crystalline KCl. The suspension was then centrifuged for 20 minutes at 30,000 rpm and the pellet discarded. The supernatant was dialyzed against 30 volumes of 10 mM Tris-Cl pH 8.0 for 90 minutes at 0°C. Ammonium sulfate was added (from a solution saturated at 0°C. and neutralized to pH 7.8 with ammonium hydroxide) in the amount of .19 ml/ml dialysate. After 10 minutes of incubation at 0° the mixture was centrifuged in a JA-20 rotor at 19,000 rpm for 15 minutes. The dark green pellet was resuspended in sucrose-Tris-histidine at 26 mg/ml (as determined by modified biuret technique). A second fraction was precipitated by adding .04 ml of saturated ammonium sulfate solution per ml of supernatant and centrifuging at 19,000 rpm for 15 minutes after a 10 minute incubation. This green pellet was also resuspended in sucrose-Tris-histidine at a protein concentration of 20 mg/ml. Both fractions were stored in liquid nitrogen.

Fractionation of cytochrome oxidase

(A) Ammonium sulfate refractionation method (from Roger Carroll, personal communication, and Carroll and Racker, 1977)

In order to increase the purity of the cytochrome oxidase used

in the reconstitution of COV the following procedure was carried out: Cytochrome oxidase prepared by the Kuboyama method was thawed out and diluted to 5 mg/ml with NaPi (pH 7.4) to a final concentration of .1 M NaPi. 20% (w/v) sodium cholate (pH 7.4) was added with stirring to give a final cholate concentration of 1.5%. Saturated ammonium sulfate solution (saturated at 0°C. and neutralized to pH 7.4 with ammonium hydroxide) was slowly added to the oxidase suspension until slight but persistant turbidity appeared (usually at about .25 saturation). The suspension was incubated at 0°C. for 30 minutes and centrifuged in a JA-20 rotor at 18,000 rpm for 25 minutes. The supernatant was brought to .35 saturation by addition of saturated ammonium sulfate solution and the precipitate was collected by centrifugation and dissolved in .1 M NaPi (pH 7.4) containing 1% (w/v) Tween 20. The final volume was 1/2 the volume of the suspension of oxidase at 5 mg/ml. Saturated ammonium sulfate was added to give slight turbidity (approximately .28 saturation) and the mixture centrifuged as before. The precipitate was dissolved in a small amount of .25 M sucrose and represented the first fraction of the oxidase. Further fractions were collected by sequential addition of ammonium sulfate solution in increments of .02 saturation, followed by a 10 minute incubation period and centrifugation each time. The precipitates from each fraction were dissolved in a small amount of .25 M sucrose. This procedure was continued until no more green color remained in the supernatant (usually at about .36 saturation). Any floating oily precipitates appearing in the supernatants after centrifugation were carefully removed by filtering the super-

natant through glass wool several times. The various fractions of the oxidase were stored in small (.5 ml) aliquots in liquid nitrogen, and later the best fractions were selected for use in further experiments by ascertaining their ability to produce COV's with high respiratory control ratios.

(B) Purification of cytochrome oxidase made by the Fowler technique

This method of purification of cytochrome oxidase was carried out according to the procedure of Kopaczyk, Perdue, and Green (1966). The 2 fractions of the oxidase were diluted to 1 mg/ml in .25 M sucrose, 10 mM Tris-Cl (pH 7.8). After approximately 10 minutes the solutions became turbid as the oxidase precipitated out of solution due to the dilution of the residual detergent in the preparation. The suspensions were centrifuged in a Sorvall rotor at 40,000 rpm for 90 minutes at 4°C. The crystal clear supernatants were discarded and the dark green pellets were resuspended in .25 M sucrose, 10 mM Tris-Cl (pH 7.5) at a protein concentration of 20 mg/ml. These pellets were not soluble, since the residual detergent had been removed. The resuspension was accomplished with the use of a glass/teflon homogenizer. The green, turbid suspensions were stored frozen in liquid nitrogen overnight and after thawing the next day the following additions were made (in the indicated order) while stirring at 0°C.: 10% (w/v) potassium deoxycholate (pH 9.0) to a final amount of 1.5 mg/mg protein, 20% (w/v) potassium cholate (pH 8.0) to a final amount of .75 mg/mg protein, .5 mM EDTA (pH 7.6) to a final concentration of 1 mM, 1.2 mg/ml of sodium dithionite powder, and 1 mg of vitamin E per 100 mg of protein. The solutions were then brought to

.15 saturation of ammonium sulfate by addition of appropriate amounts of saturated ammonium sulfate solution. The mixture was incubated in the dark at room temperature for 8 hours. After the incubation period the suspensions were centrifuged at 30,000 rpm for 18 minutes at 0°C. A green-brown pellet and green supernatant were obtained. Saturated ammonium sulfate was added to increase the saturation to .28 and the suspensions were centrifuged at 30,000 rpm for 25 minutes. The supernatants were clear and colorless and the pellets were dark green and oily in consistency. The pellets were dissolved in .25 M sucrose, 10 mM Tris-Cl, 1 mM EDTA (pH 8.0) at a protein concentration of 10-15 mg/ml and dialyzed overnight against 100 volumes of the same buffer solution at 0°C., and then frozen in liquid nitrogen.

(C) Fractionation of cytochrome oxidase into an electron transfer complex and an ionophore transfer complex (ETC/ITC; Kessler, Blondin, VandeZande, Haworth, Green, 1977)

Method 1

Cytochrome oxidase made by the method of Fowler and purified by the procedure of Kopaczyk was placed in dialysis tubing and dialyzed for 12 hours at 0°C. against about 200 volumes of 50 mM Tris-Cl (pH 8.0). The protein concentration was 10 mg/ml. While vortexing the cytochrome oxidase solution in a centrifuge tube 5 volumes of methanol containing 30 mM HCl was added. The suspension was cooled to -10°C. for 10 minutes in a centrifuge and then centrifuged in a JA-20 rotor at 8000 rpm for 10 minutes. The brown supernatant was decanted into a small beaker. The pellet, which represented the ITC fraction, was rinsed with methanol containing 30 mM HCl and 16.7% water and then resuspended by homogenization in 10 mM Tris-Cl (pH

8.0) to a final concentration of 5-10 mg/ml as determined by the Lowry technique. The supernatant was adjusted to pH 6.5 by addition of approximately 1 drop of 3.8 M sodium acetate (pH 7.7) per 10 ml of supernatant. A green precipitate appeared after this pH adjustment and was collected by centrifugation in the JA-20 rotor at 4°C. and 17,000 rpm for 20 minutes. The green pellets obtained were rinsed with 10 mM Tris-Cl (pH 8.0) and then resuspended by homogenization in .25 M sucrose (the pellets were not soluble) to a final protein concentration of 5-10 mg/ml as determined by the Lowry techinique. These precipitates represented the ETC. All aliquots of ETC and ITC were stored in liquid nitrogen.

Method 2

Cytochrome oxidase made by the method of Kuboyama was diluted with water, .2 M potassium phosphate (pH 7.4), and 10% (w/v) potassium cholate (pH 8.0) such that the final mixture contained 10 mg/ml protein, .1 M potassium phosphate, and 2% potassium cholate. A saturated solution of ammonium sulfate was added with stirring at 0°C. to give a final saturation of .25 and the pH was adjusted to 8.0 by addition of a small amount of 1 N KOH. The mixture was incubated at 4°C. for 4 days and then centrifuged in a JA-20 rotor at 17,500 rpm for 45 minutes. A light green pellet was obatined which represented the ITC fraction, and the clear green supernatant contained the ETC fraction. The supernatant was brought to .36 saturation of ammonium sulfate and the green precipitate was removed by centrifugation in the JA-20 at 18,000 rpm for 35 minutes. Both pellets (ITC and ETC) were dissolved in .25 M sucrose at 10-20 mg/ml and stored

frozen in liquid nitrogen.

Preparation of hydrophobic protein (Serrano, Kanner, Racker, 1976)

All operations were carried out at 0-4°C. Beef heart mitochondria made as previously described were treated with digitonin as previously described under the digitonin method of making SMP , except that the particles were resuspended in 75 ml of .25 M sucrose, 10 mM Tris-Cl (pH 7.4) per gram of original mitochondria after the first centrifugation. Then the suspension was centrifuged in a JA-14 rotor at 11,000 rpm for 6 minutes and the pellet resuspended in sucrose-Tris to a final volume equal to 33 ml/g original mitochondria. The mitoplasts were sonicated in 80 ml batches at the maximum power setting of a Branson W140 probe sonicator for 2 minutes. The suspension was centrifuged in a JA-20 at 18,500 rpm for 1 hour and the pellets, representing SMP , were resuspended in 10-15 ml sucrose-Tris/g mitochondria and stored frozen at -20°C. overnight. The protein concentration was adjusted to 50 mg/ml prior to freezing.

To 3 g of the SMP (60 ml) was added 40 ml of 30 mM Tris-SO_4, 1.5 mM EDTA, 3 mM $MgSO_4$, 1.5 mM dithiothreitol (pH 7.5). 12 ml of a saturated neutralized solution of ammonium sulfate was added to give a final saturation of .10, and 9 ml of a 20% (w/v) solution of sodium cholate (pH 7.5) to give a final cholate concentration of 1.5%. The mixture was stirred for 7 minutes and immediately centrifuged in a 50 Ti rotor in a Beckman L-250 ultracentrifuge at 50,000 rpm for 90 minutes. The supernatant was brought to .38 saturation of ammonium sulfate by addition of .45 ml of saturated ammonium sul-

fate solution per ml of supernatant. After centrifugation in a JA-20 rotor at 17,000 rpm for 20 minutes the saturation of the supernatant was increased to .45 by addition of .127 ml of saturated ammonium sulfate solution per ml of supernatant. After a 5 minute incubation, the mixture was again centrifuged at 17,000 rpm for 20 minutes. The supernatant was discarded and the brown pellet was resuspended by stirring with a glass rod in 23 mM Tris-SO_4, 1.2 mM dithiothreitol, 1.2 mM EDTA, 2.3 mM $MgSO_4$, 50 mM sucrose (pH 7.5). The pale, yellow-brown, clear solution was stored in liquid nitrogen in .5 ml aliquots, after adjusting the protein concentration to 30 mg/ml as determined with the biuret technique.

Preparation of oligomycin sensitivity conferring protein (OSCP) (Tzagaloff and MacLennan, 1968)

6 g of SMP made as described for the preparation of the hydrophobic protein fraction were suspended at 10 mg/ml in .35 M sodium bromide (NaBr), .25 M sucrose, 10 mM Tris-Cl, 1 mM dithiothreitol (pH 7.5) and incubated at 0°C. with slight stirring for 20 minutes. The suspension was centrifuged in a JA-20 rotor at 18,500 rpm for 90 minutes. The floating brown precipitate was resuspended in the same volume of the NaBr solution again and recentrifuged. The floating pellet was homogenized in the same final volume of .25 M sucrose, 10 mM Tris-Cl (pH 7.4) and centrifuged at 18,500 rpm for 1 hour. The pellet was rehomogenized in sucrose-Tris and the centrifugation repeated. After this centrifugation the pellet was resuspended in .3 M KCl, 1.5 mM EDTA at 15 mg/ml as determined by biuret. 1/2 vol-

ume of 1.2 N ammonium hydroxide was added with stirring and the mixture incubated with slight stirring at 0°C. for 30 minutes. The suspension was centrifuged at 18,500 rpm for 45 minutes and the supernatant decanted and set aside on ice while the pellet was resuspended in KCl-EDTA and treated with NH_4OH as before. While stirring, 10 N acetic acid was added to the combined supernatants to bring the pH to 8.0 (the amount of acetic acid added was about equivalent to 6% of the total volume of the suspension). The suspension was then centrifuged at 18,500 rpm for 30 minutes and the supernatant brought to .40 saturation of ammonium sulfate by addition of a saturated neutralized solution of ammonium sulfate. The suspension was centrifuged at 18,500 rpm for 30 minutes and the pellet dissolved in a small amount of 20 mM Tris-SO_4, 1 mM EDTA (pH 8.0). The solution was dialyzed for 4 hours against 30 volumes of this resuspension buffer, after which the dialysis solution was replaced with fresh buffer and the dialysis continued for 4 more hours, during which time the dialysate became turbid. The dialysis was centrifuged at 17,000 rpm for 25 minutes and the protein concentration in the clear yellow supernatant determined. The OSCP was frozen in 1.5 ml aliquots in liquid nitrogen.

Preparation of mitochondrial ATPase (F_1) (Horstman and Racker, 1970)

6-7 g of beef heart mitochondria made as previously described were diluted to 15 mg/ml with .25 M sucrose at 0°C. and centrifuged in a JA-20 rotor at 18,000 rpm for 20 minutes. The pelleted mitochondria were resuspended with a glass tube/teflon pestle homogenizer

to a concentration of 30 mg/ml in .15 M sucrose, 2 mM ATP, and 2 mM EDTA (pH 7.4). The suspension was sonicated in a single batch at the maximum power setting of a Branson W140 probe sonicator for 30 minutes. The temperature was allowed to rise to 45°C. during the first half of the sonication and to 52-55°C. during the second half of the sonication period. The mixture was then cooled to room temperature in a water bath and all subsequent operations were carried out at room temperature unless otherwise specified. The sonicated suspension was then centrifuged at 18,500 rpm in a JA-20 rotor for 2.5 hours. All subsequent centrifugations were also done in a JA-20 rotor. The precipitate was resuspended using homogenization in distilled water (a volume equal to the volume of the suspension at 30 mg/ml) and the sonication and centrifugation were repeated. The supernatants from these two centrifugations were both processed separately. The pH of the first supernatant (#1) was adjusted to 5.4 with 1 N acetic acid and the mixture was immediately centrifuged at 14,000 rpm for 5 minutes. The pH of the supernatant was quickly adjusted to 6.7 by addition of unneutralized 2 M Tris buffer. The total time of exposure to the acidic pH should not exceed 10 minutes. The acidification and centrifugation procedure was then repeated on supernatant #2. The next step in the procedure is a protamine precipitation, but the amount of protamine to be used is extremely critical and varies from batch to batch so a pilot precipitation must be carried out. It was found that adding more protamine than the minimum amount necessary to render the solution turbid would tend to precipitate out all the F_1, so protamine (from a freshly made .5%

solution adjusted to pH 6.0 with 1N H_2SO_4) was added to 1 ml aliquots of supernatant #1 until the minimum amount producing turbidity was found. This varied from .007-.018 ml/ml for different batches. The original preparative procedure suggests approximately .02 ml/ml. When the appropriate amount was determined, this amount of protamine was added dropwise with stirring into solution #1. After a 5 minute incubation, the suspension was centrifuged at 15,500 rpm for 5 minutes and the white precipitate was discarded. 40 ml of the protamine solution per 100 ml of supernatant was then added and the suspension centrifuged at 14,000 rpm for 15 minutes after a 5 minutes incubation. With supernatant #2, the pilot precipitation step is ommitted and 42 ml of the protamine solution per 100 ml of supernatant added in one step. After 5 minutes of incubation the suspension was centrifuged at 14,000 rpm for 15 minutes. The bright yellow pellets were combined and dissolved in a small amount (5-10 ml) of .4 M ammonium sulfate, .25 M sucrose, 10 mM Tris-SO_4, .2 mM EDTA (pH 7.5). If turbid, the suspension was centrifuged at 15,500 rpm for 5 minutes and the precipitate discarded. An equal volume of ammonium sulfate solution (saturated at room temperature and neutralized to pH 7.2 with 1 N NH_4OH) was added while stirring. The suspension was cooled on ice for 10 minutes and centrifuged at 4°C. and 15,500 rpm for 10 minutes. The pellet was resuspended in about 5 ml of .25 M sucrose, 10 mM Tris-SO_4, 2 mM EDTA, 30 mM ATP (pH 7.4) and a small aliquot removed for protein determination by Lowry or biuret assay. The protein concentration was diluted to 4 mg/ml by the addition of an appropriate amount of the same solution used to resuspend the pellet.

The solution was placed in a glass graduated cylinder and immersed in a 72°C. water bath until the temperature of the solution reached 64°C. and was then transferred to a 64°C. water bath for the remainder of the 4 minute heating period. After being cooled in a water bath to 30°C. the suspension was centrifuged at room temperature at 16,000 rpm for 10 minutes. An equal volume of a saturated ammonium sulfate solution was then added to the supernatant and the suspension was cooled on ice for 10 minutes and centrifuged at 4°C. and 15,500 rpm for 5 minutes. The white precipitate was resuspended in 10 ml of .25 M sucrose, 10 mM Tris-SO_4, 2 mM EDTA, 4 mM ATP (pH 7.4) and stored frozen in .5 ml aliquots in liquid nitrogen. When used for experiments, a vial of F_1 was quickly thawed and kept at room temperature until being returned to storage.

Preparation of complex V (Hatefi, Haavik, Jurtshuk, 1961; Hatefi, Stiggall, Galante, Hanstein, 1974)

All operations were carried out at 0-4°C. Beef heart or rat liver mitochondria were thawed at room temperature, diluted with .25 M sucrose to a protein concentration of 30 mg/ml, and centrifuged in a JA-20 rotor at 19,000 rpm for 30 minutes. The sedimented mitochondria were suspended in 50 mM Tris-Cl, .66 M sucrose, 1 mM histidine (pH 8.0) by homogenization with a glass/teflon homogenizer to a final protein concentration of 23 mg/ml. .3 mg of potassium deoxycholate/mg protein were added from a 10% (w/v) solution (pH 9.0), and then 72 g/l of crystalline KCl was added over a period of 15 minutes with continuous stirring. After complete dissolution of the

KCl the suspension was centrifuged for 45 minutes at 19,000 rpm (all centrifugations were done in the JA-20 rotor). According to the original published procedure the supernatant is supposed to be red in color, but in 5 preparations, this was never the case, and the supernatant was always a clear brown color. .25 volumes of distilled water was added to the supernatant and the suspension was recentrifuged for 1 hour. The supernatant (again, supposedly clear red but actually clear brown) was dialyzed against 20 volumes of 10 mM Tris-Cl (pH 8.0) for 3-4 hours, during which time the dialysate became cloudy. The dialysate was then centrifuged at 19,000 rpm for 2.5 hours (the actual procedure suggested centrifugation in a Spinco #30 rotor for 90 minutes at 30,000 rpm, or 78,000 xg). The supernatant was decanted and the brown pellet (which should be red in color according to the original procedure) was discarded. Instead of the Sephadex treatment suggested by the original published procedure, the supernatant was dialyzed overnight against 20 volumes of 10 mM Tris-Acetate (pH 7.5), with the dialysis solution being replaced with fresh solution after 6 hours of dialysis. The dialysate was brought to .42 saturation of ammonium sulfate by the addition of a saturated, neutralized solution of ammonium sulfate and was centrifuged at 19,000 rpm for 35 minutes. The pellet was resuspended in .25 M sucrose, 10 mM Tris-acetate (pH 8.0) at a protein concentration of 20 mg/ml as determined by the biuret reaction. Potassium cholate was then slowly added while stirring from a 20% (w/v) solution (pH 8.0) to a final concentration of .365 mg cholate/mg protein. The solution was then brought to .24-.25 saturation by addition of a

saturated solution of ammonium sulfate, at which time a white precipitate formed and was removed by centrifugation for 35 minutes at 19,000 rpm. The clear yellow supernatant was brought to .42 saturation by addition of more ammonium sulfate solution, and the yellow gelations pellet which was obtained after centrifugation at 19,000 rpm for 35 minutes was dissolved in a small amount of .25 M sucrose, 10 mM Tris-acetate (pH 8.0) to a final protein concentration of 15-25 mg/ml and stored in small aliquots in liquid nitrogen. This solution represented the complex V preparation.

The appearance of the beef heart mitochondria and rat liver mitochondria preparations was similar throughout the protein extractions. However, there were some differences at the ammonium sulfate fractionation stage. With the rat liver preparation, the supernatant was still yellow after the .42 saturation centrifugation, so a second fraction of rat liver complex V was recovered by increasing the saturation of ammonium sulfate to .50 and recentrifuging the suspension. The final supernatant was completely clear, and the yellow precipitate representing the second (.42-.50) fraction of the complex V was recovered as a yellow gelatinous floating layer. This layer was best removed by its adherance to the tip of a J-shaped Pasteur pipet, with subsequent dissolution in .25 M sucrose, 10 mM Tris-acetate (pH 8.0).

With the beef heart preparation, the supernatant was clear after the .42 saturation centrifugation, and the pellet was a yellow gelatinous floating layer. However, the precipitation at .24-.25 saturation was variable, and if a clear yellow supernatant and white pellet

were not obtained, after the .25 saturation centrifugation or a yellow gelatinous floating layer was not obtained after the .42 saturation centrifugation, the fractionation procedure with potassium cholate and ammonium sulfate was repeated on the material obtained after the .42 saturation centrifugation. After the cholate addition, the ammonium sulfate saturation was gradually increased until a white precipitate and clear yellow supernatant were obtained after centrifugation. It was necessary to increase the ammonium sulfate saturation as high as .31 for this to occur. Then the saturation was increased to .42 and after centrifugation the floating yellow precipitate was removed and dissolved in .25 M sucrose, 10 mM Tris-acetate (pH 8.0) as described above.

Preparation of the adenine nucleotide transporter (Shertzer and Racker, 1976)

SMP were made from beef heart mitochondria by the osmotic shock method detailed previously. All operations were carried out at 0-4°C. 3.6 g of SMP were incubated at 30 mg/ml in 2% (w/v) sodium cholate, 1 mM dithiothreitol, 50 M EDTA, 125 mM sucrose, .4 M ammonium sulfate, and 10 mM Tricine-KOH (pH 8.0) for 30 minutes with stirring. The insoluble residue was removed by centrifugation at 50,000 rpm in a Beckman 50Ti rotor for 1 hour. The supernatant was brought to .32 saturation of ammonium sulfate by addition of an appropriate amount of a saturated neutralized solution of ammonium sulfate, and then centrifuged at 19,000 rpm in a JA-20 rotor for 30 minutes. The precipitate was discarded and the supernatant was

brought to .40 saturation with ammonium sulfate and the precipitate obtained after centrifugation in a JA-20 for 30 minutes at 19,000 rpm was also discarded. The saturation of the supernatant was then increased to .45 and the yellow-brown pellet obtained after centrifugation was dissolved in a small amount of 125 mM sucrose, 1 mM dithiothreitol, 10 mM Tricine-KOH (pH 8.0) at a protein concentration of approximately 10 mg/ml as determined by the biuret technique. The supernatant was brought to .50 saturation by addition of more ammonium sulfate solution and a second fraction of the adenine nucleotide transporter was obtained as another yellow pellet which was also dissolved in sucrose-dithiothreitol-Tricine buffer at about 10 mg/ml. Both fractions of the adenine nucleotide transporter were stored frozen in liquid nitrogen in small aliquots.

Preparation of the phosphate transporter from mitochondria (Banerjee, Shertzer, Kanner, Racker, 1977)

Beef heart mitochondria were prepared in the usual manner. A-particles (SMP made by sonication in the presence of ammonia and EDTA) were made according to the procedure of Fessenden and Racker (1966) as follows: 5 g of mitochondria were thawed at room temperature and then placed on ice. The mitochondria were diluted to 17 mg/ml with .25 M sucrose, 10 mM Tris-Cl (pH 7.4) and centrifuged in a JA-20 rotor at 15,000 rpm for 20 minutes. The pelleted mitochondria were resuspended in 48 mM sucrose, .62 mM EDTA (pH 7.45) using a glass/teflon homogenizer to a final protein concentration of 27.5 mg/ml. An aliquot of this suspension which could be centrifuged in a single batch in a Beckman 50 Ti rotor (120 ml) was then brought

to pH 9.2 by addition of 1 N NH_4OH (approximately .01 ml/ml of suspension) with continuous stirring and pH monitoring. The suspension was then sonicated for 2 minutes at the maximum power setting of a Branson W140 probe sonicator in 30 ml batches. The sonicated solution was centrifuged in a JA-20 rotor at 18,000 rpm for 10 minutes and the supernatant was carefully decanted and centrifuged in a 50 Ti rotor at 50,000 rpm for 45 minutes. The dark green-brown pellets were resuspended in .25 M sucrose, 1 mM EDTA (pH 7.5) by homogenization. Any remaining mitochondrial suspension was processed to this point and the resuspended dark green-brown pellets were pooled and the volume adjusted to 120 ml by addition of .25 M sucrose, 1 mM EDTA (pH 7.5). The suspension was then centrifuged in a 50 Ti rotor at 50,000 rpm for 20 minutes. If the supernatant was clear further washing of the suspension was omitted, otherwise the pellets should be resuspended and centrifuged again. The final pellet represents the A-particles, and was resuspended in 5% glycerol, 2 mM dithiothreitol, 10 mM EDTA, 50 mM potassium phosphate (pH 8.0). The A-particles were stored frozen in liquid nitrogen.

All further operations were carried out at 0-4°C. The A-particles were thawed and diluted to 2.25 mg/ml in 5% glycerol, 10 mM EDTA, 2 mM dithiothreitol, 50 mM potassium phosphate (KPi) (pH 8.0). Triton X-100 was added with stirring to a final concentration of 1%. This mixture was stirred for 30 minutes and centrifuged in a JA-20 rotor at 19,000 rpm for 5 hours (160,000g x hr.). The dark brown pellet was discarded and to the clear yellow supernatant was added SM-2 Biobeads which had been incubated overnight in 5% glycerol,

10 mM EDTA, 2 mM dithiothreitol, 50 mM KPi (pH 8.0) and drained on a Buchner funnel. 12 g of Biobeads were added per 90 mg of A-particles with which the preparation was started. The suspension was gently stirred for 2 hours and then centrifuged in the JA-20 at 15,000 rpm for 10 minutes. The supernatant was filtered through Whatman #1 filter paper in a Buchner funnel and then twice through glass wool. To the filtered supernatant was added an equal volume of saturated ammonium sulfate solution (pH 7.4). The suspension was incubated for 10 minutes and then centrifuged in a JA-20 rotor for 15 minutes at 19,000 rpm. The light brown, friable, floating precipitate was collected and resuspended by homogenization in 5% glycerol, .05 mM dithiothreitol, 1 mM $MgCl_2$, .5 mM EDTA, 10 mM Tricine-KOH (pH 7.5). The pellet is not soluble in the solution and is stored as a suspension in small aliquots in liquid nitrogen.

Preparation of antibodies to F_1

Antiserum to F_1 was made by injection of rabbits with a suspension of F_1. Antibodies have been made previously using mice and horses (Fessenden and Racker, 1966; E. Racker, personal communication), but the procedure for bleeding the mice is difficult and traumatic and the use of horses was precluded because of their size and expense. Rabbits and chickens have been used in the past, but give variable results (Fessenden and Racker, 1966). However rabbits were used in these experiments because of the greater practicality of the procedure. 3 rabbits weighing about 3 kg were immunized as follows: .4 ml of a suspension of highly purified F_1 (supplied by Dr. A.

Senior) and containing 7.5 mg/ml was added to 2.1 ml of 40 mM Tris-SO_4, 1 mM EDTA, 4 mM ATP (pH 7.4). 2.5 ml of a saturated solution of ammonium sulfate and 5 ml of complete Freund's adjuvant were added to this. The mixture was emulsified with a Vertise homogenizer for 10 minutes (or until a drop of the emulsion placed on the surface of distilled water remained intact). 2.5 ml of the emulsion was drawn into each of three 5 ml syringes. Using 1 1/2" 20 gauge needles, 1.25 ml of the emulsion was injected into the inner aspect of the thigh muscle of each leg of the 3 rabbits. This was repeated 3 times at 1 week intervals. For the last 2 sets of these injections water was substituted for the saturated solution of ammonium sulfate beacuse of possible irritant effects of the ammonium sulfate.

10 days after the last injection the rabbits were test-bled by nicking a vein in the ear and removing several ml of blood. The blood was placed in test tubes at 4°C. overnight and the tubes rimmed with a wooden stick to allow clot retraction. The blood was then centrifuged in a JA-20 rotor at 15,500 rpm for 15 minutes. The serum was decanted and recentrifuged at 18,000 rpm for 15 minutes to remove any residual blood cells. The antibody titers were then checked using the Ouchterlony double diffusion assay (O. Ouchterlony, 1967). because the titers were low, the rabbits were given a booster injection of F_1 1 week later, and the blood removed from them by cardiac puncture 10 days after the booster injection. The blood was processed by centrifugation as above and the antiserum was stored frozen at -20°C.

Isolation of the immunoglobulin fraction of antiserum to F_1 (P. Stelos, 1967)

An equal volume of saturated neutralized ammonium sulfate solution was slowly added to an aliquot of antiserum with continuous stirring at 0°C. The suspension was then incubated at 4°C. overnight and then centrifuged at 16,000 rpm for 20 minutes in a JA-20 rotor. The pellet was resuspended in 60% saturated ammonium sulfate solution (pH 6.5) and recentrifuged. The precipitate was dissolved in a volume of .85% NaCl equal to the original volume of the antiserum. An equal volume of a saturated solution of ammonium sulfate was added and after 15 minutes of incubation at 0°C. the suspension was centrifuged as before. The pellet was dissolved in a small amount of .9% NaCl, 10 mM HEPES (pH 7.2) and dialyzed against approximately 30 volumes of this buffer for about 36 hours, with 2 changes of buffer at 12 hour intervals. The protein concentration was determined by the biuret method and the immunoglobulins were stored at -20°C.

Drying phospholipids for preparation of liposomes

Phospholipids were stored as a 4:1 chloroform:methanol solution at -20°C. under nitrogen. The phospholipids generally used for making liposomes and proteoliposomes were soy asolectin which had been acetone-extracted to remove neutral lipids as previously described. Generally the phospholipid solution contained about 100 mg/ml of phospholipid. The appropriate amount of the phospholipid

solution was pipetted into a pyrex test tube and dried by blowing a stream of nitrogen on the aliquot of phospholipid solution. To allow more complete drying, the phospholipids were then dissolved in a small amount of room temperature diethyl ether (.5 ml/100 mg) and dried again.

Phospholipid vesicles (PLV)

Liposomes containing no proteins were made by one of two methods.

Method 1

An appropriate salt and buffer solution (usually 40 mM K_2SO_4, 10 mM HEPES, pH 7.0) was added to the dried phospholipids in an amount so as to give a final phospholipid concentration of 20 mg/ml. The total volume of a suspension to be sonicated in a single batch should not exceed 3-4 ml, or the suspension would take a very long time to clarify. The suspension was vortexed for about 2 minutes to partially suspend the phospholipids and then sonicated in an L & R type 210 (or equivalent) bath sonicator at room temperature until the suspension became clear (nonturbid). The length of time required for this sonication depends on the effectiveness of the sonic action at dispersing the phospholipids, which necessitates tuning the sonicator for maximum visible agitation of the suspension. Upon clarification (after 5-20 minutes of sonication) the suspension of liposomes (PLV) was placed on ice until used.

Method 2

This method takes longer because of the time required for dialysis but was used when liposomes and proteoliposomes were being com-

pared in an experiment because this method of making the PLV more closely parallels the methods of formation for most of the proteoliposomes used. Distilled water and sodium cholate (from a 20% (w/v) solution, pH 7.4) were added to an aliquot of dried phospholipids to give a final phospholipid concentration of 80 mg/ml and 2% cholate. The volume of the phospholipids was neglected in this calculation. The tube was vortexed for about 1 minute to partially solubilize the phospholipids. The suspension was then sonicated at room temperature under nitrogen at the maximum tunable sonic action of the L & R type 210 (or equivalent) bath sonicator. Sonication continued until a clear (nonturbid) suspension was obtained. The maximum volume should not exceed 4-5 ml per batch or the sonication takes excessively long times. All sonication was done in pyrex tubes to minimize breakage. Only one tube was ever sonicated in the bath at a time. While phospholipid suspensions were always sonicated at room temperature, protein-containing suspensions were generally sonicated at 0°C. After sonication the suspension was diluted to 40 mg/ml by addition of an appropriate buffer solution (usually 80 mM K_2SO_4, 10 mM HEPES, pH 7.0). After mixing well the suspension was placed in dialysis tubing both ends tied tightly with braided nylon string, minimizing any trapped air in the tubing. The liposomes were then dialyzed approximately 16 hours by either of the following methods:

Dialysis

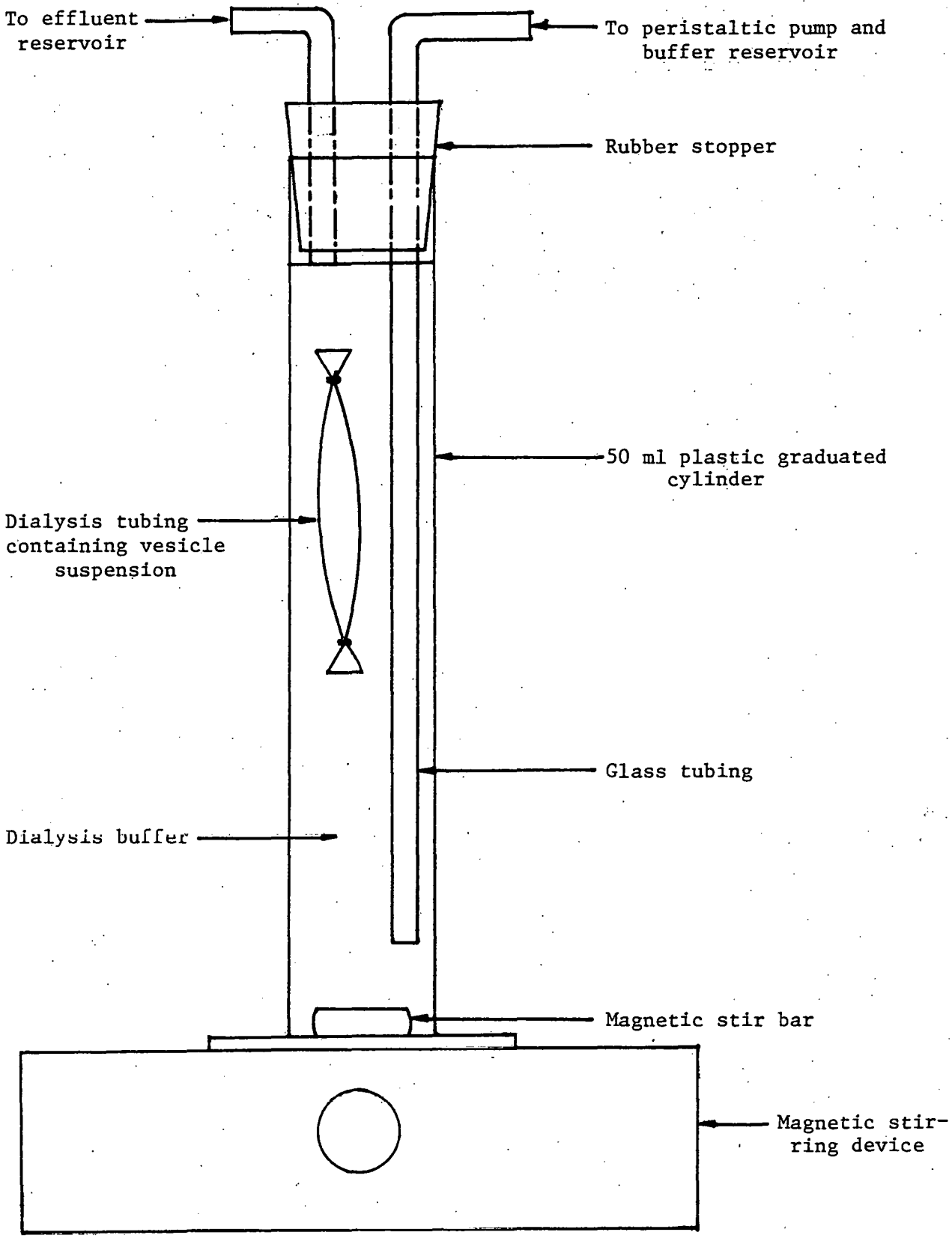
The dialysis tubing, after being tied shut with braided nylon string, was placed in a beaker or flask which contained approximately 100-300 volumes of buffer solution (usually 40 mM K_2SO_4, 10 mM HEPES,

pH 7.0) and gently stirred at 0°C. for 6-10 hours. The dialysis solution was then replaced with fresh solution and dialysis was continued for an additional 8-10 hours.

Flow dialysis

Because the above method of dialysis often required buffer changes at inconvenient times a flow dialysis was devised (see Figure 1). The system consisted of a 50 ml chamber (a plastic graduated cylinder with spout cut off so as to create a completely round cross section at the top) with a stir bar in the bottom. The dialysis solution was pumped through a tube in a 2-hole rubber stopper in the top of the chamber into the bottom of the chamber by a Sage model 375A peristaltic pump at a flow rate of approximately 35 ml/hr. The solution effluxed from the top of the chamber through a tube in the other hole of the rubber stopper and into a container. The chamber was placed on a small magnetic stir plate which allowed continuous stirring of the chamber. Stirring was absolutely essential for effective dialysis to occur. The entire assembly was housed in a refrigerator so that the operation could be carried out at 4°C. Generally dialysis of a suspension of vesicles was accomplished by placing the suspension in dialysis tubing as usual and placing the tubing in the chamber (which had been filled with the appropriate dialysis solution) and pumping fresh dialysis solution through the chamber at a rate of 35-45 ml/hour for 12-16 hours. The peristaltic pump had 4 channels, so up to 4 chambers could be operated simultaneously.

Figure 1. FLOW DIALYSIS APPARATUS



Preparation of cytochrome oxidase vesicles (COV)

4 different methods are detailed below for the formation of COV. The method generally employed was cholate dialysis, since it gave the best respiratory control ratios. When multiple batches of COV had to be made simultaneously to vary certain parameters such as the buffer solution components or cytochrome oxidase type or concentration, it was more convenient to use the cholate dilution technique. This method obviates the need for multiple dialysis chambers and dialysis solutions, and is also much more rapid than dialysis. The lysolecithin incorporation method was only used for the preparation of large-volume COV since it was the only effective means of forming large-volume COV with good respiratory control. It was not used for standard preparation of COV because of low activity due to the low concentrations of cytochrome oxidase which must be used. The phosphatidyl serine incorporation technique was used only in attempts to form right-side out systems of oxidative phosphorylation since selective incorporation of cytochrome oxidase and hydrophobic protein into the same vesicles can be achieved with this technique (Eytan and Racker, 1977). Relatively poor respiratory control ratios were obtained with the Phosphatidyl serine incorporation method.

Method 1 - cholate dialysis

COV were made by cholate dialysis according to the methods of Hinkle, Kim, and Racker (1972). Acetone-extracted asolectin was dried in a pyrex tube as previously described, and sonicated to clarity after the addition of sodium cholate and water as in the

second method of making PLV , except that the volume of cytochrome oxidase which is to be added is subtracted from the volume of water added prior to sonication. The amount of cytochrome oxidase generally used was between .125 mg and .625 mg per ml of final COV suspension (at 40 mg/ml phospholipid concentration). After the buffer was added to the sonicated phospholipids (again this was usually 80 mM K_2SO_4, 20 mM HEPES (pH 7.0)) the appropriate amount of cytochrome oxidase was added and the suspension vortexed about 10 seconds and then sonicated in the bath sonicator at 0°C. for approximately 20 seconds. After the sonication the vesicle suspension was dialyzed as previously described. When making COV which could be energized so as to form an internally positive membrane potential (usually referred to as inside-out energized vesicles by analogy with mitochondrial orientation of cytochrome oxidase and membrane potential), .75 mg/ml of cytochrome c was added at the time the cytochrome oxidase was was added to the suspension. The volume of the cytochrome c, which was added from a solution of 30 mg/ml, was also subtracted from the volume of water added to the phospholipids prior to the first sonication. After dialysis, the vesicle suspension was treated by Sephadex filtration to remove excess external cytochrome c as will be described later in this section.

Method 2 - cholate dilution (Racker, Chien, Kandrach, 1975)

A more rapid method of making COV was the cholate dilution method of Racker et al. Asolectin was dried in the usual manner and sonicated to clarity at a phospholipid concentration of 80 mg/ml and a cholate concentration of 1.4-1.6%. Then the phospholipids were

diluted to 40 mg/ml by addition of an equal volume of buffer (80 mM K_2SO_4, 20 mM HEPES (pH 7.0)), and cytochrome oxidase was added. The usual amount of cytochrome oxidase added was .25 mg/ml of suspension. The suspension was incubated at least 1 hour at 0°C., and up to several days. The vesicles were formed at the time of assay by dilution of at least 25-fold (and the usual dilution factor was about 125) into the appropriate assay mixture. COV made by the dilution technique were used primarily used for oxygen consumption studies and therefore were diluted by addition to 3.0 ml of buffer in an oxygen electrode chamber.

Method 3 - lysolecithin incorporation (Eytan, Matheson, Racker, 1975, 1976)

Lysolecithin was added to the asolectin solution so as to comprise 10% of the total phospholipid in the solution. The solution was then dried in the usual manner. Buffer solution was then added to give a final concentration of 20 mg phospholipid per ml of suspension. After vortexing for 1-2 minutes, the suspension was sonicated to clarity. Cytochrome oxidase was added to the suspension to a final concentration of .03-.10 mg/ml and the suspension was mixed by vortexing and incubated at 4°C. for about 20 hours.

Method 4 - Phosphatidyl serine incorporation (Eytan and Racker, 1977)

Phospholipids were dried as described after adding a solution of phosphatidyl serine to comprise 30% of the total phospholipids. The phospholipids were sonicated to clarity after addition of 50 mM potassium phosphate, .5 mM EDTA (pH 7.0) to give a final phospholipid concentration of approximately 7.5 mg/ml. After sonication an equal

volume of the same buffer was added as well as .125-.500 mg of cytochrome oxidase. The mixture was incubated for 30 minutes at room temperature and the vesicles then stored on ice until used.

Preparation of ATP-$^{32}P_i$ exchange vesicles (Racker, Chien, Kandrach, 1975)

Asolectin was dried in a pyrex test tube. Sodium cholate (from a 20% (w/v) solution (pH 7.4)) and water were added to the phospholipids to give a final cholate concentration of 2.25% and a phospholipid concentration of approximately 75 mg/ml. After vortexing the tube 1 minute to partially solubilize the phospholipids the suspension was sonicated to clarity. 1.25 volumes of 8 mM Tris-SO_4, .4 mM EDTA, .8 mM $MgSO_4$, .4 mM dithiothreitol (pH 7.5) were added. Hydrophobic protein was then added from a solution of 30 mg/ml to a final concentration of 4 mg/ml. The final phospholipid concentration was 30 mg/ml and the final cholate concentration was .85%. The mixture was incubated at 0°C. for 3 hours and the vesicles then assayed for ATP-$^{32}P_i$ exchange by dilution into an appropriate assay mixture as will be detailed later.

Preparation of phosphorylating cytochrome oxidase vesicles (PCOV)

PCOV were made basically according to the preparative procedure of Racker and Kandrach (1973) with a few modifications. Ethanol-extracted asolectin was dried in a pyrex test tube. Sodium cholate was added from a 20% (w/v) solution to a final concentration of 2.8% and water to give a final phospholipid concentration of 68 mg/ml. An equal volume of 10 mM Tricine-KOH, 40 mM ammonium sulfate, 2 mM

dithiothreitol, .2 mM EDTA, 50 mM sucrose (pH 8.0) was added and the tube vortexed 2 minutes and then sonicated to clarity in the usual manner. The following additions were then made: hydrophobic protein from a 30 mg/ml solution (.133 ml added per ml of sonicated suspension), cytochrome c from a 30 mg/ml solution (.06 ml/ml of sonicated suspension), and cytochrome oxidase from a 25 mg/ml solution (.012 ml/ml of sonicated suspension. The final concentrations of the components were: hydrophobic protein - 3.3 mg/ml, cytochrome c - 1.5 mg/ml, cytochrome oxidase - .25 mg/ml, cholate - 1.17%, and phospholipid - 28 mg/ml. After mixing briefly the suspension was sonicated at 0°C. and then dialyzed in the usual manner against 10 mM Tricine-KOH, 1 mM dithiothreitol, 10% (v/v) methanol, .2 mM EDTA, .1 mM ATP (pH 8.0). The PCOV were subsequently treated to remove external cytochrome c and assayed for oxidative phosphorylation as described elsewhere in the Methods section.

Preparation of complex V-containing cytochrome oxidase vesicles (Complex V-COV)

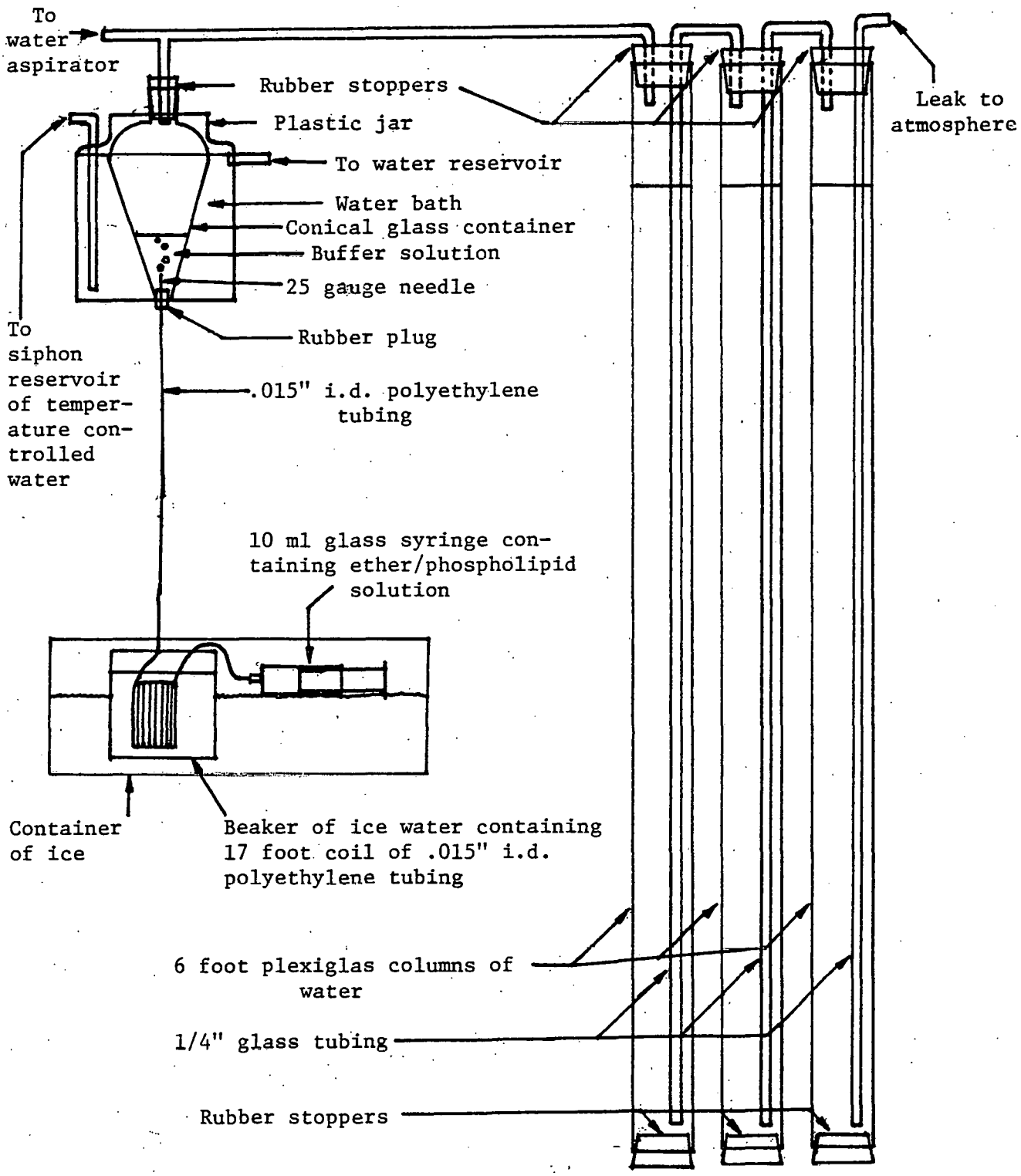
These vesicles were made in the same manner as inside-out (cytochrome c containing) COV, except that in addition to cytochrome c and cytochrome oxidase, complex V was added just before dialysis. As with the other proteins the volume of the complex V added was subtracted from the volume of water added to the phospholipids prior to sonication. The buffer most often used for the preparation of complex V-COV was 80 mM K_2SO_4, 20 mM HEPES (pH 7.0), and the dialysis solution was therefore 40 mM K_2SO_4, 10 mM HEPES (pH 7.0). Complex V

was added from a solution containing 25 mg/ml to a final concentration of .6-.8 mg/ml.

Preparation of large-volume liposomes

Following the ether vaporization techniques of Deamer and Bangham (1976) a device was developed which allowed the continuous infusion of an ether solution of phospholipids into an aqueous solution under partial vacuum, which caused vaporization of the ether solution at room temperature. A vaporization chamber was constructed by grinding away the bottom tip of a conical 50 ml graduated pyrex container and fitting it with a small rubber plug (see Figure 2). The top of the chamber was fitted with a one-hole rubber stopper with a tube which could be connected to vacuum. The chamber was placed in a plastic container through which water could be circulated for temperature control. A 25-gauge hollow needle was inserted through a rubber plug in the center of the bottom of the water-bath container and through the rubber plug in the bottom of the vaporization chamber. The needle was connected to 17 feet of coiled .015" i.d. polyethylene Intra-Medic tubing which was placed in an ice bath below the chamber. The other end of the polyethylene tubing was fitted over a 25-gauge needle attached to a 10 ml glass syringe, which was also kept on ice. The vacuum was produced by a water aspirator and was regulated by a leak to the atmosphere through 3 6-foot plastic columns filled with water and connected in series so that air was drawn into the system against a head of pressure determined by the total height of the water in the 3 columns.

Figure 2. SCHEMATIC DIAGRAM OF LARGE-VOLUME LIPOSOME APPARATUS



The thin tubing provided flow resistance which limited the rate of influx of the ether solution of phospholipids, which was contained in the glass syringe to a rate which produces effective liposome formation. The vacuum allows the vaporization to take place at room temperature or 4°C. so that proteoliposomes can be made without damage to the proteins because of heat. The vacuum also provides a constant driving force for the injection of the ether phospholipid solution.

From 1 to 5 ml of buffer solution was placed in the vaporization chamber, and room temperature water was circulated around it. 10 ml of diethyl ether containing approximately 1.4 mg/ml of phospholipids was drawn up in the 10 ml syringe after wetting the ground glass plunger with distilled water to seal it. Vacuum was applied to the chamber and the syringe connected to the polyethylene tubing and placed on ice. The vacuum was adjusted by varying the height of the water in the columns until an injection rate of .5-1.0 ml/minute was obtained. After the 10 ml of ether solution had been injected the syringe was removed from the tubing and air was drawn through the tubing and bubbled through the suspension for 5 minutes to remove residual ether. The liposomes were removed from the chamber with a Pasteur pipet.

Large-volume cytochrome oxidase vesicles

These vesicles were made using a combination of the ether vaporization technique and the lysolecithin incorporation technique. The large-volume liposomes were made as described above using either

90% asolectin, 10% lysolecithin, or 42% phosphatidyl choline, 42% phosphatidyl ethanolamine, 6% cardiolipin and 10% lysolecithin in ether solution at 1.4 mg/ml. After formation of the vesicles in 2.5 ml of aqueous buffer (usually 40 mM K_2SO_4, 10 mM HEPES (pH 7.0)) the suspension was cooled to 0°C. and .25 mg of cytochrome oxidase was added. The suspension was then incubated at 4°C. for approximately 20 hours.

Large-volume PCOV, hydrophobic protein-containing vesicles, and complex V-COV were also made using the above techniques and adding the appropriate proteins. Details of these experiments will be discussed in the RESULTS section.

Other vesicle preparations

For testing the polylysine/protamine precipitation separatory technique red blood cell ghost vesicles and sarcoplasmic reticulum vesicles were prepared. The red blood cell ghost vesicles were prepared by sonicating a suspension of red blood cell ghosts (made according to Dodge, Mitchell, Hanahan, 1963) in 20 mM NaPi. The sonication was done for 20 minutes in an L&R bath sonicator. The sacroplasmic reticulum vesicles were made from rabbit muscle according to the method of MacLennan (1970).

Removal of external cytochrome c

Two methods of removing external cytochrome c were employed, based on the methods of Jacobs and Sanadi (1960). The Sephadex method was used for proteoliposomes and the centrifugation method

was used for SMP . Proteoliposomes were difficult to resuspend homogeneously when pelleted by centrifugation and SMP suspensions tended to clog the Sephadex columns.

Sephadex method

All operations were carried out at 0-4°C. Sephadex G-150 was pre-equilibrated with a buffer with a high ionic strength. For COV and complex V-COV, .15 M KCl, 10 mM HEPES (pH 7.0) was usually used. In experiments where no potassium was desired, NaCl was substituted for KCl. For PCOV 10 mM Tricine-KOH, 1 mM dithiothreitol, .2 mM EDTA, .15 M KCl (pH 8.0) was used. The vesicle suspension was removed from dialysis and carefully layered on top of a column of Sephadex. In general the shape of the column was not critical as long as the total volume of the Sephadex it contained was at least 10 times the volume of vesicle suspension to be treated. The vesicles were allowed to pass through the column by gravity and eluant was added frequently to the top of the column. The suspension separated into a fast-moving pink to orange band which reprsented the vesicle fraction, and a slower-moving red to pink band which represented the external cytochrome c. The vesicles were collected by visually following their progress in the column and stopping the collection of the effluent before the cytochrome c band started to leave the column. The cytochrome c was flushed from the column and the column was then ready for reuse. The volume of the suspension was measured both prior to and after Sephadex filtration, and there was generally about 2-fold increase in volume. The vesicles were stored on ice until used.

Centrifugation method

SMP were diluted into 15-20 volumes of .125 M KCl, 10 mM HEPES, .5 mM sodium succinate (pH 7.2) and centrifuged at 40,000 rpm in a Sorvall type A-841 rotor for 1 hour. The external cytochrome c remained in the supernatant and the pellet was resuspended in MSH with a glass/teflon homogenizer at a concentration of 10-20 mg/ml.

Measurement of ion uptake

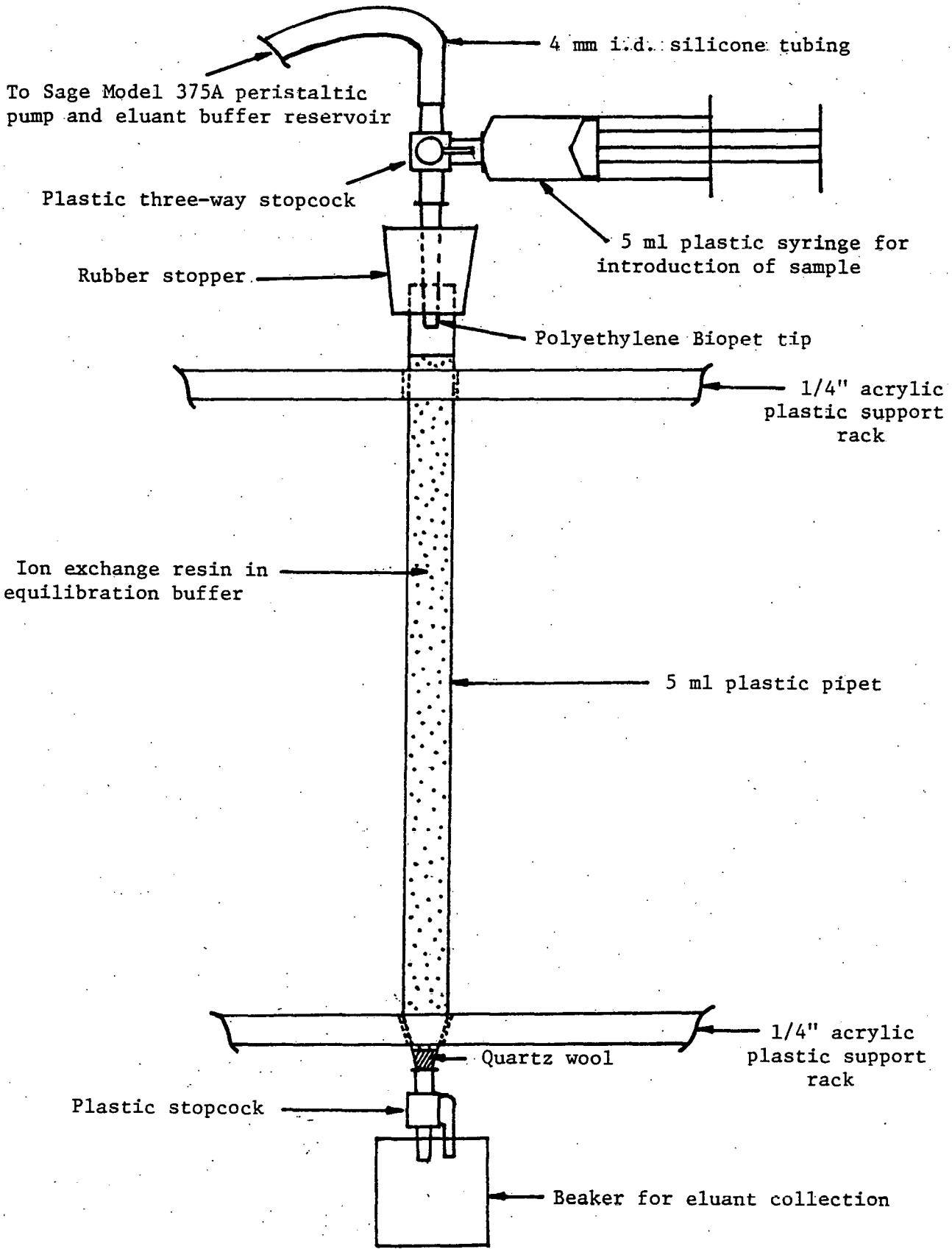
Ion transport in vesicular systems such as SMP or proteoliposomes was measured by one or two techniques. The first involves the use of ion exchange columns to remove external cations of a given type so that the fraction taken up can be measured. The second method involves precipitating the vesicles using basic polypeptides such as protamine or polylysine and subsequent separation of the aggregated vesicles from the medium using a modified millipore filtration technique. The ion exchange technique was used in earlier experiments before the development of the aggregation-filtration method. It suffers from the disadvantages of being more difficult to process many samples (a separate ion exchange column is needed for each sample), retention of up to 20-40% of the particles in the column, and of high background activity when anions are present which bind the transported cation with high affinity (such as ATP with Mn^{++} or Ca^{++}). The aggregation-filtration method was the method of choice for studying transport in vesicular systems and was used in all later experiments.

Ion exchange

Measurement of cation (Rb^+, Mn^{++}, Ca^{++}) uptake in SMP or proteoliposomes was accomplished by first converting Dowex 50-W cation exchange resin from the hydrogen to the sodium form. A quantity of resin (5-6 ml per sample to be assayed) was placed in a beaker and washed with 2-3 volumes of distilled water several times. Then the water was decanted and 2-3 volumes of 2 N NaOH was added and the resin stirred for about 5 minutes. The NaOH was decanted and replaced with fresh 2 N NaOH and the stirring continued until the resin turned a deep orange color (which indicated complete conversion to the sodium form). The resin was washed with distilled water several times to remove all residual NaOH and then washed twice with 2-3 volumes of the buffer to be used in the experiment. This was usually 10 mM HEPES, 40 mM K_2SO_4 (pH 7.0) for proteoliposomes and MSH for SMP. 5-6 ml of the resin was placed in 5 ml plastic pipets plugged with a small amount of glass wool at the tip. The pipets were held vertically in a plastic rack and were fitted with stopcocks at the bottom. Care was taken in packing the columns to avoid any air pockets in the resin. The buffer level in the columns was 1.0 ml above the top of the resin. The top of the column was fitted with a three-way stopcock through which a sample could be introduced to the column via a 5 ml syringe, and eluant subsequently pumped through the column by a Sage mode 375A peristaltic pump (see Figure 3).

Samples consisting of 2-4 mg of SMP protein or 4-12 mg of proteoliposomes were incubated in medium containing appropriate buffer, salt, substrate, and substances required for cation uptake in a final

Figure 3. DETAILS OF ION EXCHANGE COLUMN



volume of 2.0 ml. Uptake was initiated by the addition of an aliquot of radioactive plus carrier cation (usually about 1 μCi per sample). The samples were then incubated for a variable length of time (0-30 minutes) and oxygenated by continuous slow bubbling of oxygen into the samples via a series of Pasteur pipets connected to an oxygen tank. At the end of the incubation time the sample was quickly drawn up into a 5 ml syringe, air bubbles expelled, and then forced into the top of the ion exchange column over about 5 seconds. The stop-cock was then turned so as to admit eluant to the top of the column and the peristaltic pump was simultaneously started. Eluant was pumped through the column at a flow rate of 14 ml/minute for 30-40 seconds, or until approximately 8-10 ml of eluant had been pumped through the column. The external radioactively labelled cations were thus removed by the resin and the eluted volume contained the vesicles and any transported cation. In a given experiment all the eluted samples were normalized to the same final volume and an aliquot of each removed for gamma or scintillation counting for quantitation of the uptake. Because of the time-consuming nature of the elution of the radioactive cations from the resin using HCl washing, the plastic columns of resin were disposed of after being used for a single sample rather than being reconverted for reuse.

Protamine filtration

Later experiments with cation uptake by SMP and proteoliposomes were quantitated using the more convenient technique of aggregation of the vesicles with basic polypeptides followed by modified

Millipore filtration.

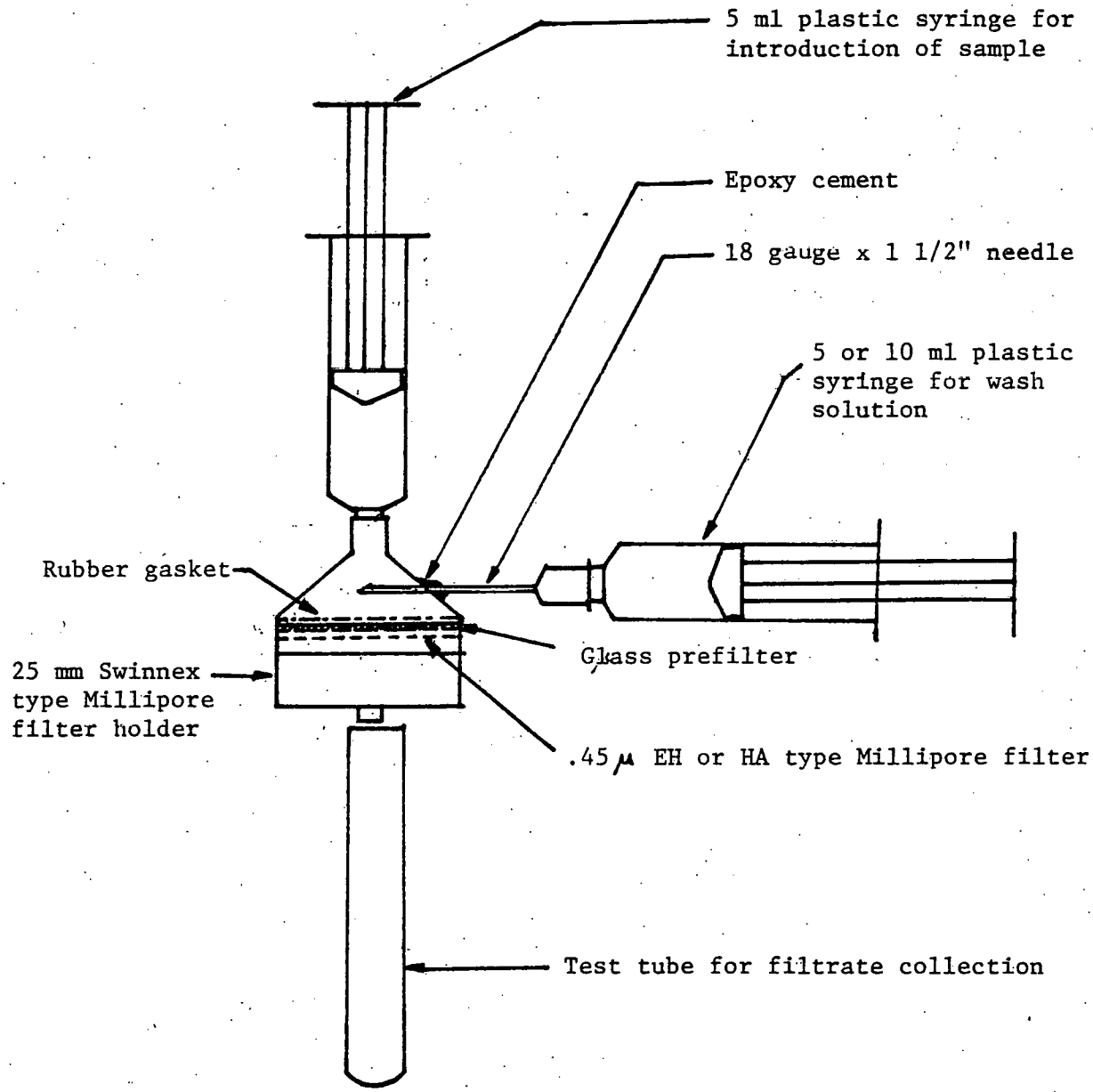
For any given type of vesicles the optimum amount of polylysine or protamine which had to be added to the sample of vesicles to form filterable aggregates had to be determined. This was most conveniently done by monitoring the light scattering of the filtered suspension. Samples were made containing the solution and amount of vesicles to be used in subsequent uptake experiments. In general 1-12 mg of the vesicles to be assayed were added to a volume of appropriate buffer solution such that the final volume after the addition of the protamine or polylysine would be 2.0 ml. Varying amounts of protamine or polylysine were then added to the samples (0-1.5 mg) from a stock solution of 4 mg/ml. The suspension was agitated 15-30 seconds and poured into a 5 ml syringe attached to a Swinnex type Millipore filter holder containing a glass prefilter and a .45 μ Millipore filter. After filtration the filtrate was assayed by placing 1.0 ml of it in a 1 ml cuvette and reading absorbance against a blank solution containing no vesicles in a Beckman DU-2 spectrophotometer. The wavelength used depended on the turbidity of the vesicle suspension but was between 450 and 500 nm. More turbid solutions scattered more light at a given wavelength and the wavelength was accordingly adjusted to keep the filtered samples with no protamine or polylysine on readable absorbance scale. The amount of polypeptide to be added to a given sample was the minimum amount which gave a filtrate with essentially no absorbance, indicating therefore complete removal of the vesicles from the medium.

When the optimum amount of precipitating polypeptide for the

samples had been determined, the uptake experiment was done as follows: An aliquot of vesicles (2-4 mg for SMP , and 8-12 mg for proteoliposomes) was added to a 20 ml plastic scintillation vial containing buffer, salt, and substances required for the uptake (or inhibitors). Then substrate and radioactively labelled cation were added in that order and mixed by brief agitation. The sample was then placed in a mechanical shaker bath to insure adequate oxygenation and agitated for a predetermined length of time (up to 60 minutes). At the end of the incubation time, the appropriate amount of protamine or polylysine (usually about .8 mg for proteoliposomes and about .4 mg for SMP) was added from a stock solution of 4 mg/ml, bringing the final volume of the sample to 2.0 ml. The sample was agitated by hand for 15 seconds to insure complete aggregation of the vesicles and then filtered through a glass prefilter and a .45 μ Millipore filter. The filter was washed by forcing 5-10 ml of buffer (usually containing 10 mM of the carrier cation) through it. The filtration and washing was done by either of 2 techniques.

The first filtration technique involved the use of a modified Swinnex Millipore filter holder (see Figure4) which contained an 18 gauge x 1 1/2" needle (bevel up) inserted into the side of the filter holder above the filter and cemented in place with epoxy. The wash solution was drawn into a 5 or 10 ml syringe which was then plugged into the needle. This was referred to as the "side-wash" technique. The sample to be filtered was poured into a 5 ml syringe attached to the top of the filter holder and forced through the filter with a plunger while just enough pressure was exerted with

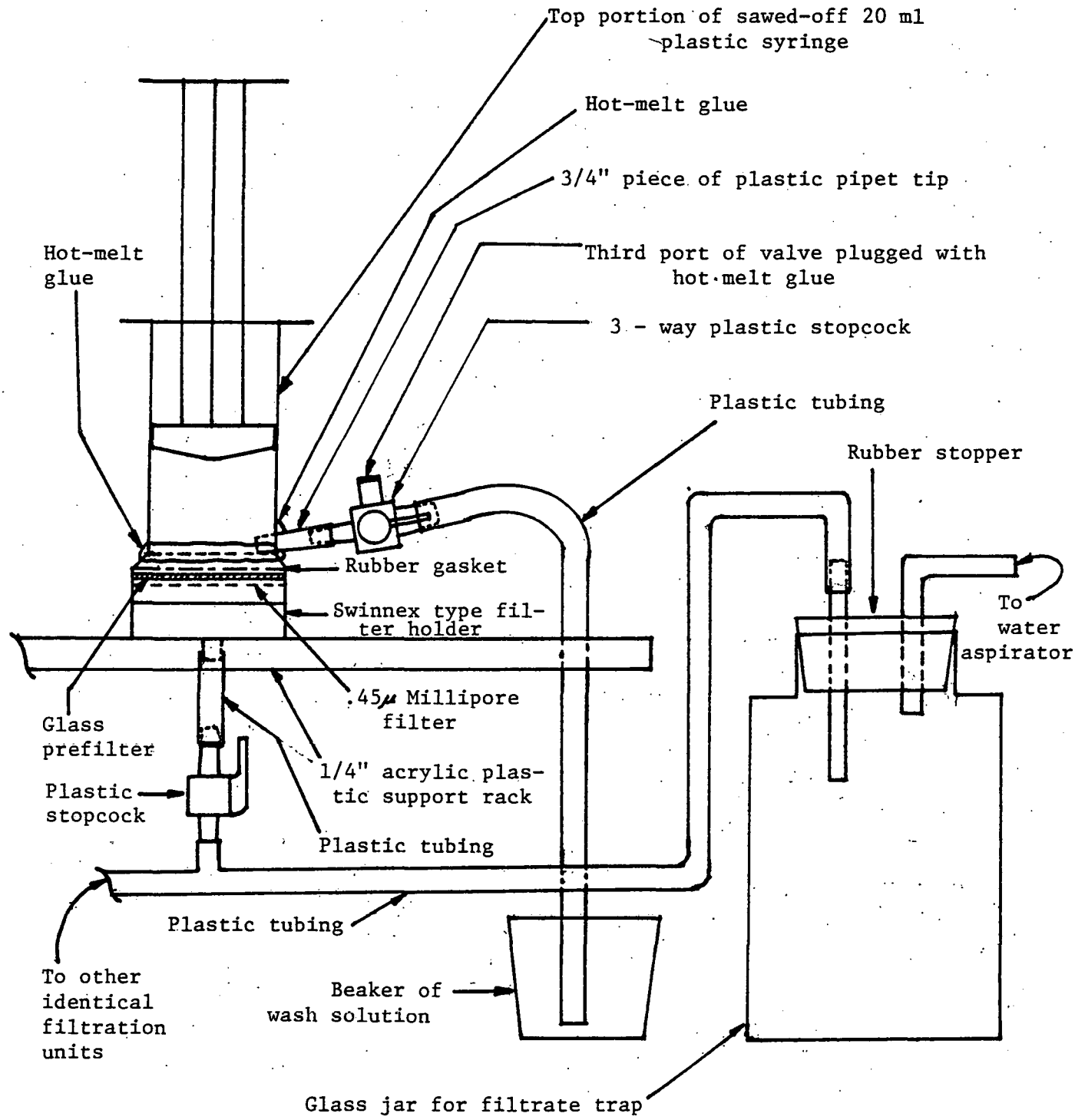
Figure 4. "SIDE-WASH" FILTRATION DEVICE



the other hand on the wash syringe to prevent reflux of the sample into the wash syringe. Immediately after filtration the wash was manually injected over about 5 seconds. The uptake was quantitated by counting the filters using appropriate techniques.

A second method employed in later experiments involved the use of a filtration device which gave more reliable and reproducible results. The filtration device consisted of a series of Swinnex type filter holders which had the tops sawed off just beyond the gasket-holding surface and 20 ml syringe barrels were glued in place on the top with hot-melt glue (see Figure 5). A tube connected to the bottom of the filter holder contained a plastic valve which allowed application of a water-aspirator vacuum through a filtrate collecting trap to an individual given filter holder. A plastic pipet tip was inserted about 1/8" into the barrel of the syringe just above the top of the filter holder and served as an inlet for the wash solution and a stop for the plunger. The wash flow was also controlled by a plastic valve. Operation of the device was as follows: Samples for uptake of cations were prepared and incubated as previously described for the "side-wash" technique. At the appropriate time for filtration of a given sample the lower valve was opened on a filter holder, which had been fitted with a gasket, glass prefilter, and .45 μ Millipore filter, thus applying vacuum to the filter. The protamine was added to the sample and the suspension agitated manually for 10-15 seconds and then poured into the syringe barrel of the filter holder. The sample vial was quickly rinsed with 2 ml of wash buffer which was also poured into the filter holder. A plunger was quickly

Figure 5. VACUUM ASSISTED FILTRATION DEVICE

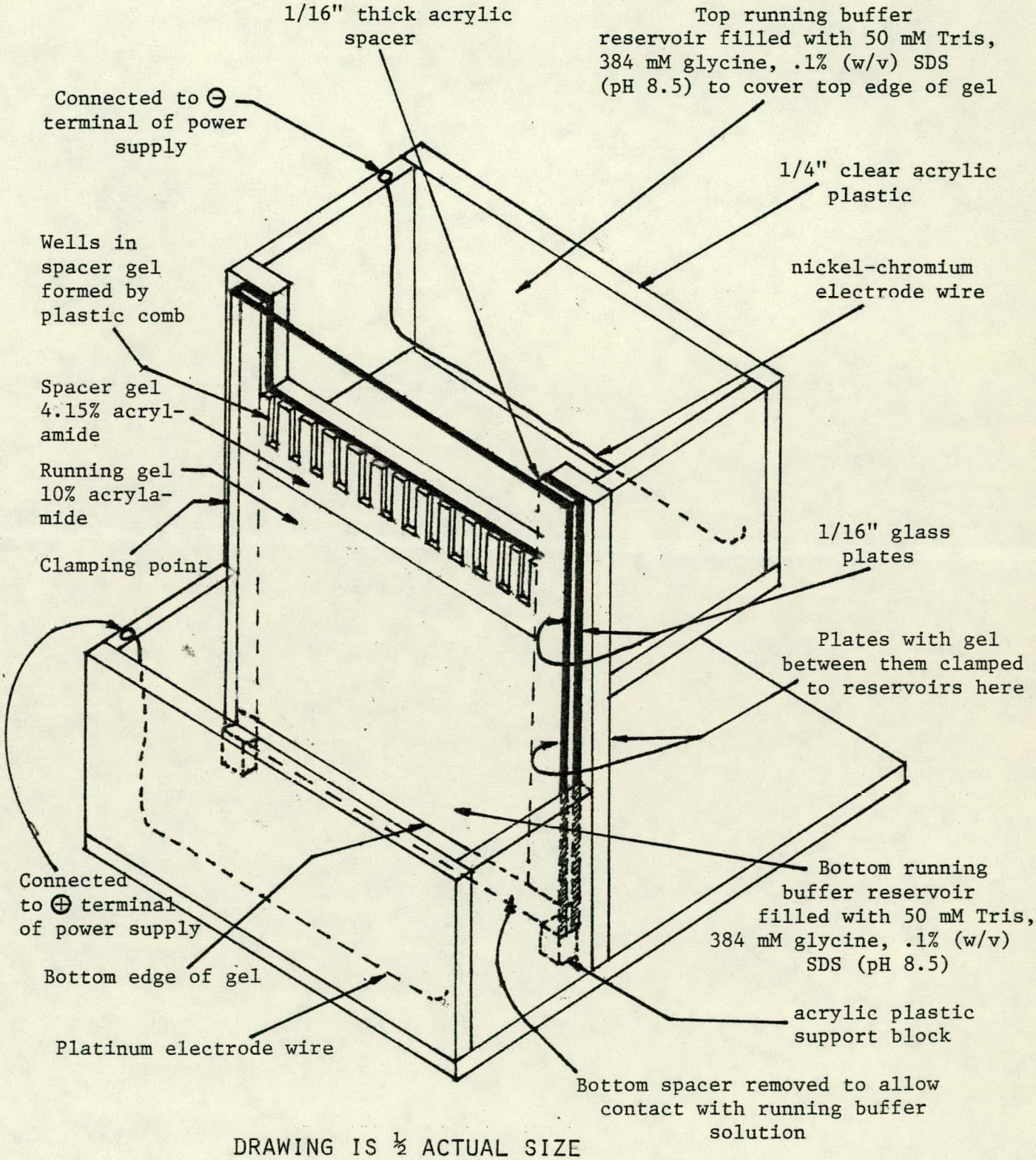


inserted into the syringe and the sample forced through the filter. During this time the wash valve was closed. As soon as the plunger was forced all the way down the wash valve was quickly opened and 5 ml of wash drawn into the syringe barrel. The valve was closed and the wash forced through the filter. This wash was then repeated. The entire filtration and wash procedure takes about 30 seconds. Generally the experiments were done by initiating the samples 1 minute apart so that samples could then be filtered sequentially.

Gel electrophoresis

Polyacrylamide gel electrophoresis of proteins was done using 10% polyacrylamide gels according to the method of Weber and Osborne (1969, 1975). A slab gel electrophoresis was constructed following the design of Reid and Bieleski (1968) (see Figure 6). Two glass plates (6 1/4" x 5 1/2" x 1/16" and 6 1/4" x 4 3/4" x 1/16") were clamped together with 1/4" x 1/16" acrylic plastic spacers coated with Cello-Seal between them so as to form a compartment for the slab gel. A 10% polyacrylamide gel was made by adding 3.75 ml of 3 M Tris-Cl (pH 8.9), 10 ml of 30% (w/v) acrylamide, .8% methyl bisacrylamide, 15.5 ml of water, .3 ml of 10% sodium dodecyl sulfate (SDS), and .015 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) to a 50 ml beaker. .18 ml of freshly made 10% (w/v) ammonium persulfate was added and mixed well. The solution was immediately poured between the glass plated until they were filled to within 1/2" of the top. A 1/16" acrylic spacer was inserted into the top of the plates to a depth of 1". The gel was allowed to polymerize

Figure 6. POLYACRYLAMIDE GEL ELECTROPHORESIS APPARATUS



DRAWING IS ½ ACTUAL SIZE

45 minutes to 1 hour. The spacer at the top of the gel was carefully removed. A 4.15% spacer gel was made by mixing 2.5 ml of .5 M Tris-Cl (pH 6.8), 1.4 ml of 30% acrylamide, .8% methyl bisacrylamide, .1 ml of 10% SDS, .005 ml of TEMED and 6 ml of water in a small beaker. .1 ml of the 10% ammonium persulfate catalyst was added to this mixture and a comb spacer placed in the top of the gel to create 12 individual wells at the top of the gel. The spacer gel was quickly poured into the top of the gel to fill the cavities around the comb spacer, with care being taken to avoid any trapped air bubbbles.

After allowing 30-60 minutes for the spacer gel to polymerize, the comb spacer and the bottom spacer were carefully removed from between the plates. The plates were fastened to the buffer reservoirs shown in the figure and buffer (50 mM Tris, 384 mM glycine, .1% (w/v) SDS, pH 8.5) was placed in the upper and lower reservoirs such that the upper and lower surface of the gel was in uniform contact with the liquid. The platinum electrode wires were connected (negative to the top reservoir, positivc to the bottom reservoir) to a 300 volt filtered DC power supply, which was connected to a variac for voltage control. A milliammeter was connected in series with the gel to monitor the current.

The protein samples were 1 mg/ml in 50 mM Tris, 5% mercaptoethanol, 2% SDS, and 5% glycerol. To each ml of sample was added .05 ml of .1% bromthymol blue as a marker. .05 ml samples (50 μg of protein) were injected into each well using a tuberculin syringe. The voltage source was adjusted by means of the variac to allow a current flow of 1.5-2.0 ma per well used. The current was stopped when the marker

dye was visible at the bottom edge of the gel (3-5 hours later) and the gel was removed from between the plates in water. The gel was then stained by incubation at 37°C. in 150-200 ml of 3 1/2% (v/v) acetic acid, 50% (v/v) methanol containing .20-.25% (w/v) Coomassie brilliant blue stain for about 12 hours. The gel was then destained at room temperature in 3 1/2% acetic acid, 50% methanol for 12 hours and subsequently swollen in 7% (v/v) acetic acid for 24 hours. The gels were stored in 7% acetic acid solution at room temperature.

Radioactive counting techniques

Calcium-45. For counting of liquid samples (e.g., aliquots of eluant from ion exchange columns, filtrates from "side-wash" filtered samples, and standard solutions for quantitation), .1 or .2 ml of the liquid was placed in a plastic scintillation vial with 1.0 ml of a solution of 1 part Beckman Biosolve and 2 parts toluene-Omnifluor scintillation fluid. This mixture was vortexed to solubilize the aqueous sample. 9 ml of toluene containing 4 g/l of Omnifluor (New England Nuclear) was added and the sample capped and vortexed to mix thoroughly. The samples were then counted using a Beckman LS-235 scintillation counter with either a 0-600 or a wide ^{14}C window.

For counting of filters, the filters were dried in a drying oven overnight and then added (both the glass prefilter and the Millipore filter) to a scintillation vial. 10 ml of toluene-Omnifluor scintillation fluid was then added and the vial capped and vortexed. The samples were then counted in the scintillation counter.

Manganese-54. For counting of liquid samples, .5 ml of the sample

plus 1.5 ml of water were placed in a plastic gamma-counting vial and counted in a Beckman Biogamma counter. The manganese samples were counted using a window of 0-400. For counting of filters, the filters were placed in the counting vials while still wet and counted the same way.

Rubidium-86. These samples were counted either by gamma or scintillation counting as described above except that for gamma counting a window of 0-560 was used and for scintillation counting a wide open window was used.

Sodium-22. These samples were counted using the same technique described for Manganese-54 except that a window of 0-1000 was used.

Carbon-14. Samples containing ^{14}C were counted in the same manner as the ^{45}Ca samples with a wide ^{14}C window.

Phosphorus-32. The solution in which ^{32}P was extracted for measurements of oxidative phosphorylation and ATP-$^{32}P_i$ exchange reacted with the toluene-Omnifluor scintillation fluid to produce a yellow color and some turbidity so all ^{32}P samples were counted by Cerenkov counting (Gelsema, deLigny, Luten, Vossenberg, 1975). 1.0 ml of the aqueous sample containing ^{32}P was added to a plastic scintillation vial containing 10 ml of 2 M sucrose. After mixing well, the samples were counted in the scintillation counter with a wide open window. ^{32}P uptake in vesicles was measured by counting the filters as described for ^{45}Ca except that a wide open window was used.

Volume determinations of proteoliposomes

All operations were carried out at 0-4°C. Proteoliposomes were

made in the usual manner for the cholate dialysis technique except that the suspension contained either 10 mM sucrose and 2 μCi/ml of ^{14}C-sucrose or 2 mM RbCl and 10 μCi/ml of ^{86}Rb/ml in addition to 40 mM K_2SO_4, 10 mM HEPES (pH 7.0). The suspension was dialyzed against approximately 10-20 volumes of this same buffer for 4-8 hours. An aliquot of the dialysis solution was retained for later counting as a standard and the dialysis solution was changed. The dialysis continued as before and the dialysis solution was changed 2-3 more times. This series of dialysis changes was to insure complete removal of the cholate in the presence of the radioactive label so that the label would be trapped in the internal volume of the vesicles in proportion to the fraction of the total volume which it represented. The suspension was then dialyzed against 100-200 volumes of dialysis buffer which was identical except that it did not contain any radioactive label. The suspension was dialyzed for 6-8 hours and the dialysis solution changed. Dialysis was continued in this manner until no radioactivity could be detected in the dialysis solution. This usually required a total of 3-4 changes of dialysis solution. The internal volume of the vesicle suspension was judged by counting aliquots of the suspension and comparing this to the standard.

Flow dialysis for testing vesicle lysis

A flow dialysis chamber of the type used by Colowick and Womack (1969) was set up with stirring of two compartments on either side of a dialysis membrane. .8 ml of a suspension of vesicles (20 mg/ml

phospholipid) which had been labelled by trapping of ^{14}C-sucrose inside with dialysis to remove external label was placed in the upper chamber of the device. Buffer, water or substances to be tested for lytic effect (detergents, protamine, polylysine) was added to the suspension to bring the final volume to 1.2 ml. 40 mM K_2SO_4, 10 mM HEPES, 20 mM sucrose (pH 7.0) was pumped through the lower chamber by a peristaltic pump at a flow rate of .36 ml/minute, and the effluent was collected in timed aliquots. The rate of appearance of the radioactive label in the effluent solution was used to judge the degree of lytic effects.

Measurement of oxidative phosphorylation

Oxidative phosphorylation was measured according to the method of Racker and Kandrach (1973) with some modifications due primarily to the use of a larger volume oxygen electrode chamber. .5 ml of a suspension of PCOV which had been treated to remove external cytochrome c (usually by the Sephadex filtration method) and containing .5-.8 mg of protein was added to a test tube containing .5 ml of 80 mM potassium phosphate, 2 mg/ml bovine serum albumin, 2 mM $MgSO_4$ (pH 8.0). .05 ml of 20 mM Na-ATP (pH 7.0), .066 mg of OSCP (.012 ml of a 5.5 mg/ml solution), and .05 mg of F_1 (.018 ml of a 2.8 mg/ml solution) were added to the vesicle suspension and incubated for 10 minutes at room temperature. .1 mg of polylysine, M.W. 70,000, was then added to 2.0 ml of room temperature, air-saturated buffer which contained 25 mM D-glucose, .3 mM EDTA, 5μM antimycin A, 5.6 mM $MgSO_4$, 2 mg/ml bovine serum albumin, and 5 mM Tris-SO_4 (pH 6.9). .05 ml of

20 mM Na-ATP (pH 7.0), .05 ml of .2 M potassium phosphate (pH 7.0) containing approximately 2 x 10^8 cpm of $^{32}P_i$, and 86 units (.02 ml of a suspension containing 4310 u./ml) of hexokinase were added in that order. After a 3 minute incubation at room temperature the reaction was initiated by addition of .1 ml of .5 M Tris-ascorbate (pH 6.8-7.0) containing 10 μg of phenazine methosulfate (PMS). The final volume was 3.37 ml. The reaction was terminated after 10 minutes by the addition of .3 ml of 50% (w/v) trichloroacetic acid. Two parallel aliquots were run with each sample to allow quantitation of the P:O ratio. The first of these was identical to the sample except for the addition of .01 ml of oligomycin (from a 1.3 mg/ml solution in 95% ethanol) during the initial incubation of the vesicles with F_1 and OSCP. This was to allow a determination of the background, or nonextractable $^{32}P_i$ which could be subtracted from the total counts in the sample to give the actual amount of esterified $^{32}P_i$. An alternate method was to terminate the reaction of the parallel sample immediately after initiation with PMS-ascorbate. It should be noted that the PMS-ascorbate solution must be kept in the dark to prevent breakdown of the photosensitive PMS. The second parallel aliquot contained the same amount of carrier phosphate, but no ^{32}P, and was used to quantitate oxygen consumption. This was necessary because of the tendency of ^{32}P to adhere to the lucite oxygen electrode chamber. The sample for oxygen consumption determination was added to the 2.0 ml of buffer directly in the 3.4 ml chamber of the oxygen electrode, and oxygen consumption was monitored after initiation with PMS-ascorbate using a Yellow Springs type 5331

oxygen electrode connected to a polarizing voltage source (.8 volts) and a Hewlett Packard model 7100BM strip chart recorder. After trichloroacetic acid termination of the reaction in the ^{32}P-containing samples they were centrifuged at 10,000 rpm for 10 minutes in a JA-20 rotor to remove phospholipids and protein. .5 ml of the clear supernatant was added to a 15 ml plastic test tube containing 1.5 ml of 2.5% (w/v) ammonium molybdate, and .3 ml of 6 N H_2SO_4. The solution immediately turned dark blue in color. After about 5 minutes, 4 ml of isobutanol was added and the mixture vigorously shaken. Separation of the isobutanol and aqueous phases was allowed to occur by incubating several minutes. The blue color (representing the reduced phospho-molybdate complex) partitions into the upper isobutanol phase, which is carefully removed with a pipet. The aqueous phase is re-extracted with 1.0 ml of isobutanol and then 1.0 ml of the aqueous phase is carefully removed and added to a scintillation vial containing 10 ml of 2 M sucrose for Cerenkov counting. A standard was made by removal of .01 ml of the remaining TCA-treated supernatant, dilution with buffer to 1.0 ml, and addition of this to 10 ml of 2 M sucrose. From the concomitant oxygen consumption measurements the P:O ratio was calculated.

Measurement of ATPase activity

An ATPase assay was developed which was convenient, allowed processing of many samples, and obviated the need for the use of ^{32}P-containing compounds. The technique is a combination of the P_i assay of Chen, Toribara, Warner (1956) and the molybdate-isobutanol

extraction procedure of Rose and Ochoa (1955). 20-50 μg of protein (ATPase) or .5-1 mg of SMP was added to 1 ml of a solution containing either 5 mM $MgSO_4$, 20 mM Tris-SO_4 (pH 7.7) for ATPase or MSH containing 5 mM $MgSO_4$ for SMP. The reaction was carried out at room temperature, and was initiated by the addition of .025 ml of .2 M Na-ATP (pH 7.2). The reaction was terminated after 1.5-2.5 minutes by addition of .1 ml of 50% TCA. The test tubes were immediately placed on ice to prevent any spontaneous breakdown of ATP and centrifuged at 0°C. in a JA-20 rotor at 10,000 rpm for 10 minutes. .5 ml of the supernatant was added to 3.5 ml of water in a 15 ml plastic test tube at room temperature. Also a series of standards were prepared (usually 5) containing from 0-20 μg of phosphorus in 4 ml fo water. 4 ml of freshly prepared reagent consisting of 1 part 10% ascorbic acid, 1 part 6 N H_2SO_4, 1 part 2.5% ammonium molybdate and 2 parts water was added to each tube. The samples were incubated at room temperature for 15-30 minutes until a discernible blue color change occurs in the standards. The color reaction is then terminated in the samples and standards sequentially in the same order in which the reagent was added to initiate the reaction so that each sample will have been incubated the same length of time. Termination is accomplished by addition of 4 ml of isobutanol followed by vigorous shaking. The blue color partitions into the upper isobutanol phase, which is removed with a pipet and placed in another tube as soon as possible to prevent the continuing color reaction in the aqueous phase from altering it.

The isobutanol phase was placed in a quartz cuvette and its

absorbance read at 820 nm using a Beckman DU-2 spectrophotometer. By comparing to the standard curve and control samples which were terminated with TCA at t = 0, quantitation of the P_i cleavage from the ATP possible.

Measurement of ATP-$^{32}P_i$ exchange

ATP-$^{32}P_i$ exchange was measured in both ATPase containing vesicles and in SMP according to the method of Kagawa and Racker (1971), and Racker, Chien, and Kandrach (1975).

ATP-$^{32}P_i$ exchange vesicles

.04-.05 ml of hydrophobic protein containing vesicles prepared as previously described was diluted into 1.0 ml of 50 mM KCl, 20 mM Tris-SO_4, 2 mM $MgSO_4$, .5 mM EDTA (pH 8.0) containing 2.5 mg/ml of BSA. Coupling factors were then added to the suspension, which was incubated at room temperature. Generally 20-30 μg of F_1 and 55 μg of OSCP were added and the mixture incubated for 5-10 minutes. For experiments involving antibodies to F_1 the appropriate serum (in .1 ml volume) was added at this time and incubated for an additional 10 minutes. The exchange reaction was initiated by the addition of .12 ml of .1 M Na-ATP, .1 M $MgSO_4$, .125 M KP_i (pH 8.0), containing approximately 10^8 cpm of $^{32}P_i$/ml. The suspension was incubated at room temperature for 20 minutes and terminated by the addition of .1 ml of 50% TCA. The samples were immediately placed on ice and then centrifuged in a JA-20 rotor at 10,000 rpm for 10 minutes. .3-.5 ml of the supernatant was added to .3 ml of 6 N H_2SO_4, 1.0 ml

of 2.5% ammonium molybdate, and .5 ml of water. The mixture was incubated at room temperature for 10 minutes with occasional agitation, and then extracted with 4 ml of isobutanol. After removal of the isobutanol phase a second extraction with 1.0 ml of isobutanol was carried out, and 1.0 ml of the aqueous phase was added to 10 ml of 2 M sucrose for scintillation counting. A standard was made from .01 ml of the TCA supernatant and nonextractable phosphate was determined from a parallel sample containing .01 ml of an ethanolic solution of oligomycin at 1.3 mg/ml.

SMP

.5 mg SMP protein was incubated at room temperature with .1 ml of antiserum or immunoglobulins (when used) for 10 minutes. Then 10-15 μmoles of Na-ATP, 10-15 μmole of $MgSO_4$, 15-20 μmoles of KP_i were then added in .4-.8 ml of MSH to initiate the exchange reaction. After 10-20 minutes of incubation at room temperature the reaction was terminated by the addition of .1 volumes of 50% TCA. The samples were cooled on ice and centrifuged as described above, and then .3 ml of the supernatant was added to .5 ml of 6 N H_2SO_4 plus 2 ml of 2.5% ammonium molybdate. After incubation at room temperature for 15 minutes, extraction with isobutanol and counting of the samples was carried out as described.

Assay of uncoupler binding

To assay the uncoupler binding capability of complex V, 3 mg of the fraction of complex V to be tested was diluted to a final volume

of .2 ml by the addition of 1 mM histidine, 50 mM Tris-SO_4 (pH 8.0) and placed in a 3" x 1/4" piece of dialysis tubing which had one end tied shut. The tubing was placed in a 4 ml glass test tube containing 3.0 ml of the same buffer with 3.5 nmole/ml of CCCP or FCCP (added from a 2.0 mM solution in 95% ethanol). A control sample containing buffer and dialysis tubing without the protein was prepared. The samples were incubated at 4°C. for 24 hours. Absorbance of aliquots of the dialysis solution from each sample was read using a Beckman DU-2 spectrophotometer at 375 nm (for CCCP) or 362 nm (for FCCP), and quantitation of the amount of uncoupler bound was determined from comparison with the absorbance of solutions containing known amounts of uncoupler.

Density gradient separation of vesicles

A density gradient forming device was constructed, consisting of two 50 ml plastic graduated cylinders connected at the bottom by a plastic tube containing a valve. Equal volumes of buffer (10 mM HEPES, 40 mM K_2SO_4, pH 7.0 for COV , 10 mM Tricine-KOH, 50 mM sucrose, .5 mM EDTA, 1 mM dithiothreitol, pH 8.0 for PCOV) containing the maximum and minimum amounts of ficoll desired for the gradient range (generally 0-2% ficoll for the minimum density and 10-20% for the maximum density) were placed in each chamber of the device. Either 18 or 5 ml was the total volume in each chamber, depending on whether a 40 ml centrifuge tube or a 10 ml centrifuge tube was to be used. Both chambers were magnetically stirred and the valve between them was opened at which time a peristaltic pump began pumping buffer out

of the high density chamber and into a polycarbonate centrifuge tube at a rate of .5-1.0 ml/minute. This was continued until all the liquid had been removed from both chambers and pumped into the centrifuge tube. A vesicle suspension of either COV or PCOV from which the external cytochrome c had been removed by Sephadex filtration was carefully layered on top of the gradient using a Pasteur pipet. The volume of the vesicle suspension was 5-7 ml for a 40 ml tube and approximately 1.0 ml for a 10 ml gradient. The tube was carefully tilted and placed in a JA-20 fixed-angle rotor and centrifuged at 19,000 rpm for 24 hours at 4°C. The centrifuge was stopped without the brake to minimize swirling effects. The vesicle fractions were visible as bands in the tube and were removed carefully using a J-shaped Pasteur pipet. The fractionated vesicles were stored at 0°C. until used.

Fluorescence measurements with dicarbocyanine dyes

Qualitative measurements of membrane potential in energized proteoliposomes and SMP , using the fluorescent dicarbocyanine dyes Di-S-C_3- (5) and Di-O-C_3-(5) according to the methods of Sims, Waggoner, Wang and Hoffman (1974), and Laris, Bahr, and Chaffee (1975), were carried out as follows:

SMP . The following substances were added to a quartz cuvette in a final volume of 1.2-1.3 ml, .9-1.0 ml of MSH, 1 μmole of ADP, 1 μmole of $MgSO_4$, 14 nmole of antimycin A, 50 μmole of Tris-ascorbate (pH 7.0), and either .25 mg of cytochrome c (for right side out energization) or 1 μg of PMS and 28 μg of protamine (for inside

out energization). The cuvette was placed in an Aminco-Keirs spectrophosphorimeter with Aminco corrected spectra attachment and magnetically stirred. .012 ml of a 21.3 μM solution of Di-S-C_3-(5) in 100% ethanol was added to the cuvette. Excitation was at 620 nm and emission was read at 670 nm. The fluorescence was monitored on a Mosley Model 7030AM Autograf recorder with a 50 second/inch sweep. .35-.65 mg of SMP was added from a 10 mg/ml suspension which had been treated by centrifugation to remove external cytochrome c. After a steady state fluorescence level was reached the sample was de-energized by the addition of .01 ml of .5 mM CCCP in 95% ethanol, and the fluorescence level was monitored until a new steady-state level was reached.

When the Di-O-C_3-(5) dye was used the same procedure was employed except that the amounts of substances added to the cuvette were .9-1.0 ml of MSH, 10 μmole of Tris-ascorbate, 1 μmole of ADP, 1 μmole of $MgSO_4$, 10 nmole of antimycin A, 150 μg cytochrome c (or 20 μg protamine plus .5 μg PMS), .005-.025 ml of Di-O-C_3-(5) (from an 81 μM solution in 100% ethanol) .2-.6 mg of SMP (from a 10 mg/ml suspension), and .01 ml of .25 mM CCCP for de-energization. The excitation and emission wavelengths were 560 nm and 600 nm respectively.

Proteoliposomes. The Di-S-C_3-(5) dye was used in the same manner as for SMP except that the following amounts of substances were used: 1.0 ml of 40 mM K_2SO_4, 10 mM HEPES (pH 7.0), 50 mole of Tris-ascorbate, and either (a) .5 mg of cytochrome c, .05 ml of proteoliposomes from a Sephadex-treated suspension at 20 mg/ml, and .01 ml of 21.3 μM Di-S-C_3-(5), for right side out energization; or (b) 40

μg of protamine, .5 μg of PMS, .003 ml of the proteoliposome suspension, and .025 ml of 21.3 μM Di-S-C_3-(5) for inside out energization. The final sample volume was 1.2-1.4 ml and was constant for all samples in a given experiment. The proteoliposomes were de-energized by the addition of .01 ml of .5 mM CCCP in 95% ethanol.

Oxidative sidedness assays

(A) PMS-Ascorbate

A solution was prepared consisting of 29 ml of MSH, 1.5 ml of .5 M Tris-ascorbate (pH 7.0), .1 ml of 1.2 mM antimycin A, .1 ml of 20 mM CCCP, and .012 ml of PMS (from a 5 mg/ml solution). A second solution was made with the same components except that 1.0 ml of polylysine (from a 4 mg/ml solution) was added. Both solutions were incubated at room temperature in the dark (due to the photosensitivity of PMS) for about 1 hour. 3.1 ml of the first solution and .05 ml of cytochrome c (from a 30 mg/ml solution) were added to a 3.4 ml lucite chamber containing a Yellow Springs Model 5331 oxygen electrode which was attached to a polarizing voltage source and Hewlett Packard model 7100BM strip chart recorder. The chamber had a cooling jacket through which room temperature water was circulated to minimize temperature variations, and was slowly stirred with a magnetic stir bar. After a brief equilibration period .2 mg of SMP protein (or .5-1.0 mg of proteoliposomes) was added and the rate of oxygen consumption obtained. The contents of the chamber were removed and the chamber was thoroughly washed with ethanol and then water. This measurement was generally repeated several times. 3.1 ml of the

solution containing polylysine was then added to the chamber and after equilibration was reached a quantity of SMP or proteoliposomes equal to that used in the previous determinations was added to the chamber and the rate of oxygen consumption again obtained. After repeating this measurement several times the percentage of inside out oxidation was calculated from the rate of the oxygen consumption with polylysine to the rate with cytochrome c.

(B) Succinate

2.9 ml of MSH, .1 ml of .1 M sodium succinate, .05 ml of cytochrome c (from a 30 mg/ml solution), .06 ml of .2 M KP_i, and .01 ml of .3 M $MgSO_4$ were added to the oxygen electrode chamber and allowed to equilibrate several minutes. 1-3 mg of SMP were added and the oxidation rate obtained. After cleaning the chamber 2.9 ml of MSH, .1 ml of .1 M sodium succinate, .06 ml of KP_i, .01 ml of .3 M $MgSO_4$, and .05 ml of protamine (from a 4 mg/ml solution) were added and the oxidation rate of an equal amount of SMP measured under these conditions. The percentage inside out was calculated from the ratio of the rate with protamine to the rate with cytochrome c.

Measurement of respiratory control ratios in proteoliposomes

All assays of respiratory control ratios (RCR's) were done at room temperature. The chart recorder was adjusted to zero oxygen by placing buffer in the chamber which had been bubbled with nitrogen for 15-20 minutes and the gain of the recorder was adjusted so that air-saturated buffer would give a reading which was on scale. 3.0 ml of the buffer in which the COV were made was placed in the

3.4 ml oxygen electrode chamber, and slowly stirred with a magnetic stir bar. .05 ml of a 30 mg/ml solution of cytochrome c and .15 ml of .5 M Tris-ascorbate (pH 6.8-7.0) was added to the chamber. Any background oxidation rate was noted (with K_2SO_4-HEPES this was usually zero but it varied with other buffer solutions). .025-.05 ml of a suspension of COV was added to the chamber and oxidation proceeded with a constant rate of oxygen consumption. When oxidation had continued long enough to obtain an accurate slope from the chart recorder .01 ml of an ethanolic solution of 10-20 mM CCCP was added and the uncoupler-stimulated rate of oxidation recorded. The sample was then withdrawn and the chamber thoroughly washed with 95% ethanol and then with distilled water. The slopes of the lines representing graphically the rates of oxygen consumption were used to determine the RCR as follows:

$$\text{RCR} = \frac{(\text{oxidation rate of uncoupled COV} - \text{background oxidation rate})}{(\text{initial oxidation rate of COV} - \text{background oxidation rate})}$$

I. RECONSTITUTION OF CYTOCHROME OXIDASE VESICLES

Introduction

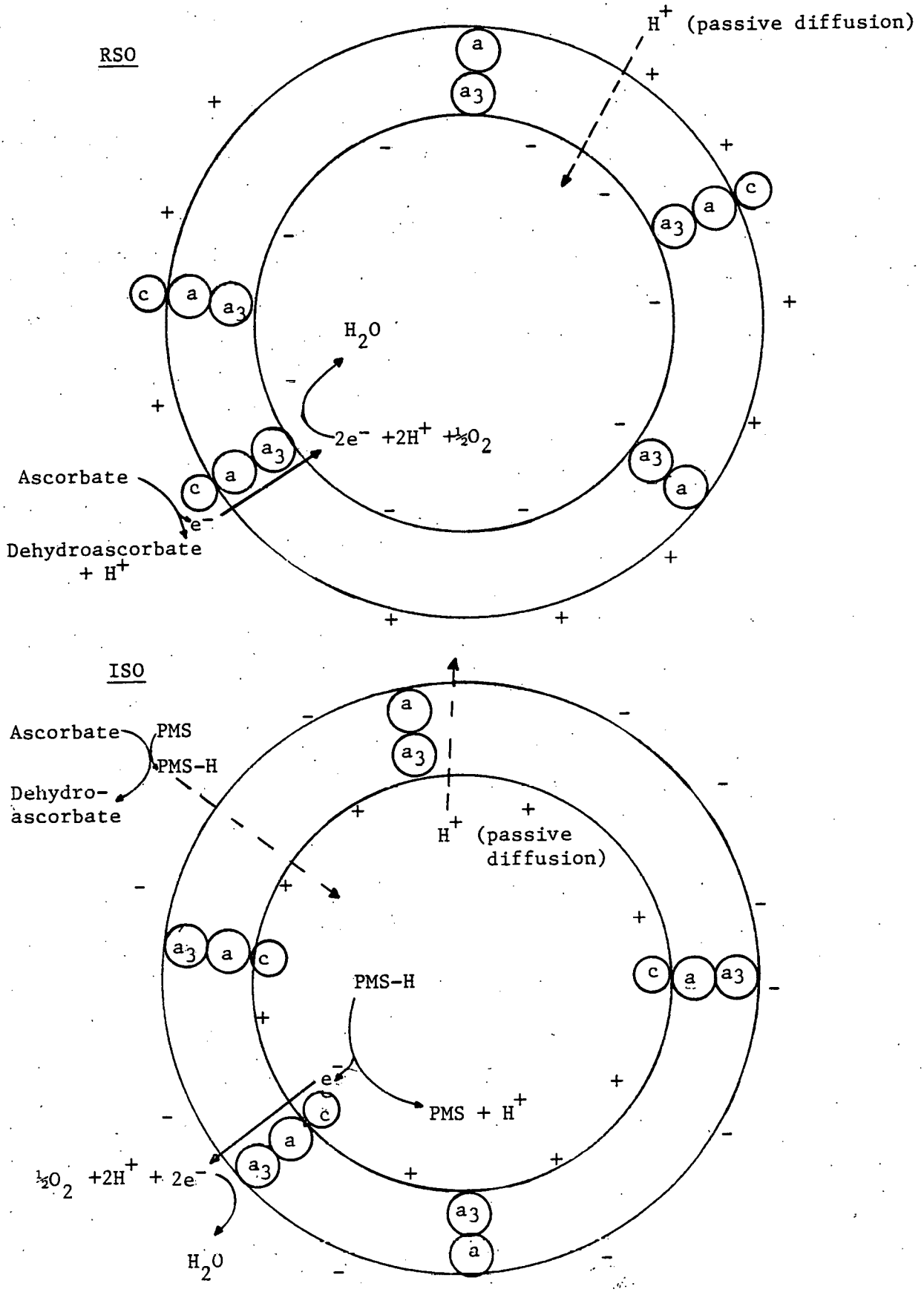
Cytochrome oxidase, also known as complex IV (Hatefi, Haavik, Griffiths, 1961, 1962), is the terminal enzyme in the respiratory chain of the mitochondrion and catalyzes the transfer of electrons from cytochrome c to molecular oxygen resulting in the formation of water (Hatefi, Haavik, Fowler, Griffiths, 1962). A number of preparations for the extraction of cytochrome from the mitochondrial membrane have been developed (Kuboyama, Yong, King, 1972; Yonetani, 1966; Fowler, Richardson, Hatefi, 1962; Hochli, Hackenbrock, 1978) along with various purification procedures (Kopaczyk, Perdue, Green, 1966; Carroll, Racker, 1977). The enzyme is hydrophobic in nature and is closely associated with mitochondrial phospholipids (Carroll, Racker, 1977; Marsh, Watts, Maschke, Knowles, 1978; Jost, Capaldi, Vanderkooi, Griffith, 1973).

Purified enzyme has been reconstituted in a functional manner in vesicles known as cytochrome oxidase vesicles (COV) in which the enzyme is incorporated in the phospholipid bilayer membrane of artificial phospholipid vesicles (Hinkle, Kim, Racker, 1972; Racker, 1972). These vesicles catalyze the transfer of reducing equivalents from an appropriate reductant (genrally ascorbate plus cytochrome c) to molecular oxygen, generating in the process a transmembrane proton gradient (Racker, 1974; Skulachev, 1974a, 1974b). The reconstitution of cytochrome oxidase vesicles has been accomplished by a number of different methods. The oxidase is located in a transmembranous orientation with the cytochrome c

reactive portion of the molecule on one side of the membrane and the O_2-reactive portion of the molecule on the other side of the membrane. (Reuben, Telford, Carroll, 1976; Racker, Loyter, Christiansen, 1971; Erecinska, Wilson, 1978). Because of this vectorial arrangement electron transport by cytochrome oxidase is anisotropic and leads to formation of a membrane potential. The membrane potential retards electron transport and tends to limit the oxidative rate (Racker, 1974; Miller, Racker, 1976). This causes the phenomenon of respiratory control in COV, as dissipation of the proton gradient by an uncoupler will remove the inhibitory effects of the membrane potential on the electron transport process and thereby stimulate the rate of respiration (Hinkle, 1970a; 1970b; Hinkle, 1973). Inclusion of cytochrome c in the interior of COV induces random bidirectional orientation of the oxidase in the membrane and allows either energization with external cytochrome c and ascorbate of the mitochondrially oriented oxidase (cytochrome c-reactive portion of the oxidase on the outside of the vesicle) or energization with membrane permeable PMS as a carrier of reducing equivalents from externally located impermeant ascorbate to the internal cytochrome c (Carroll, Racker, 1977; Skulachev, 1974a). The two modes of energization are referred to as right side out (RSO) and inside out (ISO), respectively. The energization schemes are shown in Figure 7. This provides a system of proton gradient generation which is readily controlled with regard to polarity.

Among methods used for the reconstitution of COV are the cholate dialysis method, where a solubilized suspension of phospholipids and

Figure 7. ENERGIZATION OF RSO AND ISO COV



cytochrome oxidase with Na-cholate is dialyzed for detergent removal resulting in COV formation (Hinkle, Kim, Racker, 1972; Racker, 1972). Another method is the cholate dilution procedure, where a solubilized suspension forms COV when diluted so as to reduce the detergent concentration (Racker, Chien, Kandrach, 1975). Incorporation into preformed vesicles containing lysolecithin or phosphatidyl serine has also been reported (Eytan, Matheson, Racker, 1975; 1976; Eytan, Racker, 1977; Eytan, Broza, 1978a; 1978b). Sonication of a mixture of phospholipids and cytochrome oxidase has also been used to form COV (Racker, 1973). Vesicles prepared by all of these methods demonstrate the property of respiratory control. The oxidase has been co-reconstituted in vesicles with other enzyme systems in a functional manner, including bacteriorhodopsin (Hellingwerf, Arents, VanDam, 1976), hydrophobic mitochondrial proteins (Racker, 1972), and ATPase (Racker and Kandrach, 1971; 1973). Investigations of the phenomenon of respiratory control in COV have produced evidence consistent with the concept that the stimulation of respiration by uncouplers is in fact related to inhibition of the electron transport reaction by the proton gradient generated by the oxidative process (Hinkle, Kim, Racker, 1972; Hinkle, 1970; 1973). Measurements have been made of the transmembrane potential generated by cytochrome oxidase both in vesicles (Drachev et al, 1974) and in planar phospholipid bilayers (Drachev et al, 1976). These measurements are consistent with the proposed scheme of membrane potential generation. The inhibition of the redox reaction by the formation of a transmembrane potential has also been demonstrated in reconstituted vesicular systems containing complex III (Wielburski, Nelson, 1978). Cytochrome oxidase is known to

consist of 6 peptide subunits (Keirns, Yang, Gilmour, 1971; Kessler, Blondin, VandeZande, Haworth, Green, 1977; Erecinska, Wilson, 1978). Recent work indicates that subunit II is probably the site of reaction with cytochrome c and is located on the outside of the mitochondrial membrane (Frey, Chan, Schatz, 1978; Briggs, Capaldi, 1978). There is also recent evidence for specific interactions of cations with the oxidase molecule, particularly potassium and calcium (Saris, Wikstrom, Saari, 1975; Wikstrom, 1974; Wikstrom, Harmon, Ingledew, Chance, 1976; Wikstrom, Saari, 1977). The mechanism of action of cytochrome oxidase is still a subject of much debate (Erecinska, Wilson, 1978; Wikstrom, Harmon, Ingledew, Chance, 1976). It has been proposed that in addition to functioning as a transporter of electrons cytochrome oxidase may be involved in active pumping of protons against an electrochemical gradient (Wikstrom, Saari, 1977; Wikstrom, 1977a; 1977b; Krab, Wikstrom, 1978). In RSO energized COV this would involve the addition of electrogenic proton transport outward by the oxidase in a stoichiometric fashion, tending to augment the formation of the proton gradient resulting from electron transport and proton consumption within the vesicle. Although there has been some dispute concerning this model (Moyle, Mitchell, 1978), other workers have corroborated the finding (Brand, Harper, Nicholls, Ingledew, 1978). This does not change the overall picture of the formation of a proton gradient in COV and the relation of the proton gradient to the phenomenon of respiratory control, but does give evidence of a new type of ion pumping mechanism coupled conformationally to the energy of electron transport (Krab, Wikstrom, 1978; Wikstrom, 1977a), and may have relevance to ion transporting systems to be discussed later.

Experimental evidence indicates that cytochrome oxidase is only associated with a small fraction of the total phospholipid vesicles in a suspension of COV made by conventional techniques (Carroll, Racker, 1977; Eytan, Broza, 1978). This fact suggests that there is less disparity between the protein:phospholipid ratios in COV and mitochondria than would be indicated from the amounts of phospholipid and cytochrome oxidase in the suspensions.

The controllable mode of the energization of COV with generation of transmembrane proton gradients and the compatibility of the enzyme with other proteins makes these vesicles an ideal system for the study of ion transport.

Results

Because of the importance of functional cytochrome oxidase vesicles as an energy source for reconstituted systems of mitochondrial oxidative phosphorylation and calcium transport initial experimental work was directed toward the development of active preparations of COV. The activity of the preparations was judged by oxygen consumption secondary to substrate oxidation, and the stimulation of respiration by uncouplers (respiratory control).

The initial preparation of cytochrome oxidase was done according to the previously outlined method of Kuboyama (1972). The heme to protein ratio of this preparation (referred to as batch 1) was determined from the difference in the $O.D._{605}$ between the reduced and oxidized form of the oxidase and was calculated to be 10.9 nmole heme a/mg protein. This was judged to be an adequately pure preparation to begin the production of COV. COV were prepared according to both the cholate dialysis method of Hinkle,Kim and Racker (1972) and the dilution procedure of Racker, Chien, and Kandrach (1975). Various salts were used in the vesicle solutions in an effort to optimize the RCR. As can be seen from Table 1, K_2SO_4-HEPES and KP_i worked better than KCl-HEPES (which had been suggested by Dr. Chris Miller, Cornell). The background oxidations obtained with K_2SO_4-HEPES were generally zero whereas those obtained with the KP_i buffer were not, so K_2SO_4-HEPES was selected as the best solution for general use. The dialysis method consistently produced COV with higher RCR than the dilution method.

It can also be seen that slightly higher cholate concentrations

Table 1.

CONDITIONS FOR RECONSTITUTION OF COV

Mode of preparation:		Dilution				Dialysis
Buffer	[cholate]: .7%	.75%	.85%	.95%	1.0%	1.0%
	Respiratory Control Ratios (RCR'S)					
50 mM KP_i	2.64±.44(2)	2.52±.23(2)	2.77±.38(3)	3.37±.26(3)	3.30±.62(4)	4.41±.54(2)
	-	-	-	-	3.22±.30(3)	4.72±.02(2)
40 mM K_2SO_4-	-	-	-	-	3.20±.15(3)	4.29±.23(2)
10 mM HEPES	-	-	-	-	3.81±.29(4)	-
40 mM KCl-	1.72±.23(2)	-	-	-	2.17±.10(2)	-
10 mM HEPES						

COV were made as described under METHODS using the buffers and final cholate concentrations shown above. Final asolectin concentration was 40 mg/ml, pH was 7.0, and cytochrome oxidase (batch 1) concentration was 417 µg/ml. The different samples of COV were made in .6 ml aliquots and were assayed for RCR by placing .025 ml of the vesicle suspension in an oxygen electrode chamber with 3.0 ml of appropriate buffer, .15 ml of .5 M Tris-ascorbate (pH 6.8), .75 mg of cytochrome c and dividing the respiration rate obtained into the rate obtained after the addition of .01 ml of 16 mM CCCP in ethanol. The RCR's given above represent the mean ± one standard deviation of the number of observations in parentheses.

(around 1%) gave better RCR's than the concentrations recommended by Racker, Chien, and Kandrach (1975) with the dilution procedure. It was also found that sucrose, EDTA, and histidine seemed to have some deleterious effects on the RCR's of COV. This was of concern in later reconstitution experiments where these components were occasionally used. The data is shown in Table 2. A second batch of cytochrome oxidase was extracted and then fractionated according to the method of Carroll and Racker (1977) as previously mentioned in the METHODS section. A total of four fractions were obtained. The RCR of the COV made from any particular fraction depended on the relative concentrations of phospholipid and cytochrome oxidase. Figure 8 shows the variation of RCR with [cytochrome oxidase]. Each fraction of oxidase which was tested showed an optimum protein concentration which gave the best RCR.

The oxidative activity and RCR of COV made from all the various batches of oxidase that were extracted are shown in Table 3. The cytochrome oxidase made by the method of Kuboyama (1972) shows consistently higher oxidative activity as well as RCR. The fractionation procedure of Carroll and Racker greatly improved the RCR's of the COV made from the Kuboyama oxidase, while the Kopaczyk purification of the Fowler oxidase did not seem to change the activity very much. It is noted that the fractions with greater oxidative activity per μg of protein also gave higher RCR's, indicating that some removal of protein impurities had occurred.

Polyacrylamide gel electrophoresis was done on all the various fractions of cytochrome oxidase. Figure 9 shows a gel electrophoresis pat-

Table 2.

EFFECTS OF VARIATION IN BUFFER SOLUTION ON RCR'S OF COV

$[K_2SO_4]$	[HEPES](pH 7)	[sucrose]	[EDTA]	[histidine]	[DTT]*	RCR
40 mM	10 mM	-	-	-	-	7.95
5 mM	10 mM	-	-	-	-	7.16
5 mM	10 mM	100 mM	-	-	-	5.71±1.26(2)
5 mM	10 mM	100 mM	.2 mM	-	-	3.08
5 mM	10 mM	100 mM	.2 mM	2 mM	-	1.66±.37(2)
5 mM	10 mM	100 mM	.2 mM	2 mM	1 mM	2.12±.26(4)

COV in the above experiment were made in .55 ml aliquots by the dilution technique described in the METHODS section. The final asolectin concentration was 40 mg/ml, cholate concentration was .8%, and cytochrome oxidase (batch 2, fraction 1) concentration was .225 mg/ml. .025 ml of COV were added to an oxygen electrode chamber containing 3.0 ml of the buffer in which the vesicles were made, .15 ml of .5 M Tris-ascorbate (pH 7.0), and 1.5 mg of cytochrome c. The rate of oxidation obtained was divided into the rate obtained after addition of .01 ml of 10 mM CCCP to determine the RCR's shown.

Figure 8. [CYTOCHROME OXIDASE] VERSUS RCR IN COV

COV were made by the dilution technique at a final cholate concentration of 1% and varying final cytochrome oxidase concentrations as shown. The COV made using fractions I and III of batch 2 of cytochrome oxidase were made in a final buffer concentration of 40 mM Na_2SO_4 - 10 mM HEPES (pH 7.0) and the COV made using unfractionated cytochrome oxidase from batch 1 were made in a KP_i buffer with a final concentration of 50 mM (pH 7.0). RCR's were measured as previously described, with 1.5 mg of cytochrome c per sample for the COV made from the batch 2 fractions, and .75 mg of cytochrome c per sample for the COV made from batch 1.

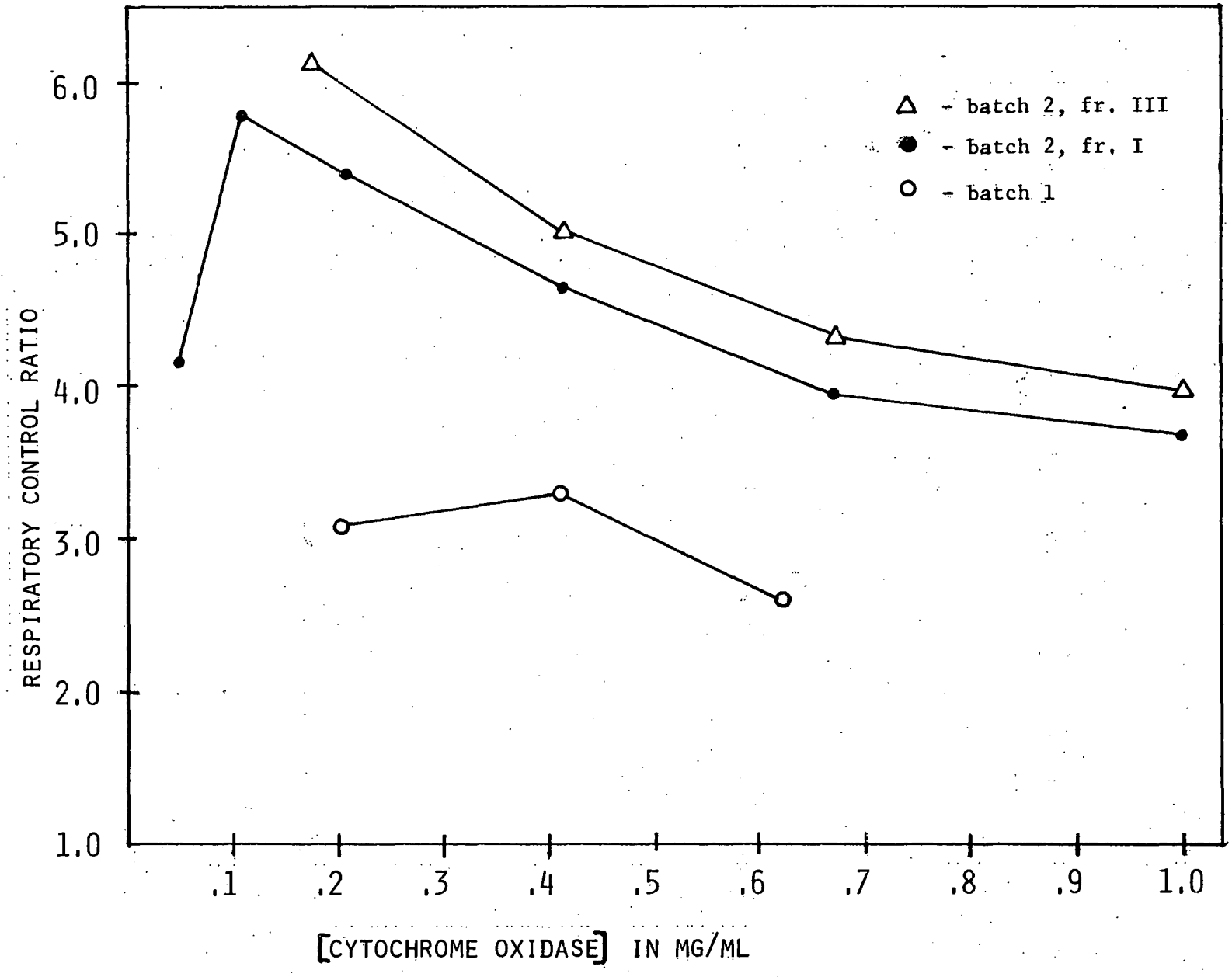

△ - batch 2, fr. III
● - batch 2, fr. I
○ - batch 1
RESPIRATORY CONTROL RATIO
6.0
5.0
4.0
3.0
2.0
1.0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
[CYTOCHROME OXIDASE] IN MG/ML

Table 3.

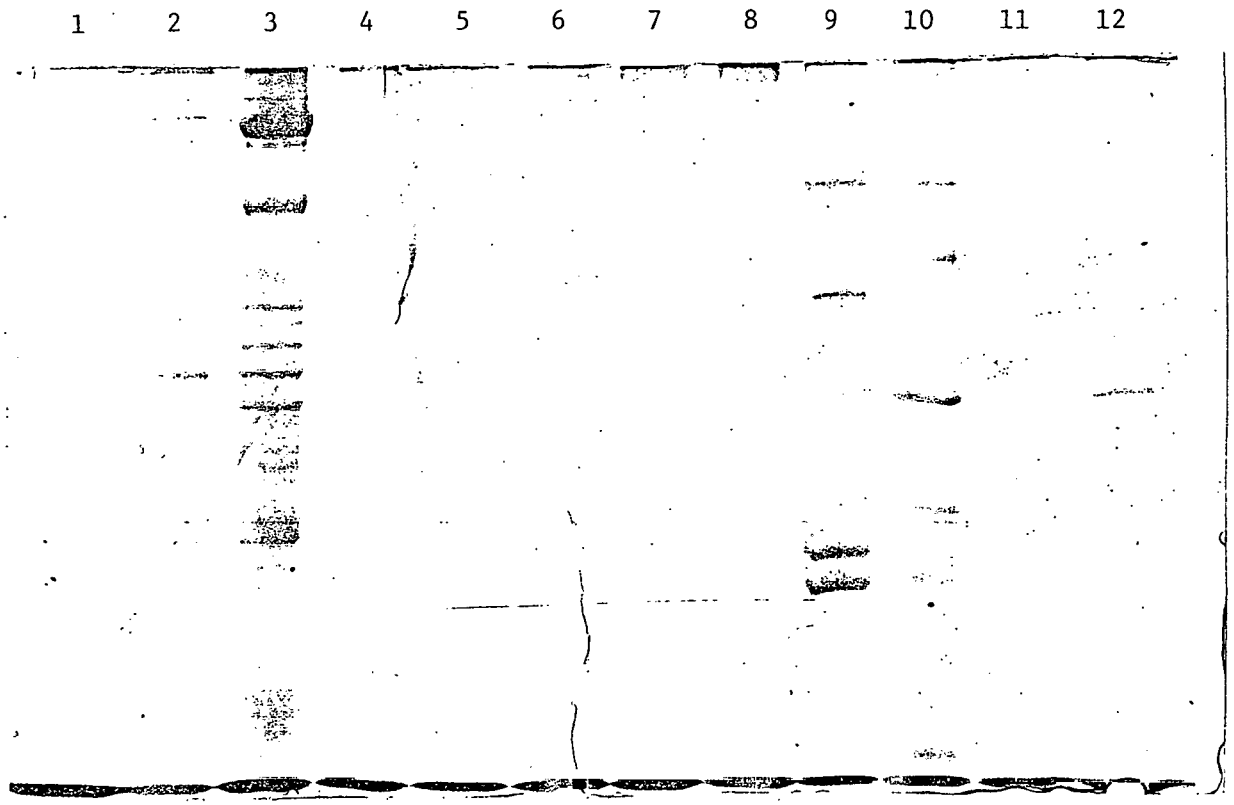
OXIDATIVE ACTIVITY OF COV MADE FROM VARIOUS CYTOCHROME OXIDASE PREPARATIONS

Cytochrome Oxidase	RCR	Max. (uncoupled) oxidation rate nmole O_2/min/µg protein
Batch 1 (unfractionated)*	3.05	4.20
Batch 2 (unfractionated)*	4.97	4.80
Batch 3 (unfractionated)*	5.44±.60(3)	4.67±.41(3)
Batch 2, fraction I **	7.50	5.24
Batch 2, fraction II **	6.11±.58(3)	6.28±.16(3)
Batch 2, fraction III **	7.52±.45(2)	6.07±.48(2)
Batch 2, fraction IV **	5.49±.42(2)	6.75±.14(2)
Fowler, fraction I #	1.15	-
Fowler, fraction II #	1.36	-
Kopaczyk, fraction I ##	1.10	1.59
Kopaczyk, fraction II ##	1.66	2.28

* Cytochrome oxidase made by the method of Kuobayama (1972)
**Cytochrome oxidase made by the method of Kuobayama and purified by the method of Racker (See METHODS section)
Cytochrome oxidase made by the method of Fowler (1962)
Cytochrome oxidase made by the method of Fowler and purified by the method of Kopaczyk (1966)

The above preparations of cytochrome oxidase were incorporated into COV and assayed for RCR using the cholate dilution technique as previously described. The final cholate concentration in the suspension was 1% and the cytochrome oxidase concentration was between 333 and 417 µg/ml of vesicle suspension. The COV were made and assayed in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0).

Figure 9. GEL ELECTROPHORESIS OF VARIOUS CYTOCHROME OXIDASE FRACTIONS (I)



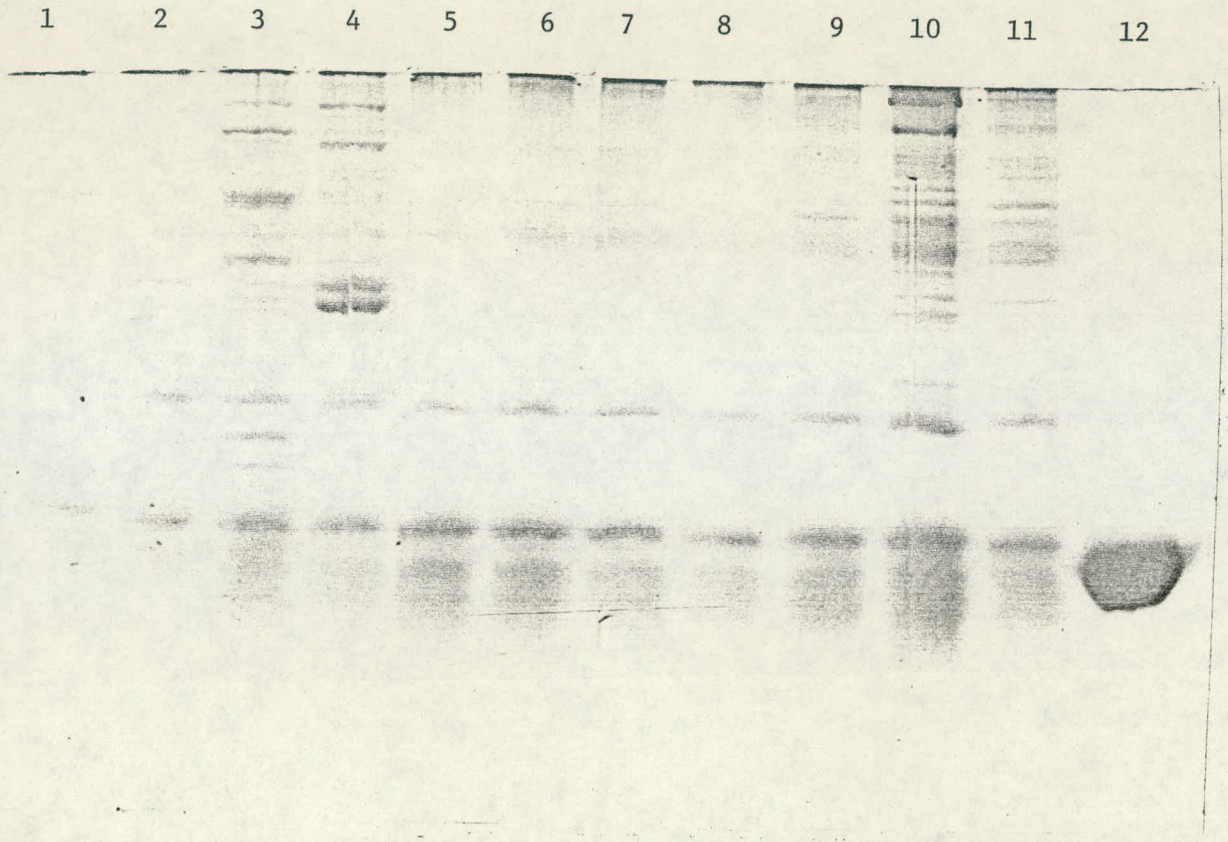
1 - Cytochrome c
2 - Cytochrome oxidase (batch 1)
3 - Cytochrome oxidase (batch 3)
4 - Cytochrome oxidase (batch 2, unfractionated)
5 - Cytochrome oxidase (batch 2, fraction I)
6 - Cytochrome oxidase (batch 2, fraction II)
7 - Cytochrome oxidase (batch 2, fraction III)
8 - Cytochrome oxidase (batch 2, fraction IV)
9 - Cytochrome oxidase (Fowler preparation, fraction I)
10 - Cytochrome oxidase (Fowler preparation, fraction II)
11 - Cytochrome oxidase (Fowler preparation, Kopaczyk purification, fraction I)
12 - Cytochrome oxidase (Fowler preparation, Kopaczyk purification, fraction II)

Polyacrylamide gel electrophoresis was done as described in the METHODS section. The gel contained 10% acrylamide and approximately 35 μg of each of the above protein fractions was used per well. The cytochrome oxidase fractions in wells 2 - 8 were prepared by the method of Koubayama as detailed in the METHODS section.

tern run on a 10% acrylamide gel. Since this type of gel works best for proteins in the molecular weight range of 10,000 - 100,000 (Weber and Osborne, 1969; 1975), it was used as a screening gel. Numerous high molecular weight impurities are evident in batches 1 and 3 of the Kuboyama oxidase, as well as in the oxidase made according to the Fowler method. Batch 2 had the cleanest pattern on gel electrophoresis, and the subsequent fractionation of batch 2 appeared to remove much of the higher molecular weight contaminants. The 15% gel of Figure 10 shows much better detail, particularly in the low molecular weight range. What appears to be a single low molecular weight band at the bottom of the gel of Figure 9 is resolved into the 3 low molecular weight subunits of the oxidase in Figure 10. (Note that the order of the proteins is reversed in Figure 10.) This band does appear to be present in all the oxidase preparations to some extent. There also appears to be some correlation between the purity of the protein and the RCR, since the higher RCR's were always found with the cleaner preparations.

Phosphatidyl serine incorporation was also used as a method of preparation of COV. It was felt that this method might be useful in the reconstitution of oxidative phosphorylation in a mitochondrial orientation, since proteins could be selectively incorporated into vesicles in this manner (Eytan, Racker, 1977). Table 4 shows the RCR's obtained using the phosphatidyl serine incorporation method. The optimum protein concentration was much lower than that used by Eytan and Racker, but despite protein optimization and variation of the type of phosphatidyl serine used, RCR's greater than between 2 and 3 were not obtained.

Figure 10. GEL ELECTROPHORESIS OF VARIOUS CYTOCHROME OXIDASE FRACTIONS (II)



1 - Cytochrome oxidase (Fowler preparation, Kopaczyk purification, fraction II)
2 - Cytochrome oxidase (Fowler preparation, Kopaczyk purification, fraction I)
3 - Cytochrome oxidase (Fowler preparation, fraction II)
4 - Cytochrome oxidase (Fowler preparation, fraction I)
5 - Cytochrome oxidase (batch 2, fraction IV)
6 - Cytochrome oxidase (batch 2, fraction III)
7 - Cytochrome oxidase (batch 2, fraction II)
8 - Cytochrome oxidase (batch 2, fraction I)
9 - Cytochrome oxidase (batch 2, unfractionated)
10 - Cytochrome oxidase (batch 3)
11 - Cytochrome oxidase (batch 1)
12 - Cytochrome c

Polyacrylamide gel electrophoresis was done according to the procedure described in the METHODS section. This gel contained 15% acrylamide and approximately 50 μg of protein per well. The cytochrome oxidase fractions in wells 5 - 11 were prepared by the method of Koubayama as detailed previously.

Table 4.

VARIATION OF CYTOCHROME OXIDASE CONCENTRATION IN COV MADE BY PHOSPHATIDYL SERINE INCORPORATION

[cytochrome oxidase]	Respiratory Control Ratio
400 µg/ml	1.69
200 µg/ml	1.47
125 µg/ml	2.19
62.5 µg/ml	2.07
37.5 µg/ml	2.26

COV were made by the phosphatidyl serine incorporation technique as described in the METHODS section. The phospholipid composition was 3 parts mitochondrial phosphatidyl ethanolamine, 1 part mitochondrial phosphatidyl choline, and 2 parts phosphatidyl serine (Brain extract, Sigma type III) at a final concentration of 3.7 mg/ml in 50 mM KP_i, .5 mM EDTA (pH 7.0). .2 ml aliquots of COV were prepared using cytochrome oxidase from batch 2, fraction I. After a 30 minute incubation at room temperature the vesicles were assayed for RCR in the usual manner.

It was desirable to produce large-volume cytochrome oxidase vesicles for the reconstitution of a mitochondrially oriented system of oxidative phosphorylation. Significant amounts of the large F_1 molecule (dia. 90 Å) must be incorporated into the interior of the vesicles for this type of orientation. However, the diameter of the COV is typically 300-1000 Å, so steric hindrance is likely to impair the trapping of F_1 within the vesicles. Therefore a procedure for the reconstitution of large-volume COV was developed. The basic technique involved the ether vaporization of a solution of phospholipids in buffer following the methods of Deamer and Bangham (1976). Because the ether vaporization only works well at about 40° C., deleterious effects on proteins could occur. By using a vacuum to lower the vaporization temperature of the ether, the vaporization process can be carried out at lower temperatures. The method of Deamer and Bangham did not allow continuous infusion of the solution, however, so only very small amounts of the vesicles could be produced. By using a piece of small diameter polyethylene tubing to provide a flow resistance, the vacuum could be used as the driving force for the ether infusion, and continuous infusion of the phospholipid solution at the optimum infusion rate for giant liposome formation was possible. Table 5 shows a summary of the three methods which were used to produce large-volume COV's. The first method involved vaporization of the ether solution in the presence of the oxidase in hope of incorporating the oxidase into the vesicles at the time of formation. The technique had limited success at low protein concentrations, but the RCR's were too low to be useful. Then the incorporation was tried in the presence of small amounts of cholate, as in the incorporation method of Eytan and Racker (1977). It was

Table 5.

7-14 mg of asolectin in ether at 1.4 mg/ml was injected into 2.5-3.0 ml of 40 mM K_2SO_4, .25 M sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.0) at a rate of .5-1.0 ml/minute at room temperature and under vacuum as described in the METHODS section. For direct incorporation, cytochrome oxidase (batch 2, fraction I) was included in the buffer at the time of ether injection. For cholate incorporation, the appropriate amount of sodium cholate was added either during vaporization or to preformed liposomes prior to incubation. For lysolecithin incorporation, a 1:1:.15:.25 ether solution of phosphatidyl choline : phosphatidyl ethanolamine : cardiolipin : lysolecithin at 1.4 mg/ml was injected into the buffer and an appropriate amount of cytochrome oxidase was added to the suspension after vaporization. The control sample for the lysolecithin incorporation technique was formed by sonicating phospholipids to clarity in the same buffer at the same concentration and adding cytochrome oxidase.

Table 5. LARGE-VOLUME CYTOCHROME OXIDASE VESICLES

Method 1		Incubation	[Cytochrome oxidase] μg/ml	[Phospholipid] mg/ml	[Cholate]	RCR
Direct incorporation		none	208.3	2.3	-	1.00
		24 hr., 4°C.	208.3	2.3	-	1.03
		none	83.3	2.0	-	1.40*
		none	62.7	2.7	-	1.52
		none	41.7	3.3	-	1.40
		none	8.3	3.0	-	1.65
Method 2						
Cholate incorporation	cholate added at formation	16 hr., 4°C., dialysis	66.7	4.7	.1%	1.00
		none	66.7	4.7	.07%	1.00
	cholate added after formation	12 hr., 4°C.	114.6	2.0	.04%	1.00*
		12 hr., 4°C.	83.3	3.3	.05%	1.00
Method 3						
Lysolecithin incorporation		20 hr., 4°C.	38.5	9.0	-	3.30*
(Standard sonication		20 hr., 4°C.	33.3	9.0	-	3.80*) #

*Buffer contained only 40 mM K_2SO_4 and 10 mM HEPES (pH 7.0)

#Control sample for lysolecithin incorporation.

found that addition of cholate either at the time of formation of the vesicles or subsequently was not conducive to the formation of COV which had respiratory control. Finally, the lysolecithin incorporation technique of Eytan and Racker was employed. Lysolecithin was used as 10% of the total phospholipid in the ether solution. After formation of large-volume, lysolecithin-containing liposomes by ether vaporization, cytochrome oxidase was added and the mixture incubated at 4° C. overnight. This method was able to produce COV with RCR's greater than 3. It was hoped that this method could subsequently be used to produce mitochondrially oriented PCOV.

In order to measure membrane potentials or transport of ions in reconstituted vesicular systems it is desirable to separate the particles from the suspending medium. Labelled cytochrome oxidase vesicles were formed with ^{86}Rb trapped inside them. As is shown in Figure 11, even after 1.5×10^6 g-minutes of centrifugation, almost half of the COV remained in the supernatant fraction. It is apparent that centrifugation is not an effective means for rapid separation of COV from suspending medium.

Filtration of COV suspensions using Millipore filters was next employed as a separatory technique. Table 6 shows the results of these experiments. COV were generally labelled by trapping of a radioactive substance which was present during the first phase of the dialysis (cholate removal phase). After the cholate had been removed and the vesicles were able to retain internal contents, a second phase of dialysis was employed to remove any label external to the vesicles. The labelled suspensions were then forced through Millipore filters of varying pore

Figure 11. CENTRIFUGATION OF ^{86}Rb-LABELLED COV

COV were labelled by the dilution procedure by incubating .6 ml of COV (made with 40 mg/ml phospholipid, 1% sodium cholate, 50 mM KP_i (pH 7.0), and 417 μg/ml cytochrome oxidase, batch 1) with .01 ml (257 μCi) of ^{86}Rb for 30 minutes and then diluting into 4 ml of 50 mM KP_i (pH 7.0) containing 515 μCi of ^{86}Rb. After 5 minutes of incubation the mixture was further diluted to a final volume of 15 ml by addition of 50 mM KP_i, and then dialyzed 3 times against 1000 volumes of this buffer for 12 hours at 0°C. to remove external label. 4 ml aliquots of COV were then mixed with 4 ml of buffer and centrifuged in a JA-20 rotor, with serial removal of aliquots of supernatant for radioactivity determination.

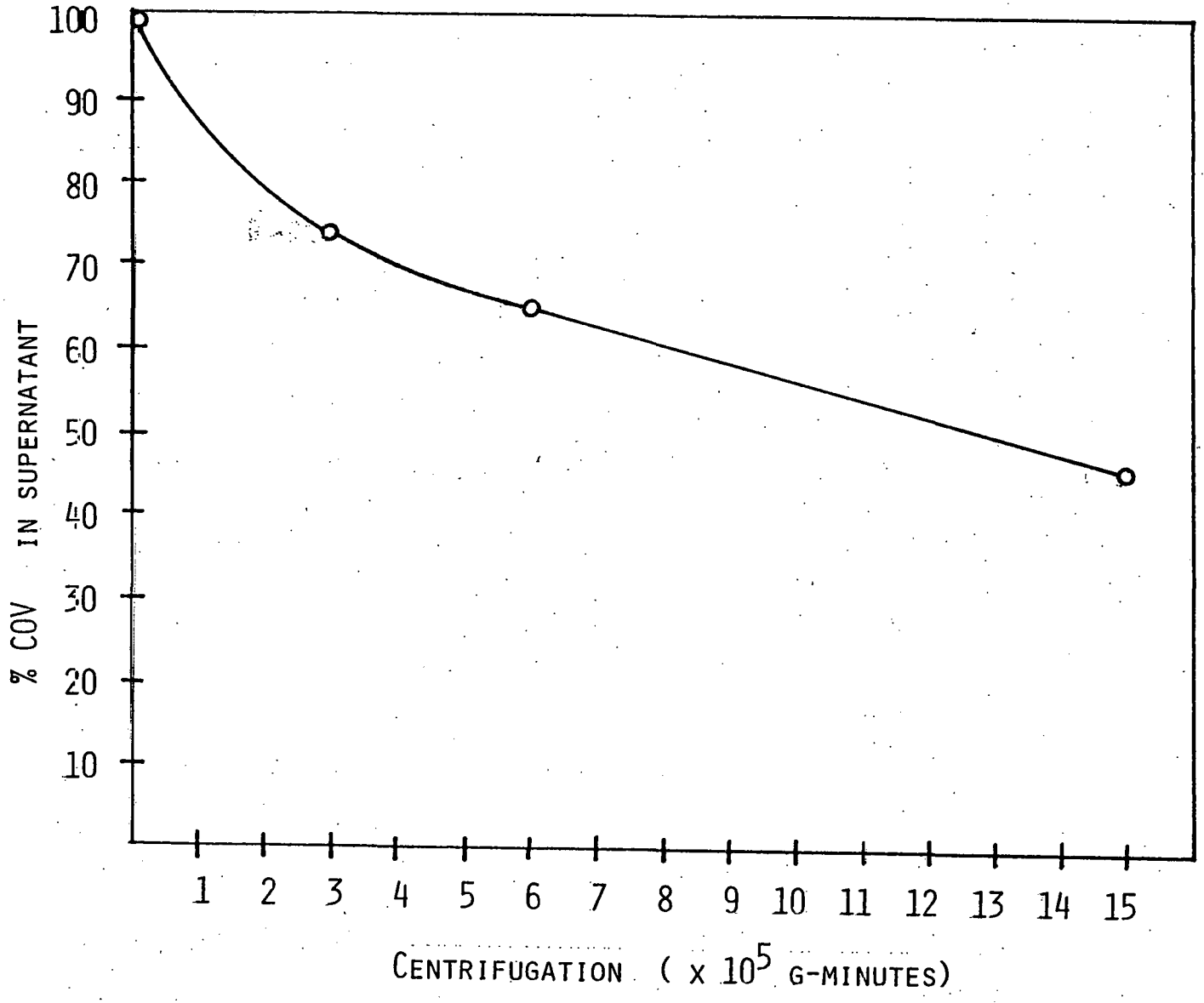

100
90
80
70
60
50
40
30
20
10
% COV IN SUPERNATANT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CENTRIFUGATION (X 10^5 G-MINUTES)

Table 6. FILTRATION OF CYTOCHROME OXIDASE VESICLES

[Cytochrome oxidase] μg/ml	Formation: Buffer	Formation: Dialysis 1	Formation: Dialysis 2	Filtration: [COV] mg/ml	Filtration: Volume	Filtration: Filter	Results: Assay	Results: % retained on filter
250	40 mM K_2SO_4, 10 mM HEPES, 20 mM sucrose, 2.35 μCi/ml of ^{14}C-sucrose	3 volumes 5-8 hours x7	139 volumes 8 hours x4	3.2	2.0 ml	.45μ	^{14}C-counting of filtrate	5.5%
312	40 mM K_2SO_4, 10 mM HEPES, 50 mM sucrose, 2.0 μCi/ml of ^{14}C-sucrose	8.3 volumes 4-8 hours x5	208 volumes 10 hours(avg) x3	4.0	2.0 ml	.45μ	^{14}C-counting of filtrate	14.0%
417	40 mM K_2SO_4, 10 mM HEPES	-	208 volumes 11 hours and 7 hours	4.0	2.0 ml	.45μ	O.D.$_{470}$ of filtrate	12.0%
833	40 mM KCl, 10 mM HEPES, 2 μCi/ml $^{86}RbCl$	833 volumes 8 hours x2	833 volumes 6-12 hours x3	1.0	1.0 ml	.22μ	^{86}Rb-counting of filtrate	58.3%
				2.0	1.0 ml	.22μ		44.7%
417	50 mM KP_i[a]	-	-	.53	3.0 ml	.05μ[b,d]	^{86}Rb-counting of filtrate	65.0%
				.53	3.0 ml	.05μ[b]		51.0%
				.53	3.0 ml	.05μ[c,d]		79.0%
				.53	3.0 ml	.05μ[c]		75.0%

Table 6.

[a] COV containing trapped ^{86}Rb as a label were formed by a combination dilution/dialysis technique as indicated in the legend of Figure 12.
[b] Filters were placed in holder with shiny side distal to the sample.
[c] Filters were placed in holder with flat side distal to the sample.
[d] Filters were prewashed with 10 ml of water.

COV were made by the dialysis technique as detailed in the METHODS section using unfractionated cytochrome oxidase from batch 2 for the upper two experiments and from batch 1 for the lower 2 experiments in the above table at the concentrations indicated. After sonication the vesicle suspensions were dialyzed against the appropriate volume of the indicated buffer solution shown under Dialysis 1. The approximate length of time of each dialysis cycle and the number of cycles (dialysis against fresh buffer solution) is also indicated under Dialysis 1. The suspensions were subsequently dialyzed against buffer containing no radioactive label as indicated under Dialysis 2. Samples were diluted in the nonradioactive buffer to the [COV] (mg/ml of phospholipid) and volume shown and then filtered through Millipore type EH filters in Swinnex type filter holders. A glass prefilter was used with the .45μ filters. The % of COV removed from the suspension was determined by radioactive counting of the filtrates and comparison with unfiltered controls, or by measuring light scattering of the filtrates at 470 nm and comparison with unfiltered controls.

size. In one experiment the O.D. of the filtrate was used to quantitate the vesicles. (The linearity of the vesicle concentration with the amount of light scattering at 470 nm is shown later in Figure 16). It is obvious from the table that the smaller pore size filters retain more of the vesicles. However, even with a .05μ filter a significant fraction of the particles passes through, and any smaller filters would be impractical since it would be too difficult to force a significant amount of suspension through them due to clogging. In fact, a suspension of COV at .53 mg/ml can barely be manually forced through the .05μ filters. In addition, having to apply such large forces for filtration might increase the tendency for the vesicles to lyse on the filter.

Table 7 shows the results of filtration of COV made by the lysolecithin incorporation technique (both large-volume and sonicated types). It can be seen that a .45μ filter (which removes 5.5-14% of dialyzed COV, or 4.5% of sonicated lysolecithin COV) removes 32.5% of the large-volume COV, indicating that these proteoliposomes are in fact larger in size. It is also noted that the RCR's of either sonicated or large-volume COV improve with filtration. This implies that the larger vesicles have lower RCR's.

In the dialysis type of labelling of COV it was assumed that the innate low permeability of proteoliposomes (which is presumably responsible for the phenomenon of respiratory control in COV because pH gradients and membrane potentials can be maintained) allows retention of the internal label during the second phase of the dialysis. The fact that vesicles could be produced in which the external dialysis solution had no radioactivity while the vesicles themselves retained significant radio-

Table 7. FILTRATION OF COV MADE BY LYSOLECITHIN INCORPORATION

COV	Respiration rate nmole O_2/min/μg protein Initial	+ CCCP	RCR	% oxidative activity removed by filtration
(1) Large-volume COV				
unfiltered suspension	2.1	6.9	3.3	
filtrate	1.2	4.7	4.0	32.5%
(2) Sonicated COV				
unfiltered suspension	1.8	6.6	3.8	
filtrate	1.4	6.3	4.5	4.5%

Large-volume COV were made by the ether vaporization technique as previously described. 13.6 mg of phospholipid solution (1:1:.15:.25 phosphatidyl choline : phosphatidyl ethanolamine : cardiolipin : lysolecithin) in 10 ml of ether was injected under vacuum into 2.5 ml of 40 mM K_2SO_4, 10 mM HEPES (pH 7.0) at 40°C. over a period of 15 minutes. Cytochrome oxidase (batch 2, fraction I) was added to a final concentration of 38.5μg/ml and the suspension was incubated at 4°C. for 20 hours. Sonicated COV were made by sonicating 13.6 mg of the same phospholipid mixture in 1.5 ml of buffer until clarified and incubating the suspension at 4°C. for 20 hours after adding cytochrome oxidase (batch 2, fraction I) to a final concentration of 33.3μg/ml. For oxidative assay .2 ml of COV was added to an oxygen electrode chamber containing 3.1 ml of 40 mM K_2SO_4-10 mM HEPES, 22.7 mM Tris-ascorbate (pH 7.0) with 1.5 mg of cytochrome c. After obtaining the initial oxidation rate .01 ml of 10 mM CCCP was added and the uncoupled rate determined. .5 ml of the suspension was diluted to 2.0 ml and filtered through a .45μ EH type Millipore filter with a glass prefilter, and .8 ml of the filtrate was assayed for oxidative activity as above. The % of oxidative activity removed by filtration was determined from the uncoupled respiration rates.

activity support this assumption. Vesicles were made with sucrose as a label (to minimize any binding to the vesicles) and after the second phase of dialysis was complete, leakage of the label with time was followed, as is shown in Figure 12. It is apparent that COV retain the internal label for long periods of time with very little efflux. The presence of hydrophobic protein such as complex V appears to make the vesicles more permeable to the label. As is expected, cholate greatly accelerates the efflux of label by increasing the permeability of the membrane.

Because of the relative impermeability of COV to small molecules, it was possible to use the labelling experiments to determine the internal volume of the vesicle suspensions. During the initial labelling (cholate dialysis phase) samples of the dialysis solution were taken for radioactive counting. After the second phase of the dialysis was complete and no further radioactivity could be detected in the dialysis solution (all external label presumably having been removed) a sample of the vesicle suspension was taken for counting. By comparing the initial and final concentrations of the radioactive substance the internal volume of the suspension could be determined. The experiments were not practical as a routine determination of volume on vesicle suspensions because of the enormous amounts of radioactive label which were required for the first dialysis phase. Table 8 shows the results of several volume determination experiments on COV and complex V-COV. The percentages shown refer to suspensions of vesicles at 40 mg/ml phospholipid concentration. By using an average of these volume measurements an estimate of concentrations of substances transported by the vesicle suspensions in later experiments could be made.

Figure 12. RATE OF LEAKAGE OF INTERNAL RADIOACTIVE LABEL FROM COV AND COMPLEX V-CONTAINING COV

^{14}C-sucrose-containing vesicles were made by dialysis. 1.5 ml of each suspension was prepared containing 40 mg/ml asolectin, 1% sodium cholate, 40 mM K_2SO_4, 10 mM HEPES, 5 mM sucrose (pH 7.0), 417 μg/ml of cytochrome oxidase (batch 3), .76 mg/ml cytochrome c, 2 μCi/ml ^{14}C-sucrose, and, in the aliquot of complex V-COV 633 μg/ml of complex V (from beef heart, batch 2). The vesicles were placed in separate dialysis tubing and dialyzed together against 20 ml of 40 mM K_2SO_4, 10 mM HEPES, 5 mM sucrose (pH 7.0) containing 2 μCi/ml of ^{14}C-sucrose.[2] The total time of dialysis was 57.5 hours, and the dialysis solution was changed at 2.5, 5, 26, 34, and 47.5 hours of dialysis. Then the suspensions were dialyzed against 500 ml of nonradioactive dialysis solution for 60 hours changed at 15, 22, and 47 hours. .3 ml aliquots of the vesicle suspensions were placed in dialysis tubing and incubated in 4 ml of nonradioactive dialysis solution for the times indicated in the Figure , with serial removal of .1 ml of the dialysis solution for radioactive counting to allow quantitation of the rate of label efflux. All dialysis was carried out at 4°C.

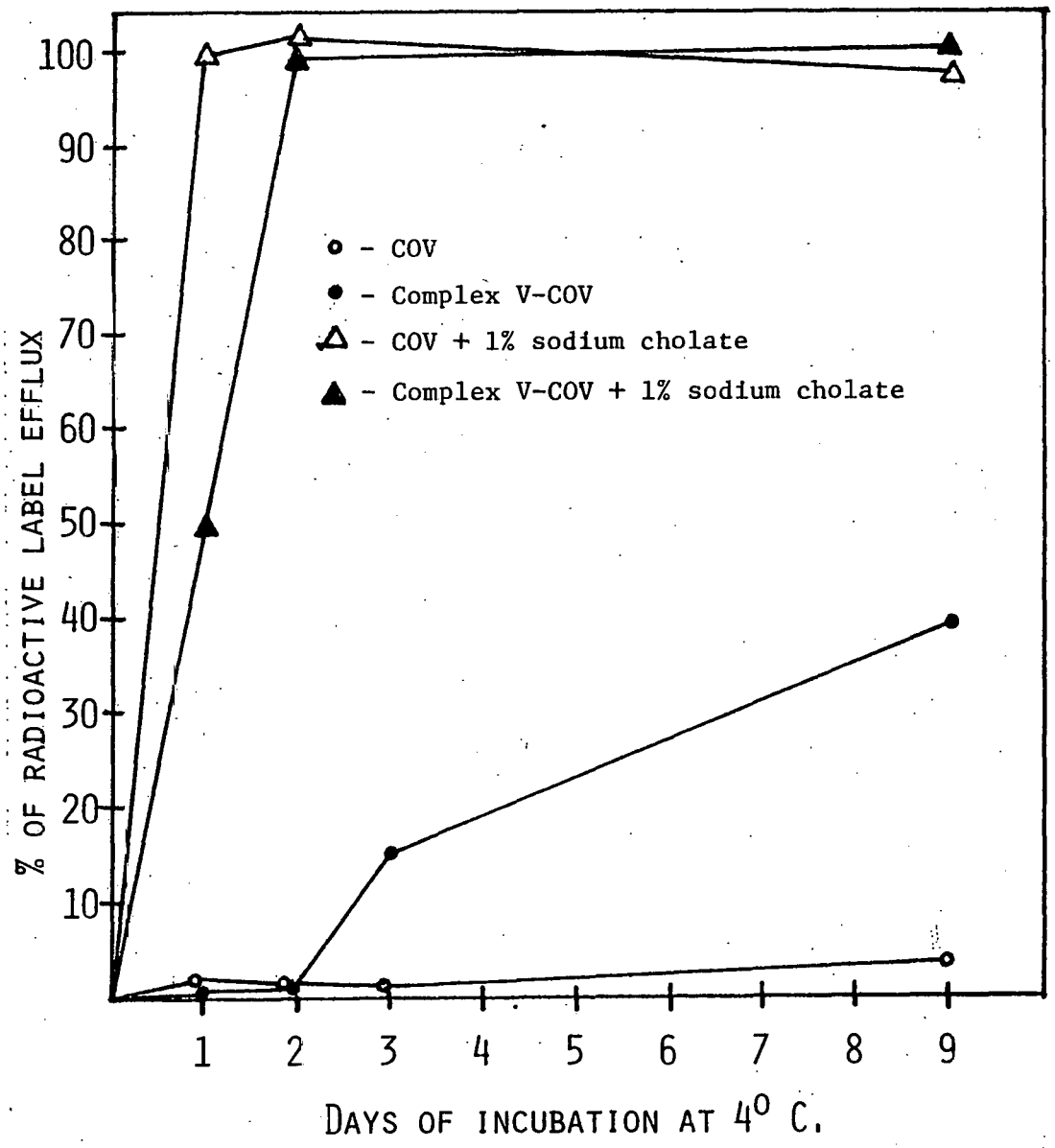

100
90
80
70
60
50
40
30
20
10
% OF RADIOACTIVE LABEL EFFLUX
- COV
- Complex V-COV
- COV + 1% sodium cholate
- Complex V-COV + 1% sodium cholate
1
2
3
4
5
6
7
8
9
DAYS OF INCUBATION AT 4° C.

Table 8.

Radioactively labelled COV were made by dialysis as previously described for determinations of internal volume. All vesicles were made in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0), with the additions shown above. After addition of the appropriate amounts of the proteins to give the final concentrations shown, the vesicle suspensions were dialyzed against the indicated volume of buffer for the lengths of time (shown per each dialysis cycle) and for the number of cycles (dialysis against fresh buffer) indicated under Dialysis 1. The vesicles were then transferred to a dialysis container with the indicated volume of nonradioactive buffer and dialyzed as shown under Dialysis 2. Aliquots of the initial labelled dialysis solution and the labelled vesicles were counted with appropriate techniques to determine the internal volume.

Table 8. INTERNAL VOLUME DETERMINATIONS OF VESICLE SUSPENSIONS

[Cytochrome oxidase] μg/ml	[Complex V] μg/ml	[Cytochrome c] μg/ml	Buffer additions	Dialysis 1	Dialysis 2	Internal volume % of suspension	Internal volume μl/mg phospholipid
417 [a]	-	-	2.5 mM RbCl, 12.5μCi/ml of ^{86}Rb	16.7 vol. 8 hours x3	188 vol. 8 hours x4	3.3%	0.83
417 [b]	-	-	10μCi/ml of ^{86}Rb	83.3 vol. 4-8 hours x3	83.3 vol. 3 hours x 3	5.3%	1.30
417 [c]	-	760	5 mM sucrose 2μCi/ml of ^{14}C-sucrose	8.3 vol. 3-12 hours x6	208 vol. 8-24 hours x4	3.3%	0.83
417 [c]	633	760	5 mM sucrose 2μCi/ml of ^{14}C-sucrose	8.3 vol. 3-12 hours x6	208 vol. 8-24 hours x4	3.9%	0.98
550 [c]	800	760	5 mM sucrose 2μCi/ml of ^{14}C-sucrose	13.3 vol. 8-24 hours x4	167 vol. 6-20 hours x4	3.3%	0.83

[a] Cytochrome oxidase from batch 2, unfractionated
[b] Cytochrome oxidase from batch 1
[c] Cytochrome oxidase from batch 3

Discussion

The reconstitution of COV was successful with regard to production of vesicles with the characteristic of respiratory control. As was demonstrated in the Results section COV produced by the cholate dialysis, cholate dilution, lysolecithin incorporation, and phosphatidyl serine incorporation techniques all showed stimulation of respiratory rate by uncouplers. Because the COV made by the dialysis technique possessed the highest RCR's, consistent with reports by other workers (Carroll and Racker, 1977), these preparations were used in most of the experimental work. Dialyzed preparations would theoretically have the higher membrane potential and pH gradient formation. The variations in buffer solutions and cytochrome oxidase concentrations and fractionation procedures as detailed earlier were used to optimize the RCR's of the COV. The electrophoresis results demonstrated that the cleaner fractions of cytochrome oxidase produced the better RCR's, probably secondary to minimization of proton leakage due to incorporation of contaminating proteins in the preparations. The oxidative activity of the preparations also correlated with the purity, which would be expected.

The development of methods for reconstitution of large-volume COV did not prove to be useful for the investigations of oxidative phosphorylation and calcium transport which were undertaken later and will be described. However the technique does demonstrate that functional activity of two membrane associated enzyme systems can indeed be reconstituted in large-volume vesicles (cytochrome oxidase and the hydrophobic protein catalyzing the ATP-$^{32}P_i$ exchange reaction), and provides

an alternative method of proteoliposome reconstitution which may prove to have usefulness in other types of experimental investigation of reconstituted systems.

The extremely small size of the COV and other proteoliposomes has been reported by others (Carroll, Racker, 1977; Eytan and Broza, 1978; Ruben, Telford, Carroll, 1976). The experiments related to development of separatory techniques for proteoliposomes clearly show the difficulty of using the conventional techniques of filtration and centrifugation. Furthermore recent studies have shown that cytochrome oxidase is actually incorporated in a small fraction (only about 20%) of the total amount of phospholipid vesicles. This has been demonstrated by fractionation of COV on ficoll density gradient centrifugation. This work also indicates that the smaller diameter vesicles are the population associated with the oxidase (Carroll, Racker, 1977; Eytan, Broza, 1978a). This means that separatory methods such as filtration would tend to be the most ineffective with the most important fraction of the vesicles. Calculation of internal concentrations of transported substances is also rendered inaccurate because of the selective incorporation of the enzyme in a subpopulation of the suspension of vesicles.

II. EFFECTS OF IONOPHORES ON COV

Introduction

The ionophore valinomycin (Brockman, Schmidt-Kastner, 1956) is known to be an uncharged cyclic polypeptide which can complex with alkalai metal ions and mediate transmembrane transport in an electrogenic mode (Moore, Pressman, 1964; Pressman, 1965; Mueller, Rudin, 1967; Pressman, Harris, Jagger, Johnson, 1967; Stefanac, Simon, 1967). The cation selectivity for valinomycin has been shown to be $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ (Mueller, Rudin, 1969; Simon, Morf, 1972; Hayden, Hladky, 1972). Gramicidin is another uncharged ionophore which mediates cation transport in an electrogenic mode by channel formation with a stoichiometry of 2 gramicidin molecules per channel (Urban, Hladky, Haydon, 1978). The selectivity of gramicidin has been shown to be $Rb^+ > Cs^+ > K^+ > Li^+ > Na^+$ (Hladky, 1974). It has been noted that uncouplers increase the respiratory rate of COV by dissipation of the membrane potential which is inhibitory to the electron transport process as previously discussed. Experiments have shown that valinomycin in the presence of potassium increases the respiratory rate of RSO energized COV (Racker, 1974). This was supposed to be due to the dissipation of the membrane potential by inward diffusion of potassium. The combination of valinomycin and nigericin is more effective than valinomycin alone, presumably because the exchange of potassium and protons allowed by nigericin causes breakdown of the pH gradient in addition to membrane potential dissipation (Racker, 1974). Uncouplers such as CCCP also cause marked stimulation of the respiratory rate and addition of valinomycin increases this somewhat.

This has been explained as due to dissipation of the pH gradient by the CCCP and dissipation of the membrane potential by valinomycin in the presence of potassium (Racker, 1972; 1974; Skulachev, 1974a). Because of this other workers have routinely used the combination of valinomycin plus a protonophoric uncoupler for the measurement of RCR's in COV (Earroll, Racker, 1977). However, the rate of oxidation is constant after addition of valinomycin, which would not be expected if inward diffusion were the sole mechanism because after equilibrium were reached the membrane potential should be re-established and the initial oxidation rate resumed. Furthermore since in COV the membrane potential results from net proton pumping, dissipation of the proton gradient by protonophoric uncouplers should eliminate both the membrane potential and the pH gradient. Thus there is some question as to the validity of the model proposed for the uncoupling of COV by valinomycin.

The addition of valinomycin to a cellular, organelle, or vesicular system followed by measurement of K^+ or Rb^+ distribution is a common technique for measurement of membrane potentials (Rottenberg, Scarpa, 1974; Puskin, Gunter, Gunter, Russell, 1976; Rottenberg, 1975; Azzone, Bragadin, Pozzan, DellAntone, 1977). It is important that the mechanism of action of valinomycin be understood for these measurements to be meaningful. Data will be discussed later which indicates that there are specific stimulatory effects of valinomycin and gramicidin on cytochrome oxidase which account for some of the findings mentioned above.

Results

It was noted that the increased rate of oxidation of COV in the presence of valinomycin and K^+ is constant and does not return to a lower rate after time for K^+ equilibration has occurred, as it should as long as electrons continue to be transported. It was found that the valinomycin-mediated stimulation of COV oxidation occurred not only when the $[K^+]$ was greater outside the vesicles (as would be expected if the breakdown of membrane potential due to K^+ influx is the mechanism), but also occurred when the $[K^+]$ was greater inside the COV or when no K^+ was present at all. This data is summarized in Table 9. The amount of stimulation is variable between different batches of cytochrome oxidase and preparations of vesicles, but is always present.

Uncouplers presumably stimulate the rate of respiration of COV by breaking down the proton gradient. Since the membrane potential and pH gradient are both produced by the net transport of protons (Skulachev, 1974b) and the vesicles are quite impermeable to most ions (Racker, 1974), dissipation of the proton gradient by a protonophore should eliminate both membrane potential and pH gradient across the membrane. Therefore, addition of valinomycin and K^+ should have little or no effect in the presence of CCCP. If any stimulation would occur, it would have to be due to a slightly positive (internally) diffusion potential resulting from the inward transport of K^+, which would quickly dissipate when equilibrium is reached. However as is illustrated by Table 10 the stimulation of respiration occurred in the presence of CCCP and is independent of the direction of flux of K^+. The stimulated rates are also con-

Table 9. STIMULATION OF COV RESPIRATION BY VALINOMYCIN

Formation buffer	Assay buffer	Initial respiration rate nmole O_2/min/μg protein	Respiration rate after addition of valinomycin nmole O_2/min/μg protein	% stimulation
K_2SO_4	Na_2SO_4	1.63	2.44	49.7%
Choline-Cl	Choline-Cl	3.34	3.71	11.1%
K_2SO_4	Choline-Cl	1.30	2.33	79.2%
Na_2SO_4	Choline-Cl	1.36	1.99	46.3%
* Na_2SO_4	Na_2SO_4	1.61	2.90	80.1%
* Na_2SO_4	Na_2SO_4	0.91	1.31	44.0%
* Na_2SO_4	Na_2SO_4	1.07	1.45	35.5%
* Na_2SO_4	Choline-Cl	1.29	1.81	40.3%
# K_2SO_4	Na_2SO_4	0.83	3.92	472.3%
# K_2SO_4	Na_2SO_4	1.26	5.19	411.9%

* Cytochrome oxidase from unfractionated batch 2
\# Cytochrome oxidase from batch 2, fraction III

COV were made in 10 mM HEPES plus the salt indicated under Formation buffer (at 40 mM concentration) at pH 7.0. The dialysis technique was used to prepare the vesicles. .025-.05 ml of the dialyzed vesicle suspension was assayed for oxygen consumption in a volume of 3.2 ml of 10 mM HEPES (pH 7.0) plus the salt (at 40 mM concentration) indicated under Assay buffer above. The assay medium also contained 23 mM Tris-ascorbate and .75-1.5 mg of cytochrome c. Vesicles assayed in choline-Cl medium were dialyzed against this medium prior to assay for 4 hours to remove external Na^+ or K^+. After obtaining the initial respiration rate 1 μg of valinomycin in .01 ml of ethanol was added to obtain the stimulated rate of respiration.

Table 10. STIMULATION OF COV RESPIRATION BY VALINOMYCIN IN THE PRESENCE OF CCCP

Cytochrome oxidase	Formation buffer	Assay buffer	Respiration rates nmole O_2/min/μg protein Initial	+CCCP	+Valinomycin	% Stimulation
batch I	Na_2SO_4	Na_2SO_4	3.90	6.62	6.73	1.0%
	K_2SO_4	K_2SO_4	1.24	3.96	4.53	14.4%
	K_2SO_4	Choline-Cl	1.15	4.23	5.18	22.5%
	Na_2SO_4	Choline-Cl	-	4.15	4.50	8.4%
batch 2, fraction I	Na_2SO_4	Na_2SO_4	1.85	7.41	10.76	45.2%
	K_2SO_4	K_2SO_4	2.03	12.16	18.48	52.0%
batch 2, fraction III	K_2SO_4	Na_2SO_4	0.97	5.01	8.20	63.7%
batch 3	Na_2SO_4	Na_2SO_4	0.81	3.81	4.05	6.3%
	Na_2SO_4	Na_2SO_4	0.73	4.66	5.25	12.7%

COV were made in 10 mM HEPES (pH 7.0) plus 40 mM of the indicated salt at a cytochrome oxidase concentration of 417 μg/ml by the dialysis method. .025-.05 ml of COV was added to 3.2 ml of assay solution containing 40 mM of the appropriate salt, 10 mM HEPES, .75-1.5 mg of cytochrome c, and 23 mM Tris-ascorbate (pH 7.0). After obtaining the initial respiration rate .01 ml of a 20 mM ethanol solution of CCCP was added and the uncoupled respiration rate determined. Finally .01 ml of ethanol containing 1 μg of valinomycin was added to obtain the stimulated rate of respiration. The % stimulation was taken as the percentage increase in the valinomycin stimulated rate over the CCCP stimulated rate.

stant with time and do not show dissipation. Stimulation occurred in the complete absence of K^+, or when the $[K^+]$ was greater inside the vesicles than outside. Because of the lack of dependence of the stimulatory effects of valinomycin on COV on the flux of K^+ it is unlikely that these stimulatory effects are due to the dissipation of the membrane potential.

A suspension of cytochrome oxidase mixed with phospholipids in an oxygen electrode chamber does not show respiratory control. This is shown in Table 11. However valinomycin stimulates the rate of oxidation of substrate. This occurred in the absence of K^+ or in the presence of lytic amounts of detergent such as Tween-20. The stimulation did not occur in the absence of added phospholipid. Furthermore the order of addition of the ingredients has an effect in that the phospholipids must be added after the oxidase for the stimulation to be observed.

As is shown in Table 12, the stimulatory effect of a constant amount of valinomycin is greater as the cytochrome oxidase concentration of the suspension decreases. This occurs in the presence of a constant amount of phospholipid. The amount of stimulation appears to be related to the relative amounts of protein and valinomycin present. Table 15 shows increasing stimulatory effect with increasing amounts of valinomycin over a 1000 fold concentration range. The effect is present to a lesser extent in the absence of K^+.

Table 13 shows the effects of various other ionophores on the respiration of COV. Gramicidin stimulates COV in the absence of K^+ or Na^+. However this stimulation is probably largely due to transport of protons

Table 11. STIMULATION OF CYTOCHROME OXIDASE BY VALINOMYCIN

$[K^+]$ mM	[Tween-20] % (v/v)	[Phospho-lipid] mg/ml	Initial respiration rate nmole O_2/min/μg protein	Additions (final concentrations)	Final respiration rate nmole O_2/min/μg prot.	% change in rate
0.0	0.0	0.66	3.65	63 μM CCCP	3.38	-7.4%
0.0	0.0	0.66	3.65	63 μM CCCP + .31 μg/ml valinomycin	4.67	+27.9%
0.0	1.0	0.66	1.68	.31 μg/ml valinomycin	2.09	+24.4%
7.3	0.0	0.66	2.29	.31 μg/ml valinomycin	2.63	+14.8%
7.3	0.0	0.00	2.87	.31 μg/ml valinomycin	2.75	-4.2%

Cytochrome oxidase was prepared as previously described (Koubayama method) and suspended in 40 mM Na_2SO_4, 10 mM HEPES, 5% Tween-20 at pH 7.0 and at a concentration of 2.5 mg/ml. .01 ml of this suspension was added to an oxygen electrode chamber containing 3.2 ml of 40 mM Na_2SO_4, 10 mM HEPES (pH 7.0), 23 mM Tris-ascorbate with .47 mg/ml of cytochrome c and the indicated amounts of KCl, Tween-20, and asolectin. After obtaining the initial respiration rate the substances shown above were added in a volume of .01 ml of 95% ethanol and the change in respiratory rate noted.

Table 12. VALINOMYCIN STIMULATION VERSUS [CYTOCHROME OXIDASE]

[Cytochrome oxidase] μg/ml	Respiration rates nmole O_2/min/μg protein Initial	+ CCCP	+ Valinomycin	% Stimulation
208	4.08	17.96	20.91	16.4%
417	3.21	11.43	12.14	6.2%
833	0.95	4.94	5.20	5.3%

COV were made by the cholate dilution method as previously described in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0) with the indicated concentrations of cytochrome oxidase (batch 2, unfractionated). After 1.5 hours of incubation at 0°C. .05 ml aliquots of the vesicles were assayed for O_2 consumption in the presence of 23 mM Tris-ascorbate and 1.5-3.0 mg of cytochrome c. The uncoupled rate was determined by addition of .01 ml of 20 mM CCCP in ethanol, and the subsequent valinomycin-stimulated rate was obtained by adding .01 ml of ethanol containing 1 μg of valinomycin. The % stimulation was determined from the increase in the valinomycin-stimulated rate over the CCCP-stimulated rate.

Table 13.

These measurements are the average of two experiments.
* Cytochrome oxidase used in these experiments was from batch 2, unfractionated.

COV were made in 10 mM HEPES plus the indicated salt (40 mM) as previously described. The pH was 7.0, the cytochrome oxidase concentration (batch 1) 417μg/ml, and formation was according to the cholate dialysis method. .025 ml of vesicles was assayed for respiration in 10 mM HEPES, 23 mM Tris-ascorbate (pH 7.0) plus 40 mM of the indicated salt. The assay mixture had a final volume of 3.2 ml and contained .75 mg of cytochrome c. After determination of the initial respiration rate .01 ml of a 20 mM ethanolic solution of CCCP was added and the uncoupled rate obtained where indicated. Subsequently, .01 ml of an ethanolic solution of the indicated ionophore (in an amount such as to give the final concentration shown above) was added and the % change in the respiration rate was noted. COV formed in Na^+ or K^+ containing buffer and assayed in choline-Cl containing buffer were dialyzed for 4 hours against the choline-Cl containing buffer prior to assay to remove external Na^+ or K^+.

Table 13. STIMULATION OF RESPIRATION OF COV BY VARIOUS IONOPHORES

Ionophore (final concentration)	Formation buffer	Assay buffer	Rates of respiration nmole O_2/min/μg protein			% change in rate
			Initial	+ CCCP	+ Ionophore	
Gramicidin						
3.1 μg/ml	Choline-Cl	Choline-Cl	3.22	-	4.68	+45.3%
3.1 μg/ml	Choline-Cl	Choline-Cl	4.26	5.92	6.23	+5.2%
*3.1 μg/ml	Na_2SO_4	Na_2SO_4	0.55	5.33	7.39	+38.6%
0.31 μg/ml	Choline-Cl	Choline-Cl	3.49#	-	4.51#	+29.2%
0.31 μg/ml	K_2SO_4	Choline-Cl	0.91	3.70	4.39	+18.6%
0.31 μg/ml	Na_2SO_4	Choline-Cl	1.32#	3.89#	4.54#	+16.7%
Nigericin						
0.31 μg/ml	Choline-Cl	Choline-Cl	3.62	-	3.57	-1.4%
0.31 μg/ml	Choline-Cl	Choline-Cl	3.25	5.28	5.28	0.0%
0.31 μg/ml	K_2SO_4	Choline-Cl	1.34	2.26	2.04	-10.8%
*0.78 μg/ml	Na_2SO_4	Na_2SO_4	0.96	5.24	4.72	-11.0%
X537A						
*3.1 μg/ml	Na_2SO_4	Na_2SO_4	0.73	-	0.93	+27.4%
*3.1 μg/ml	Na_2SO_4	Na_2SO_4	-	4.74	4.78	+0.8%
A23187						
*3.1 μg/ml	Na_2SO_4	Na_2SO_4	0.84	-	1.00	+19.0%
*3.1 μg/ml	Na_2SO_4	Na_2SO_4	-	4.74	4.70	-1.7%
Ca^{++} ligand						
*15.4 μg/ml	Na_2SO_4	Na_2SO_4	0.54	1.88	1.38	-26.6%
*15.4 μg/ml	K_2SO_4	K_2SO_4	0.67	3.32	3.09	-6.9%

by the nonspecific gramicidin (Myers, Haydon, 1972). When CCCP is used to break down the proton gradient the stimulatory effect is much less but is still present. Significant stimulation also occurs when there is internal K^+ or Na^+ but only choline externally. Therefore the stimulatory effects of gramicidin are also independent of ion fluxes.

In the absence of K^+, or with greater internal than external $[K^+]$, nigericin does not stimulate respiration of COV. X537A stimulates COV respiration only in the absence of CCCP, implying that it is in this case acting as a protonophore. In the presence of CCCP, significant stimulation does not occur. A23187 also shows this property of stimulation only in the absence of CCCP and probably acts the same way. The Ca^{++}-ligand of Vuilleumier, Gazzoti, Carafoli, and Simon (1977) does not stimulate respiration but is somewhat inhibitory.

Because valinomycin has been used to determine membrane potentials in mitochondria it is significant that there are specific stimulatory properties of valinomycin on cytochrome oxidase, as this could alter the measurements considerably. However valinomycin was not found to have any stimulatory effects on mitoplasts as depicted in Table 14. The fact that there is stimulation of COV energized so as to have an internally positive membrane potential (using PMS-ascorbate and internal cytochrome c) further indicates that breakdown of membrane potential is not the mechanism of action of valinomycin in COV.

Table 14. COMPARISON OF VALINOMYCIN-STIMULATED RESPIRATION IN COV, INSIDE-OUT COV, AND MITOPLASTS

Preparation	Rates of respiration nmole O_2/min/μg protein				
	Initial	+ CCCP	+ 1 μg valinomycin	+ 10 μg valinomycin	% Stimulation
COV (right side out energization)	2.03	12.16	-	18.48	52.0%
COV (inside out energization)	0.91	0.94	-	1.00	6.4%
.35 mg of mitoplasts	0.13	0.55	0.55	0.55	0.0%
.25 mg of mitoplasts	0.09	0.52	0.52	-	0.0%

COV were made by the dialysis method, with 417 μg/ml of cytochrome oxidase (batch 2, fractionI). Inside-out energized vesicles were made in the presence of 1.5 mg/ml of cytochrome c. Mitoplasts were made by subjecting rat liver mitochondria to digitonin treatment as described in the METHODS section to remove the outer membranes. COV were made and assayed in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0), with 23 mM Tris-ascorbate and either 1.5 mg of cytochrome c or, for inside out energization, 80 μg of protamine and 1.0 μg of PMS. Mitoplasts were assayed in 135 mM mannitol, 45 mM sucrose, 24 mM HEPES, .5 mM sodium succinate (pH 7.2) containing 1.5 mg of cytochrome c and 23 mM Tris ascorbate. Uncoupled rates were obtained by adding .01 ml of 20 mM CCCP, and valinomycin was subsequently added in .01 ml of ethanol to obtain the stimulated rates.

Table 15. VARIATION IN VALINOMYCIN STIMULATION OF COV WITH [VALINOMYCIN]

$[K^+]$ mM	Initial respiration rate nmole O_2/min/μg protein	Valinomycin added final concentration in ng/ml	Valinomycin-stimulated respiration rate nmole O_2/min/μg protein	% Stimulation
7.3	3.93	0.3	4.15	5.6%
7.3	3.93	3.4	4.30	9.4%
7.3	3.93	34.2	4.52	15.0%
7.3	3.93	342.2	4.78	21.6%
0.0	3.70	30.8	4.00	8.1%
0.0	3.70	338.8	4.05	9.5%
0.0	3.70	3418.8	4.20	13.5%

COV were made by the dialysis technique in 40 mM choline-Cl, 10 mM HEPES (pH 7.0) with 417 μg/ml of cytochrome oxidase (batch 1). .05 ml of the vesicle suspension was added to 3.2 ml of 40 mM choline-Cl, 10 mM HEPES (pH 7.0) containing 1.5 mg of cytochrome c, 23 mM Tris-ascorbate, and the indicated concentration of KCl. After obtaining the initial respiration rate, the appropriate amount of valinomycin was added in .01 ml of ethanol and the stimulated rate of respiration was determined.

Discussion

Previous explanations of the stimulatory effect of valinomycin on the respiratory rate of COV have assumed the direction of the K^+ flux to be inward with resultant collapse of the membrane potential initiating the stimulation. While this may be partly correct, such a mechanism would require a return to the initial oxidation rate after equilibrium is re-established. This fact has been generally overlooked in the past. Investigations carried out here have demonstrated a lack of dependence of the stimulatory effects on the K^+ flux direction. Indeed the stimulation was found to occur in the complete absence of K^+, under conditions when the K^+ flux would be outward instead of inward, and in solubilized cytochrome oxidase where no membrane potential production would be possible. It is noteworthy that the stimulation occurs in the presence of CCCP which dissipates the protonmotive force. Therefore it is concluded that the stimulatory effects of valinomycin on COV respiration are not solely related to the collapse of the membrane potential by a K^+ flux. Others have noted greater stimulation of respiration by the combination of valinomycin and CCCP than by CCCP alone and have stated that this is due to the fact that the valinomycin dissipates the membrane potential while the CCCP dissipates the pH gradient. Since the membrane potential and pH gradient may be both a result of net proton pumping it would seem that CCCP could dissipate both without the need for valinomycin to dissipate the membrane potential.

The stimulation observed with valinomycin was generally greater in the presence of K^+ than when no K^+ was present. It is also interesting that the stimulation occurred with batch 1 (extracted with only K^+ salts)

as well as with batches 2 and 3 (extracted only with Na^+ salts). The decrease in the stimulatory effect of valinomycin with increasing concentrations of cytochrome oxidase supports the idea of valinomycin-protein interaction.

The similar stimulatory effects of gramicidin with complete lack of such effects by nigericin suggest that the effect may be common to electrogenic K^+ ionophores, even though they operate by different ionophoric mechanisms. It has been suggested that cytochrome oxidase may contain an ionophoric polypeptide with K^+ specificity (Kessler, Blondin, VandeZande, Haworth, Green, 1977; Blondin, Kessler, Green, 1977). The effects of valinomycin and gramicidin may be related to the interaction with or substitution for this ionophore. It is also known that preparations of cytochrome oxidase contain significant amounts of bound phospholipid (Jost, Capaldi, Vanderkooi, Griffith, 1973; Marsh, Watts, Maschke, Knowles, 1978). The ionophores may allow greater accessibility of either bound or external K^+ to a catalytic site within the oxidase which is shielded by the phospholipids. The lack of effects of valinomycin on mitoplasts or (from some recent work completed by Dr. T. Gunter) mitochondria indicates that measurements of membrane potentials using valinomycin may not necessarily be invalid, but the specific effects of valinomycin on the oxidase should be taken into account in these instances. Furthermore the routine use of the combination of CCCP plus valinomycin for measuring RCR's in COV by many researchers is probably not indicated and should be re-examined in the light of these data. The lack of stimulatory effects in intact mitochondria may be related to the location of the rate-limiting step in the oxid-

ation process. While the oxidation by cytochrome oxidase is rate-limiting in the COV case, if this is not the case in mitochondria stimulation by valinomycin would not be expected. Furthermore interaction with the many other proteins present in the mitochondrial membrane may alter or prevent the interaction with these ionophores.

III. RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION

Introduction

The actual mechanism of the coupling of phosphorylation to substrate oxidation is not yet known. However there are at present three basic groups of theories which have been advanced. They are the chemical intermediate, conformational and chemiosmotic coupling theories. The chemical intermediate theory was the earliest and was set forth by Lipman in 1946 and later modified by Slater (1953) and others (Skulachev, 1972). According to this idea a reduced electron carrier combines with an unknown chemical intermediate which forms a high energy phosphate bond upon oxidation, the energy of this bond subsequently being transferred to the binding of phosphate to ADP to form ATP. The conformational theory proposed by Boyer (1964; 1974) suggests that redox energy is transferred into conformational changes in proteins which is subsequently transferred to the formation of the bond of phosphate to ADP when relaxation of the strained conformation occurs. The chemiosmotic theory of Mitchell (1961; 1966) proposes that coupling of oxidation and phosphorylation is through the protonmotive force established by a proton gradient which results from the anisotropic arrangement of the redox chain in the mitochondrial membrane (Skulachev, 1972;Racker, 1975). This last theory requires coupling of oxidation to phosphorylation to occur only in closed membrane-bound systems in which a membrane potential can be developed, and experimental evidence supports this concept (Racker and Kandrach, 1973; Sone, Yoshida, Hirata, Kagawa, 1977). Reconstitution of the third site of oxidative phosphorylation in vesicles has been achieved by co-recon-

stitution of cytochrome oxidase and the mitochondrial ATPase system (Racker and Kandrach, 1971; 1973; Kagawa, 1972). The protonmotive force generated by cytochrome oxidase appears to be responsible for the coupling of oxidation and phosphorylation. Similar results have been obtained with protonmotive forces generated in reconstituted vesicles with bacteriorhodopsin plus the ATPase system (Racker, Stoeckenius, 1973).

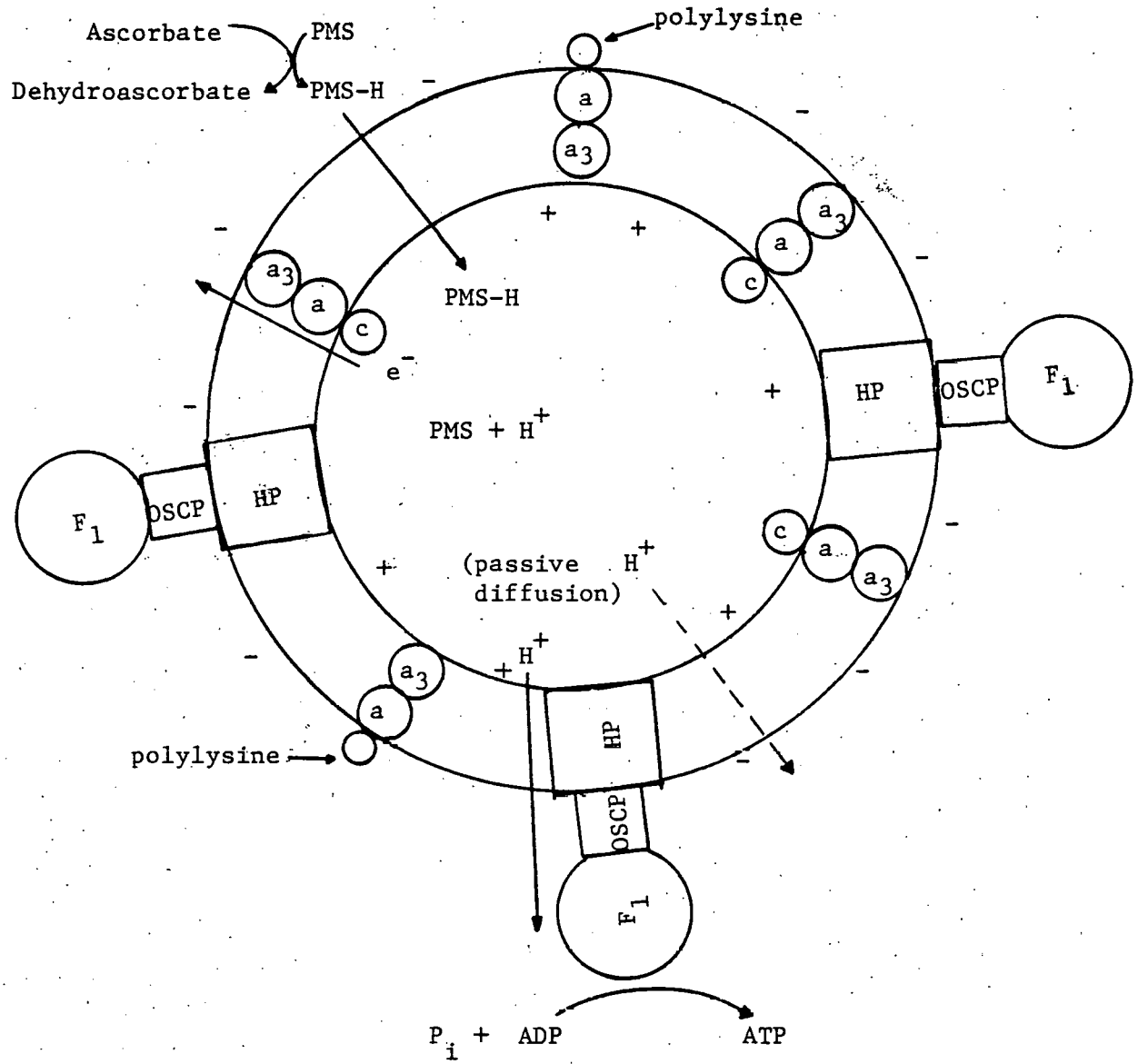
The exact stoichiometry of the number of protons translocated through the ATPase per ATP molecule synthesized is a subject of controversy, with numerous recent reports indicating that the original number of 2 protons per ATP proposed by Mitchell (1966) may be inadequate. Current experimental evidence indicates that probably 3 or 4 protons are transported per ATP synthesized (Alexandre, Reynafarje, Lehninger, 1978; Reynafarje, Lehninger, 1978; Brand, Lehninger, 1977). This would allow oxidative phosphorylation to occur with lower ambient membrane potentials than originally proposed (Mitchell, 1966).

One of the major problems associated with the reconstituted systems of oxidative phosphorylation is the low P:O ratios which have been obtained (Racker, Kandrach, 1973). This makes evaluation of the quantitative importance of the proton gradient in the coupling process rather difficult. The scheme proposed for the operation of the reconstituted system of oxidative phosphorylation in PCOV and the location of the component parts is shown in Figure 13.

To date the system has been reconstituted only in the ISO orientation, though current work is being carried out toward development of a mitochondrially-oriented system (Banerjee, Racker, in press). Quan-

Figure 13.

PCOV.



titation of the protonmotive force in a reconstituted system of ATPase has produced results consistent with the chemiosmotic theory (Kagawa, 1977). However the mechanism of oxidative phosphorylation remains controversial.

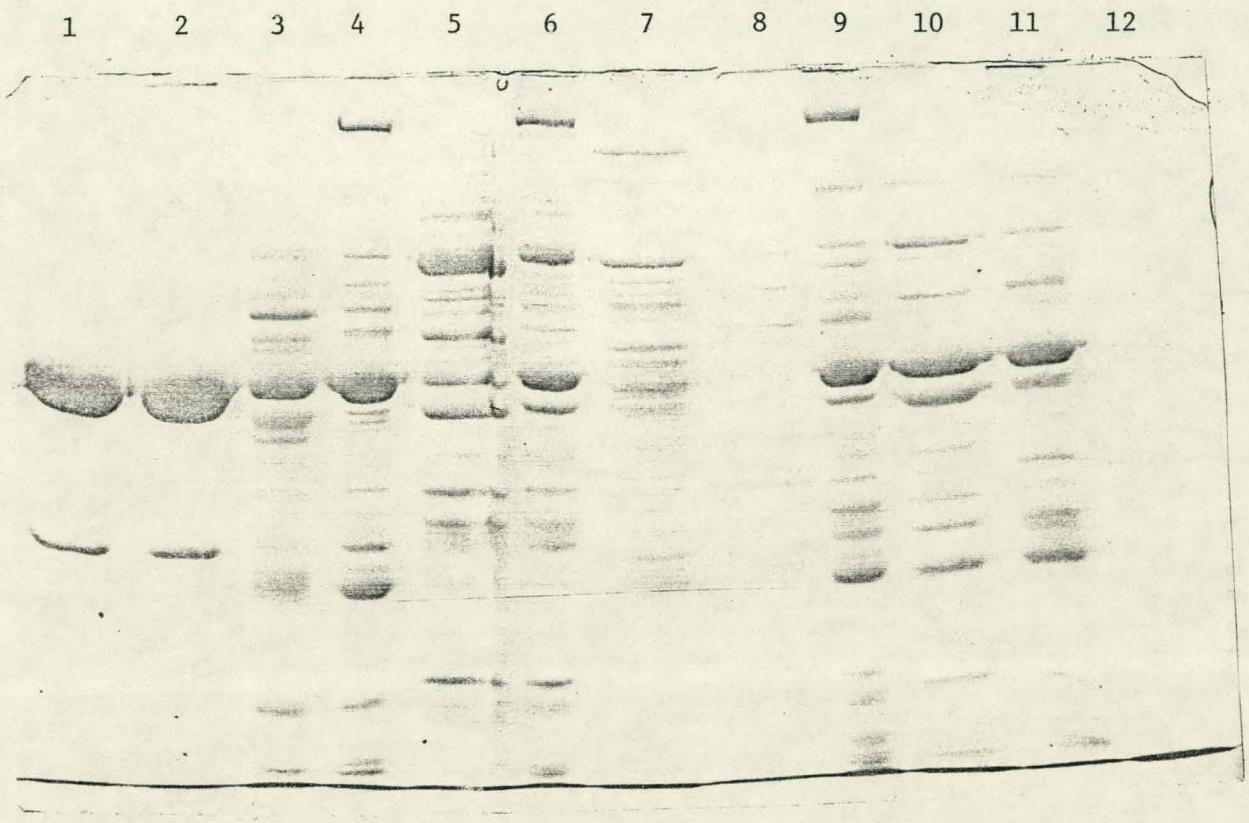
Investigations of a reconstituted system of the third site of oxidative phosphorylation has led to the development of reconstituted ion transport systems. However, resolution of the problem of the mechanism of coupling of oxidation to phosphorylation will have to await the development of techniques for reconstitution of the oxidative phosphorylation system with much more efficient coupling of the two enzymatic processes.

Results

The various components for the reconstitution of the third site of oxidative phosphorylation were extracted from beef heart according to the procedures outlined in the METHODS section. Figure 14 shows the polyacrylamide gel electrophoresis pattern of various mitochondrial proteins. It is apparent that the subunits of F_1 are common to several of the protein complexes : "OSCP", hydrophobic protein, complex V, adenine nucleotide transporter, and phosphate transporter. Since all the protein extracts are hydrophobic protein fractions, some overlap of the components would be expected. It is also noted that the hydrophobic protein and complex V preparations were fairly similar in electrophoretic patterns.

In order to allow rapid quantitation of the ATPase activity of the various protein preparations, a simple ATPase assay was developed as is detailed in the METHODS section. Basically the phosphate assay of Chen Toribara, and Warner (1956) was used to form a reduced (colored) phosphomolybdate complex. The reaction was stopped by the extraction of the complex into isobutanol, where the $O.D._{820}$ could be read to quantitate the amount of phosphate. This simple detection of phosphate cleaved from ATP was used to judge the ATPase activity. Relatively large amounts of substrate were used so that the reaction would be fairly constant for a short period of time. However the build-up of ADP would rapidly inhibit the reaction. This is shown in Figure 15. Because of this limitation of the assay, the enzyme was incubated for short periods of time only. Table 16 gives the ATPase activity of some of the mitochondrial protein extracts. The ATPase activity of the hydrophobic protein and complex V

Figure 14. GEL ELECTROPHORESIS OF VARIOUS MITOCHONDRIAL PROTEINS



1 - F_1 (batch 1)
2 - F_1 (batch 2)
3 - OSCP (batch 2)
4 - Hydrophobic protein (batch 2)
5 - Complex V (from beef heart, batch 1)
6 - Complex V (from beef heart, batch 2)
7 - Complex V (from rat liver, fraction I)
8 - Complex V (from rat liver, fraction II)
9 - Adenine nucleotide transporter (fraction I)
10 - Adenine nucleotide transporter (fraction II)
11 - Phosphate transporter
12 - cytochrome c

Polyacrylamide slab gel electrophoresis was done as described in the METHODS section. The gel contained 10% acrylamide and approximately 40 μg of each of the above protein fractions was used per well. The protein fractions were extracted from beef heart except where otherwise indicated, and all extractions were performed as outlined in the METHODS section.

Figure 15. Time Dependence of the ATPase Assay

200 μg of F_1 was incubated for the indicated length of time at room temperature in 2.2 ml of 20 mM Tris-SO_4, 11.4 mM $MgSO_4$, and 9.1 mM Na-ATP at pH 7.5. The reaction was terminated by addition of .05 ml of 50% trichloroacetic acid and centrifugation in a JA-20 rotor at 10,000 rpm for 10 minutes. .5 ml of the supernatant was added to 7.5 ml of 1% ascorbic acid, .25% ammonium molybdate and .6 N H_2SO_4 and incubated at room temperature for 30 minutes. The solution was extracted with 4 ml of isobutanol and the absorbance of the isobutanol phase read at 820 nm and compared with controls containing known amounts of P_i to quantitate the P_i cleaved from ATP. Samples were corrected for spontaneous nonenzymatic hydrolysis of ATP. The points shown represent the average for two experiments.

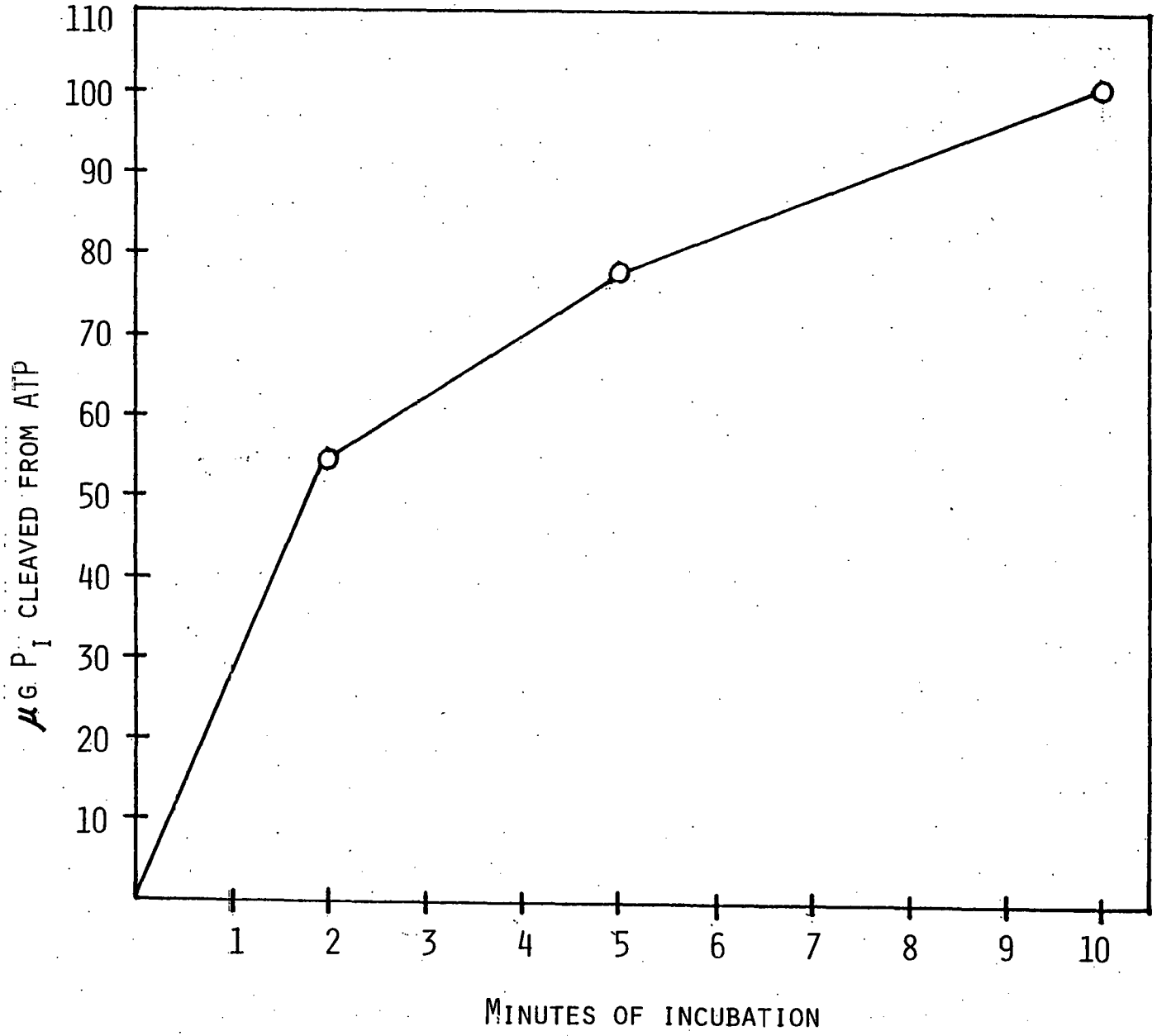

110
100
90
80
70
60
50
40
30
20
10
μG P_I CLEAVED FROM ATP
1
2
3
4
5
6
7
8
9
10
MINUTES OF INCUBATION

Table 16. ATPase Activity of Various Proteins

Protein	Rate of ATP hydrolysis μmole/mg/min
Hydrophobic protein (1)	.18
Hydrophobic protein (2)	2.0
Hydrophobic protein (1) + 250 μg asolectin	.75
F_1 (1)	22.2
F_1 (2)	15.7
F_1 (1) + 6 μg oligomycin	23.0
F_1 (1) + 20 μg OSCP (1)	19.0
F_1 (1) + 20 μg OSCP (1) + 6 μg oligomycin	17.0
Complex V (from beef heart (1))	.25
Complex V (from beef heart (1)) + 250 μg asolectin	.25
Complex V (from beef heart (1)) + 250 μg asolectin + 6 μg oligomycin	.25
Complex V (from beef heart (2))	0.0
Complex V (from rat liver, fraction I)	0.0

20 - 50 μg of the appropriate protein was incubated at room temperature for 1.5 - 2.5 minutes in 1.0 ml of 20 mM Tris-SO_4, 5 mM $MgSO_4$, and 5 mM Na-ATP at pH 7.6. The reaction was terminated by the addition of .1 ml of 50% trichloroacetic acid. After centrifugation at 0°C. .5 ml of the supernatant was added to 7.5 ml of 1% ascorbic acid, .25% ammonium molybdate, .6 N H_2SO_4 and incubated at room temperature for 15 minutes. The solution was extracted with 4 ml of isobutanol and the absorbance of the isobutanol phase was read at 820 nm and compared with standards to allow quantitation of the P_i which had been cleaved from ATP. The number in parentheses after each protein indicates the batch number of the protein.

is quite low compared to the purified ATPase. The OSCP was found to confer a slight degree of oligomycin sensitivity to F_1. The OSCP from batch 1 was insoluble, however, and might have contained some phospholipids which could have resulted in some fraction of membrane-bound ATPase which would be sensitive to oligomycin.

Having found the ATPase to be functional, the next phase of the reconstitution was to reconstitute ATP-$^{32}P_i$ exchange. Table 17 summarizes the reconstitution of the ATP-$^{32}P_i$ exchange. It can be seen that the coupling factors F_1 and OSCP stimulated the exchange. The ATP-$^{32}P_i$ exchange was completely sensitive to oligomycin. The hydrophobic protein fraction from batch 1 was not effective in the reconstitution of the exchange reaction. Later this batch of hydrophobic protein was incorporated into COV. According to Racker (1972), this should result in loss of respiratory control which can be restored by rutamycin or oligomycin. The restoration of respiratory control is due to blockage of the oligomycin-sensitive proton pore. Batch 1 of hydrophobic protein did not confer an oligomycin-sensitive loss of respiratory control to COV. Because of this a second batch of hydrophobic protein was extracted and found to be effective in the reconstitution of the ATP-$^{32}P_i$ exchange reaction. The temperature at which the assay was run was found to have a large effect on the rate of exchange as can be seen from the Table. Hydrophobic protein obtained from Dr. E. Racker did not produce as high a rate of exchange as the second batch made in this laboratory.

Large-volume ATP-$^{32}P_i$ exchange vesicles were also reconstituted using the lysolecithin incorporation technique described earlier. This was done as a test of the compatibility of the phospholipid mixture used

Table 17. Reconstitution of ATP-$^{32}P_i$ exchange

Type of vesicles	HP[a]/sample	[HP]	Added coupling factors F_1	Added coupling factors OSCP	Incubation temperature	ATP-$^{32}P_i$ exchange nmole/minute/mg HP
HP vesicles[b]	155 μg	3.9 mg/ml	-	-	30°C.	36.5 ± 0.4 (2)
HP vesicles[b]	155 μg	3.9 mg/ml	30 μg[c]	-	30°C.	78.6
HP vesicles[b]	155 μg	3.9 mg/ml	30 μg[c]	55 μg	30°C.	113.8 ± 2.8 (2)
HPvesicles[b] + 12 ng oligomycin	155 μg	3.9 mg/ml	30 μg[c]	55 μg	30°C.	0.0
HP vesicles	193 μg	3.9 mg/ml	21 μg	55 μg	30°C.	188.9 ± 5.7 (4)
Large-volume HP vesicles	80 μg	0.6 mg/ml	11 μg	25 μg	30°C.	9.8 ± 1.0 (2)
HP vesicles	170 μg	4.3 mg/ml	14 μg	28 μg	23°C.	18.3
HP vesicles[d]	170 μg	4.3 mg/ml	14 μg	28 μg	23°C.	11.6
PCOV	30 μg	5.2 mg/ml	14 μg	28 μg	23°C.	11.3
PCOV[d]	30 μg	5.2 mg/ml	14 μg	28 μg	23°C.	4.1

[a] HP = hydrophobic protein

[b] P_i in these samples was 25 μmole instead of 15 μmole.

[c] F_1 in these samples was from batch 1.

[d] HP for these experiments was obtained from E. Racker, Cornell University.

Table 17.

Vesicle Formation:

HP vesicles - Asolectin was sonicated to clarity at a concentration of 28.9 mg/ml in 4 mM Tris-SO_4 .2 mM EDTA, .4 mM $MgSO_4$, .2 mM dithiothreitol, and 1% (w/v) sodium cholate at pH 7.5. HP from batch 2 was added to give the final concentration shown above. The suspension was then incubated at 0°C. for 3 hours.

Large-volume HP vesicles - An ether solution of 9:1 asolectin:lysolecithin at 1.4 mg/ml was injected into 4 mM Tris-SO_4, .2 mM EDTA, .4 mM $MgSO_4$, .2 mM dithiothreitol (pH 7.5) at 38°C. at a rate of about .8 ml/minute under vacuum as described in the METHODS section.The final phospholipid concentration in the suspension was approximately 8 mg/ml. After addition of the appropriate amount of HP the suspension was incubated for 3 hours at 0°C.

PCOV (phosphorylating cytochrome oxidase vesicles) - A 4:1 mixture of mitochondrial phosphaditylethanolamine: phosphatidylcholine was sonicated to clarity at a phospholipid concentration of 40 mM in 4 mM Tricine-KOH, 16 mM $(NH_4)_2SO_4$, .8 mM dithiothreitol, .1 mM EDTA, 20 mM sucrose, and 1.8% (w/v) sodium cholate at pH 8.0. Cytochrome oxidase (batch 2, fraction III) was added to a final concentration of .5 mg/ml and cytochrome c to a final concentration of 2 mg/ml. After addition of HP to a concentration of 5.2 mg/ml the suspension was dialyzed overnight against 160 volumes of 10 mM Tricine-KOH, 1 mM dithiothreitol, .2 mM EDTA and 10% (v/v) methanol at pH 8.0, with one change of buffer after 6 hours. Defatted BSA was added to the suspension to a final concentration of 16 mg/ml and dithiothreitol to a final concentration of 3 mM, and .5 ml of the suspension was then layered on top of 9 ml of 10 mM Tricine-KOH, 1 mM EDTA, and 10 mM $MgCl_2$ (pH 8.0). The suspension was centrifuged in a Beckman 50 Ti rotor at 50,000 rpm for 90 minutes and the pellet resuspended in 1.6 ml of 10 mM Tricine-KOH, 1 mM EDTA, 1 mM dithiothreitol, and .25M sucrose at pH 8.0.

Assay:

An aliquot of vesicles was added to 1.0 ml of 50 mM KCl, 20 mM Tris-SO_4, 2 mM $MgSO_4$, and .5 mM EDTA (pH 8.0). F_1 (batch 2) and OSCP (batch 2) were added and the mixture incubated at the appropriate temperature for 5 - 10 minutes. Defatted BSA was added to a final concentration of 2.5 mg/ml. The reaction was initiated by addition of .12 ml of .1 M Na-ATP, .1 M $MgSO_4$, and .125 M KP_i (pH 8.0) containing 10^6 - 10^7 cpm of ^{32}P. After 20 minutes the reaction was discontinued by addition of .12 ml of 50% trichloroacetic acid and after centrifugation .3 - .5 ml of the supernatant was added to 1.0 - 1.5 ml of 2.5% ammonium molybdate and .2 - .3 ml of 6N H_2SO_4. After two extractions with isobutanol 1.0 ml of the aqueous phase was counted to quantitate the exchange.

in the large-volume vesicles with the functionality of the proteins. The reconstitution was carried out in the "inside out" mode (F_1 and OSCP on the outside of the vesicle). Because of the difficulty in reconstituting the third site of oxidative phosphorylation at this time, PCOV were also tested for ATP-$^{32}P_i$ exchange and found to be almost as active as hydrophobic protein vesicles assayed under the same conditions.

Although the exchange rates were not quite as high as those reported by Kagawa and Racker (1971), they were reasonably good and the proteins were judged to be functional in phosphorylation. The next phase of the reconstitution was the combination of the COV system with the ATP-$^{32}P_i$ exchange system to form a substrate dependent system of ADP phosphorylation.

Table 18 summarizes some of the reconstitution of oxidative phosphorylation. HP(1) only produced phosphorylating vesicles in one experiment and was later abandoned in favor of the second batch of HP, which consistently produced phosphorylating vesicles, although the rates of phosphorylation were low. The use of mitochondrial phospholipids and hydrophobic protein obtained from Dr. Racker produced no better results than the asolectin and hydrophobic protein from this laboratory. An ethanol extract of asolectin (E. Racker, A. Kandrach, personal communication) was used instead of the crude asolectin and produced slightly better results. In view of the high RCR's attainable with batch 2 of the cytochrome oxidase and the presence of ATP-$^{32}P_i$ exchange in PCOV it seemed likely that two populations of vesicles were present: COV and exchange vesicles, in which both sets of proteins were somewhat segre-

Table 18.

PCOV' were made essentially as detailed in the METHODS section with the modifications detailed in the above table. The indicated phospholipids were sonicated in a cholate solution to clarity and an equal volume of 10 mM Tricine-KOH, 40 mM $(NH_4)_2SO_4$, 2 mM dithiothreitol, .2 mM EDTA, 50 mM sucrose (pH 8.0) was added. Then cytochrome oxidase (batch 2, fraction III), cytochrome c, and hydrophobic protein were added in the appropriate amounts. At this time the suspensions were sonicated at 0°C. for the indicated lengths of time. The suspensions were then dialyzed against 10 mM Tricine-KOH, 1 mM dithiothreitol, 10% (v/v) methanol, .2 mM EDTA, .1 mM ATP (pH 8.0) overnight. The dialyzed suspension was treated to remove external cytochrome c either by passage through a G-150 Sephadex column equilibrated with 10 mM Tricine-KOH, 1 mM dithiothreitol, .2 mM EDTA, .15 M KCl (pH 8.0), or by adding 10 - 20 mg/ml of defatted BSA , layering the suspension on about 10 volumes of .25M sucrose, 10 mM Tricine-KOH, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (pH 8.0), and centrifugation in a 50 Ti rotor at 50,000 rpm for 60 - 90 minutes, followed by resuspension of the pellet in a small volume of .25M sucrose 10 mM Tricine-KOH, 1 mM EDTA, 1 mM dithiothreitol (pH 8.0).

Oxidative phosphorylation was measured using .5 ml of the vesicle suspension, preincubating with appropriate amounts of F_1 and OSCP, and making the parallel measurements of oxidation and phosphorylation in a volume of 3.4 ml as detailed in the METHODS section.

Table 18. RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION

HP type[a]	HP(1)[d]	HP(1)	HP(1)	HP(2)	HP(2)	HP(R)	HP(2)	HP(2)
[HP] - mg/ml	3.9	3.7	3.6	3.9	5.2	5.3	5.3	2.6
[cytochrome c] - mg/ml	1.9	1.8	2.5	2.0	2.0	2.0	2.0	2.2
Phospholipid type[b]	A	A	A	A	A	M	M	EE
[Phospholipid] - mg/ml	46	44	43	47	47	37	37	25
[Cholate] - %	1.3	1.7	1.7	1.8	1.8	1.8	1.8	2.0
[Cholate] - mg/mg HP	3.3	4.7	4.7	4.7	3.5	3.5	3.5	7.8
[Cytochrome oxidase] - μg/ml	390	490	600	520	330	490	490	430
[Cytochrome oxidase] - μg/mg HP	100	133	167	133	82	93	93	167
Sonication time (seconds)	20	none	15	none	30	none	none	45
Method of cytochrome c removal[c]	cent.	cent.	seph.	seph.	cent.	cent.	cent.	cent.
Respiration rate - nmole O/minute	142	34	37	61	225	75	68	322
Phosphorylation rate - nmole ATP/minute	0.0	0.0	2.3	0.0	1.8	0.3	0.7	14.1
P:O	0.0	0.0	0.062	0.0	0.008	0.004	0.010	0.044

[a] HP type = hydrophobic protein from batch 1 - (1), batch 2 - (2), or from E. Racker, Cornell University - (R)

[b] A = soy asolectin, M = 4:1 mixture of mitochondrial phosphatidyl ethanolamine : phosphatidyl choline, EE = ethanol extracted asolectin.

[c] cent. = centrifugation method, seph. = sephadex method

[d] In this experiment .43 mg/ml of F_1 (batch 1) and .36 mg/ml of OSCP (batch 1) were added to the suspension prior to dialysis.

gated. This would explain the presence of ample oxidation rates and functional phosphorylating proteins but miniscule P:O ratios, since the proton gradients of the COV were not driving the ADP phosphorylation reaction in the exchange vesicles. Instead of mixing the proteins in the suspension prior to dialysis a brief sonication was employed to try to"scramble"the proteins so as to allow a more homogeneous distribution. Better P:O ratios were obtained with this technique (compare last two columns of Table 18). Next the amounts of the hydrophobic protein and cytochrome oxidase were varied. The amounts of proteins are quite critical according to Racker and Kandrach (1973). The optimization of the protein concentrations is shown in Table 19. At optimum [HP] and [cytochrome oxidase] the P:O rose to .133. It was also found that using the Sephadex method of cytochrome c removal instead of the centrifugation method gave higher P:O ratios (See Table 20).

The P:O ratios were still not high enough to allow any quantitation of the protonmotive forces to be meaningful. The problem was thought to be still a matter of nonhomogeneity of the protein distribution in the vesicles. It had been shown by Carroll and Racker (1977) that a population of COV can be separated into two separate groups of vesicles: phospholipid vesicles devoid of any cytochrome oxidase and vesicles containing the oxidase. The separation of the two populations was effected by density gradient centrifugation. PCOV were run on a ficoll density gradient as described in the legend of Table 20 in order to separate the denser proteoliposomes (hopefully those containing the cytochrome oxidase plus hydrophobic protein) from the lighter fraction (COV, hydrophobic protein vesicles, or phospholipid vesicles). Three

Table 19. OPTIMIZATION OF [HYDROPHOBIC PROTEIN] AND [CYTOCHROME OXIDASE] IN PCOV

[HP] mg/ml	[Cholate] % - mg/mg HP	[cytochrome c] mg/ml	[cytochrome oxidase] μg/ml - μg/mg HP	Respiration rate nmole O/min.	Phosphorylation rate nmole ATP/ min.	P:O
1.8	1.5 - 8.4	0.9	95 - 52	111	4.0	0.036
3.8	1.5 - 4.0	1.9	190 - 50	121	7.1	0.059
7.2	1.4 - 1.9	2.5	260 - 36	110	2.1	0.019
3.4	1.2 - 3.5	1.5	125 - 35	149	17.0	0.113
3.4	1.2 - 3.5	1.5	250 - 75	177	23.4	0.133
3.4	1.2 - 3.5	1.5	500 - 150	304	29.4	0.097

PCOV were made as previously described with the amounts of cholate, cytochrome c, cytochrome oxidase (batch 2, fraction III), and Hydrophobic protein (batch 2) indicated above. Ethanol extracted soy asolectin was the type of phospholipid used in these experiments and the final concentration was between 24 and 28 mg/ml of suspension. The suspensions were sonicated at 0°C. for 30 - 45 seconds in a bath sonicator prior to dialysis. After dialysis the external cytochrome c was removed by the sephadex technique. After addition of the coupling factors F_1 (batch 2) and OSCP (batch 2) .5 ml aliquots of the vesicle suspensions were assayed for oxidative phosphorylation as detailed in the METHODS section.

Table 20. FICOLL DENSITY GRADIENT PURIFICATION OF PCOV

Cytochrome c removal	Ficoll gradient fraction	Respiration rate nmole O/min.	Phosphorylation rate nmole ATP/min.	P:O
Sephadex	---	53	4.4	.084
Centrifugation	---	106	1.6	.015
Sephadex	3-4% ficoll	86	0.0	.000
Sephadex	5-6% ficoll	111	5.6	.051
Sephadex	7-8% ficoll	98	18.5	.189
*Sephadex	7-8% ficoll	98	20.9	.213

*The ATP added to this sample was increased from 2 μmoles to 20 μmoles at the time of assay for phosphorylation.

PCOV were made as previously described with 26 mg/ml ethanol extracted asolectin, 4.6 mg/ml hydrophobic protein (batch 2), 1.5% sodium cholate, 2.3 mg/ml cytochrome c, 240 μg/ml cytochrome oxidase (batch 2 fraction III). The suspension was sonicated in a bath sonicator for 45 seconds prior to dialysis. External cytochrome c was removed from one aliquot by the centrifugation method previously described and from another aliquot by the Sephadex method. P:O ratios were then measured on the two aliquots in the usual manner. 7 ml of the Sephadex treated suspension was layered on top of a linear density gradient containing 10 mM Tricine-KOH, 50 mM sucrose, .5 mM EDTA, and 1 mM dithiothreitol (pH 8.0). The volume of the gradient was 32 ml and the ficoll concentration ranged from 0% at the top of the tube to 20% at the bottom. The gradient was centrifuged in a JA-20 rotor at 19,000 rpm for 36 hours at 0°C. Three bands were visible after centrifugation and were removed using J-shaped Pasteur pipets. Each fraction of the gradient was assayed for oxidative phosphorylation in the usual manner.

bands were visible on the ficoll gradient. The upper most band was pink in color and located at 3-4% ficoll; the middle band was yellow-orange, and was located at 5-6% ficoll; and the lower band was yellowish and slightly turbid, located at 7-8% ficoll. The P:O ratios of the various fractions are shown in Table 20. The lower band had by far the most phosphorylation coupled to oxidation. The upper band had oxidative activity but no phosphorylation. This was consistent with the idea that the heavier vesicles contained both proteins.

However despite all the variables which were optimized in the reconstitution of the third site of oxidative phosphorylation, the highest attainable P:O ratio was 0.213, which was still not high enough to allow meaningful quantitation of the role of protonmotive force in oxidative phosphorylation.

Attempts were made to produce a right side out (mitochondrially oriented) reconstituted system of oxidative phosphorylation. This was undertaken because of the availability of cation ionophores, which would allow quantitation of the membrane potential from the Nernst distribution after cation uptake had reached steady state values in respiring vesicles. Cation uptake would only occur in vesicles with an inside negative membrane potential which would be produced by the cytochrome oxidase in a mitochondrially oriented system.

Because of the large diameter of F_1 (90 Å) compared to the inside diameter of the COV made by conventional techniques (200-400 Å), inclusion of adequate amounts of F_1 inside these vesicles to reconstitute oxidative phosphorylation was felt to be sterically unfavorable. For this reason these experiments involved the use of the large-volume lipo-

somes previously discussed. The fact that both ATP-$^{32}P_i$ exchange and cytochrome oxidase activity with respiratory control were reconstituted in the large-volume liposomes indicated that the proteins were compatible with the lysolecithin-asolectin phospholipid membrane system of the large-volume liposomes. Several experiments were carried out with the large-volume liposomes in which F_1 and OSCP were placed in the vaporization chamber at the time of formation of the vesicles, with external proteins subsequently being removed by centrifugation. After this, hydrophobic protein and cytochrome oxidase were added to the lysolecithin-containing vesicles and incorporated by overnight incubation. In some experiments $^{32}P_i$, hexokinase, glucose, and ADP were included at the time of vesicle formation (so that the substrates for ATP formation and assay were retained inside the vesicles with the F_1), and in other experiments the phosphate transporter and adenine nucleotide transporter (isolated as previously described) were incorporated along with the hydrophobic proteins and the cytochrome oxidase so that the substrates for phosphorylation could be added externally and the ATP formed could also be measured externally. However despite variation in buffers, amounts of protein, and phospholipids no phosphorylation coupled to oxidation was obtained in any of these experiments. More recently it has been found by Banerjee and Racker (in press) that ATPase incorporation into the interior of phospholipid vesicles requires a very alkaline pH (about 10) at the time of vesicle formation.

Although the experiments leading to the reconstitution of oxidative phosphorylation did not produce a system which was tightly coupled enough to allow quantitation of the protonmotive forces involved in oxidative

phosphorylation, it did produce a technology for formation of proteoliposomes which could produce and maintain protonmotive forces across their membranes in a controlled fashion. This seemed to be an ideal system for the study of ion transport in vesicles. The only impediment to using cytochrome oxidase and/or ATPase vesicles as ion transporting systems was the difficulty of separating the vesicles from the suspending medium to quantitate the transport. Because of this problem a technique was developed which allowed rapid and complete separation of proteoliposomes from suspending media.

Discussion

The original goal of the investigative efforts presented in this dissertation was examination of the role of the protonmotive force in a reconstituted system of oxidative phosphorylation. The experimental work was directed at production of functional vesicles coupling oxidation and phosphorylation of ADP and at methods of measurement of the pH gradients and membrane potentials developed by these systems. Most of the methods of measurement of protonmotive force depended on the separation of the vesicles from the suspension media, and it was for this reason that the physical characteristics of the vesicles and various separatory techniques were investigated to such a great extent.

The development of functional cytochrome oxidase vesicles represented the first step in the process of the reconstitution of oxidative phosphorylation. The methods of Racker and Kandrach (1973) were generally followed in this work. The protein extractions of F_1, OSCP, and HP were in general successful and the ATP-$^{32}P_i$ exchange reaction was reconstituted without difficulty. However when the oxidative and phosphorylating systems were co-reconstituted the results were generally poorer than those obtained by previous workers. The introduction of sonic mixing of the proteins prior to dialysis greatly improved the results, as did ficoll gradient separation of the vesicles to enrich the population of vesicles containing both proteins. Despite multiple preparations of proteins and the use of preparations from Dr. E. Racker P:O ratios greater than about 0.2 were not obtained. Since the reconstitution is so strongly dependent on the incorporation of both oxida-

tive and phosphorylating enzymes in the same vesicles, and the proteins when assayed separately seemed to be adequately functional, it appears that the difficulty was in the co-reconstitutive process. The use of purified mitochondrial phospholipids instead of asolectin did not significantly improve the co-reconstitution. The reason for the lack of success with the technique is not clear. It is possible that a difference in the type of sonicators used was responsible, though the use of several different sonicators did not appear to make a difference. Even though the proteins possessed adequate individual functional capabilities it is possible that subtle differences in the extraction procedures caused alterations which interfered with co-reconstitution. Certainly the OSCP was not extracted as a pure protein (as judged from gel electrophoresis patterns) though since this was added at the time of assay it probably did not affect the reconstitution process. However, the HP preparations also contained impurities when compared with previous published results (Serrano, Kanner, Racker, 1976), and this may have caused the faulty co-reconstitution.

Other difficulties were subsequently encountered which would have made measurements of the protonmotive force difficult in any event. One of these is the previously mentioned tendency of the proteins to incorporate into only a fraction of the phospholipid vesicles. This makes volume determinations for calculation of accumulated concentrations of ions quite inaccurate. Also calibration of dicarbocyanine dyes for measurement of internally positive membrane potentials in the anticipated range (50-200 mV) was found to be difficult and inaccurate (Dr. A. Waggoner,

personal communication). A method of measurement using the lipophilic anion tetraphenyl boron was tried but required precipitation of the TPB from the solutions, which was found to be experimentally impossible at the low concentrations which had to be used to prevent uncoupling. (R. Rosier, unpublished observations). Preliminary measurements of membrane potentials in COV were made using equilibration of Rb^+ in the presence of very low concentrations of valinomycin (lower than those which cause a stimulatory effect on respiration) with ion exchange columns to remove external cation. Calculations gave membrane potentials in the range of 40 mV (based on the use of the total internal volume of the suspension and corrections for both external and internal binding), for a number of different experiments. Because of the uncertainty of the internal volume of the active fraction of the suspension the actual membrane potential may have been somewhat higher, although these results compare favorably with the results of others (Drachev et al, 1974; 1976). Reconstitution of RSO (mitochondrially oriented) PCOV was unsuccessful, however, as previously described in the METHODS section.

Recently a system of measurement of membrane potentials using sensitive SCN^- and NO_3^- electrodes has been introduced for internally positive membrane potentials (Kell, John, Sorgato, Ferguson, 1978). Such a system would be readily applicable to the measurement of membrane potentials in oxidative phosphorylation if the difficulties of co-reconstitution and volume determination could be overcome.

The development of functional membrane potential generating systems of proteoliposomes did provide a vehicle for the study of ion transport in simplified reconstituted systems. The method of measurement of ATPase

activity which was used for assay of various preparations of ATPase was another useful development of the investigations of oxidative phosphorylation. This assay has advantages over previously used assays (Kagawa, Racker, 1966; Pullman, Penefsky, Datta, Racker, 1960) in that it does not require the use of radioactive $^{32}P_i$, is inexpensive, and allows for rapid processing of many samples conveniently. The primary drawback of this method is the dependence of the assay on the incubation time. For the experimental purposes of this work, relative amounts of ATPase activity were of more concern than absolute numbers, and since all samples were incubated for the same lengths of time, this was not a problem. A subsequent improvement in the technique was developed which circumvents this difficulty. A lower concentration of ATP is used in conjunction with an ATP regeneragint system (phosphoenolpyruvate plus pyruvate kinase) to ensure a constant ATP concentration without time dependence. With this modification the ATPase assay is more accurate and applicable to the determination of absolute rather than relative ATPase activity.

IV. AGGREGATION OF VESICLES WITH BASIC POLYPEPTIDES

Introduction

The study of ion transport in reconstituted vesicular systems requires methods of separating the vesicles from the suspending medium after the transport has taken place so that it can be quantitated. COV have been shown to be extremely small in size, ranging from 250-1000 Å in diameter (Ruben, Telford, Carroll, 1976; Racker, 1972). Filtration with conventional filtration techniques often gives satisfactory results where the vesicle diameter is larger than the pore diameter of the filter. However the amount of vesicular material which can be separated by filters of small pore size is limited because of clogging of the filters and decreases with decreasing pore size. In addition it is often desirable to work with vesicles whose diameter is less than the smallest available pore size for conventional filters. Since it has been shown that the cytochrome oxidase in COV associates with the smallest vesicles (Carroll, Racker, 1977; Eytan, Broza, 1978b) filtration or centrifugation techniques run the risk of losing a significant fraction of the active vesicles even when most of the vesicles are recovered in the pellet or on the filter. With small vesicles centrifugation is a slow technique and rapid time points cannot be resolved.

Ion exchange columns have been used for quantitation of ion uptake in vesicles (Gasko, Knowles, Shertzer, Suolinna, Racker, 1976). The disadvantages of this technique are (1) retention of some of the particles in the columns (2) time consuming and expensive set-up of equipment for processing reasonable numbers of samples (3) interference with

measurements by any substances which are used in the samples which bind significant amounts of the ions (4) lack of usefulness of the technique for quantitation of the transport of nonionized substances (5) minimum time resolution of 45 to 60 seconds (6) dilution of samples so that very small amounts of ion transport are measured with poor accuracy.

The aggregration of vesicles with basic polypeptides such as protamine and polylysine provides a method of separation which is rapid, inexpensive, complete, and versatile. The method is effective for use in combination with either centrifugation or filtration, and can be used for a wide variety of natural and artificial membranes.

Results

It was necessary to achieve separation of vesicles in order to measure membrane potentials and ion transport in SMP and proteoliposomes. Ion exchange columns were used in early experiments, but recovery of vesicles was found to be only 60-90% (R. Rosier, unpublished observations), and quite variable. Furthermore in the presence of highly charged proteins such as polylysine which are used in the selective energization of SMP and COV with PMS and ascorbate, even higher percentages of the vesicles are lost due to retention in the columns of ion exchange resin. When substances such as ATP are included in the samples during divalent cation transport large amounts of cation are not removed by the resin and result in very high background activities observed in the eluant solution. Setting up and reconverting the resins to the appropriate ionic form is time consuming, and processing a sample takes a minimum column transit time of about 1 minute, which does not allow resolution of shorter transport times.

For these reasons ion exchange resins were not found to be satisfactory as a separatory technique. The results discussed earlier indicate that centrifugation and filtration of proteoliposomes is impractical because of their extremely small size. However it was noted that when basic polypeptides such as polylysine were added to suspensions of proteoliposomes the turbidity of the suspensions greatly increased, and with large amounts of polylysine macroscopically visible aggregrates of the particles appeared.

It was also noted that the light scattering of a suspension of vesicles was essentially directly proportional to the concentration of

the vesicles in the suspension, particularly at low concentrations. Figure 16 shows a plot of the optical density of various suspensions of vesicles versus concentration. Aggregration of vesicles with basic polypeptides produced suspensions of particles which were readily filterable using conventional Millipore filtration techniques. Since the objective was removal of as many vesicles from the suspensions as possible (optimally greater than 95%), monitoring of optical density of the filtrates provided an easy and rapid assay for the presence of unfiltered particles. Obviously the size of the particles increases with aggregration so that the optical density is not a quantitative measure of the amount of unfiltered vesicles. However since the end point of the procedure is complete removal of vesicles and aggregation increases the effective size of the light scattering particles (thereby increasing the O.D. of the suspension), the sensitivity of the light scattering measurements of residual particles in the filtrates is actually increased by the aggregration. Thus measurements of the optical density of the filtrates provides a sensitive measure of the completeness of the filtration.

Figure 17 and 18 show the optical density of filtrates of suspensions of vesicles as a function of the amount of polylysine added. There is an optimum amount of polylysine which causes essentially complete removal of aggregated vesicles by a .45μ Millipore filter with a glass prefilter to prevent clogging. This optimum amount of polylysine is slightly different for each type of vesicle tested, as can be seen from the Figures. At larger amounts of polylysine the light scattering of

Figure 16. OPTICAL DENSITY OF SUSPENSIONS OF VESICLES VERSUS CONCENTRATION

SR-vesicles, RBC-vesicles, COV , SMP , and Liposomes were prepared as described in the METHODS section. The protein concentration of the SR-vesicles, RBC-vesicles, and SMP was measured by the biuret technique . The vesicles were suspended in the following buffers: SR-vesicles, .25 M sucrose, 10 mM Tris, 1 mM histidine (pH 8.0); RBC vesicles, 9 mM NaP_i (pH 7.4); SMP , MSH (pH 7.2); COV and liposomes, 40 mM K_2SO_4, 10 mM HEPES (pH 7.0). The phospholipid concentration of the liposomes and COV was 40 mg/ml. Suspensions of the particles were diluted to the appropriate concentrations of protein or phospholipid and the $O.D._{500}$ was read on a Beckman DU-2 spectrophotometer (or the $O.D._{470}$ for COV and liposomes).

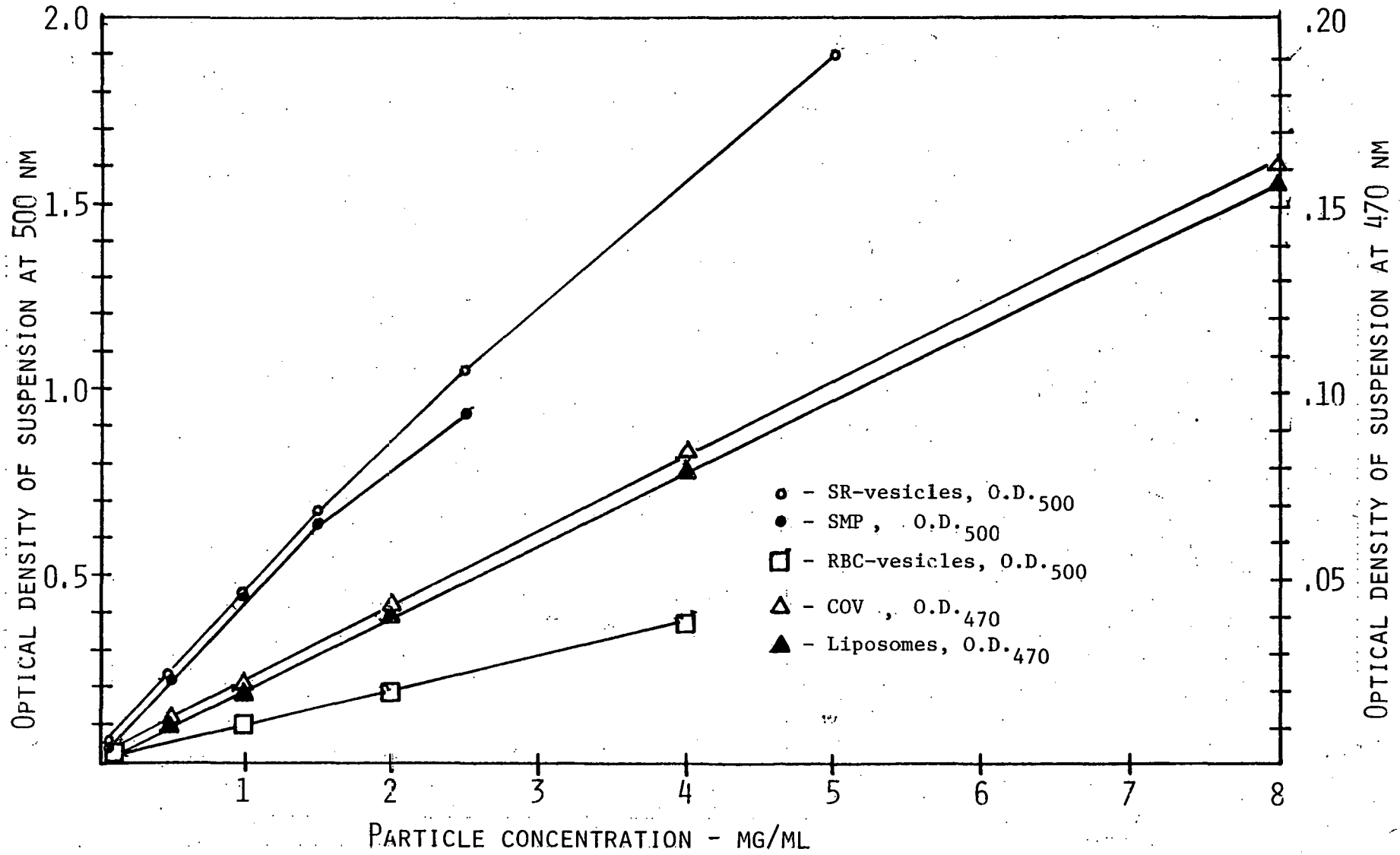

2.0
1.5
1.0
0.5
.20
.15
.10
.05
1
2
3
4
5
6
7
8
OPTICAL DENSITY OF SUSPENSION AT 500 NM
OPTICAL DENSITY OF SUSPENSION AT 470 NM
PARTICLE CONCENTRATION - MG/ML
- SR-vesicles, O.D.500
- SMP , O.D.500
- RBC-vesicles, O.D.500
- COV , O.D.470
- Liposomes, O.D.470

Figure 17. OPTICAL DENSITY OF FILTRATES OF SR-VESICLE, SMP, AND RBC-VESICLE SUSPENSIONS WITH VARYING AMOUNTS OF POLYLYSINE

SR-vesicles, SMP and RBC-vesicles were prepared as previously described and suspended in the media given in the legend of Figure 16. 2 ml samples were made containing 5 mg of SMP or SR-vesicle protein, or 4 mg of RBC-vesicle protein in the appropriate buffer. Varying amounts of polylysine were also included in each sample (MW 70,000). After several minutes of incubation at room temperature the samples were filtered through a .45 μEH type Millipore filter with a glass prefilter, and the optical density of the filtrate was read at 500 nm.

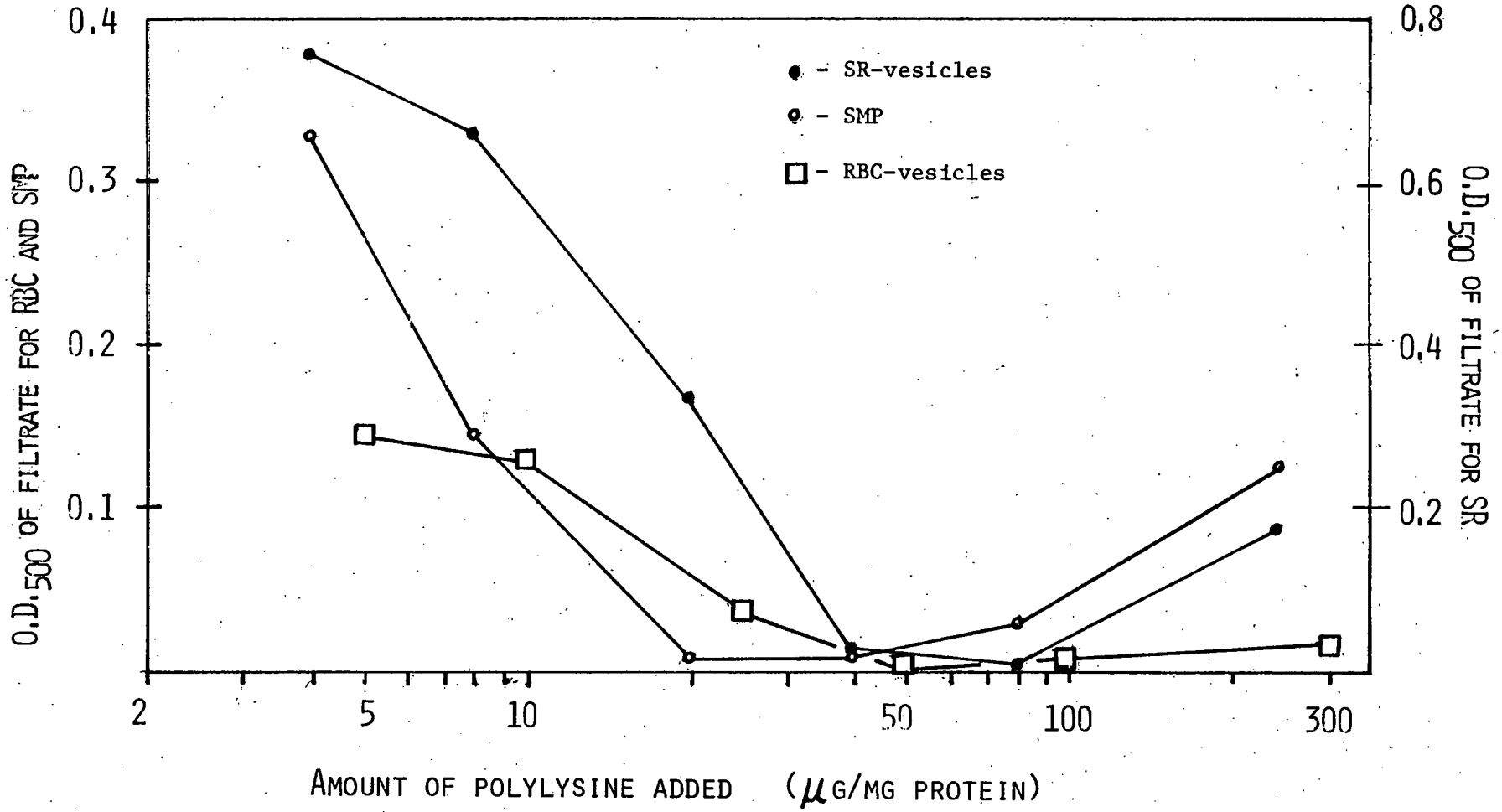
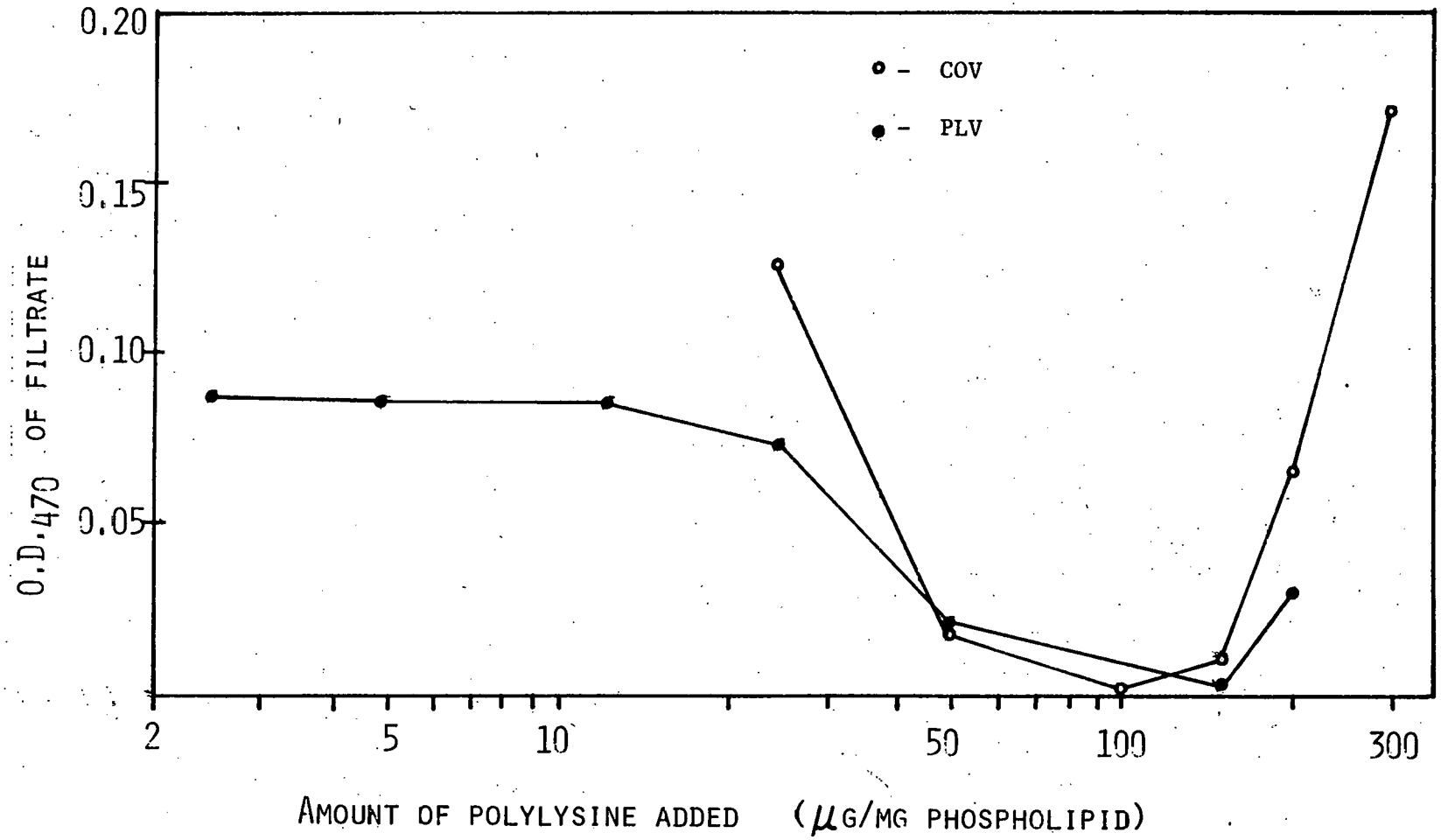

0.4
0.3
0.2
0.1
0.8
0.6
0.4
0.2
2
5
10
50
100
300
● - SR-vesicles
○ - SMP
□ - RBC-vesicles
O.D.500 OF FILTRATE FOR RBC AND SMP
O.D.500 OF FILTRATE FOR SR
AMOUNT OF POLYLYSINE ADDED (μG/MG PROTEIN)

Figure 18. OPTICAL DENSITY OF FILTRATES OF COV AND LIPOSOME SUSPENSIONS WITH VARYING AMOUNTS OF POLYLYSINE

COV and liposomes (PLV) were made as previously described in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0). The cholate dialysis procedure was used for the formation of the vesicles. The cytochrome oxidase was from batch 2 (unfractionated) and was at a final concentration of 417 μg/ml in the COV suspension. 8 mg of vesicles (phospholipid weight) was placed in 2 ml aliquots of buffer containing varying amounts of polylysine (MW 70,000). After incubation for several minutes at room temperature, the suspensions were filtered through a .45μ EH type Millipore filter and the optical density of the filtrates were read at 470 nm.

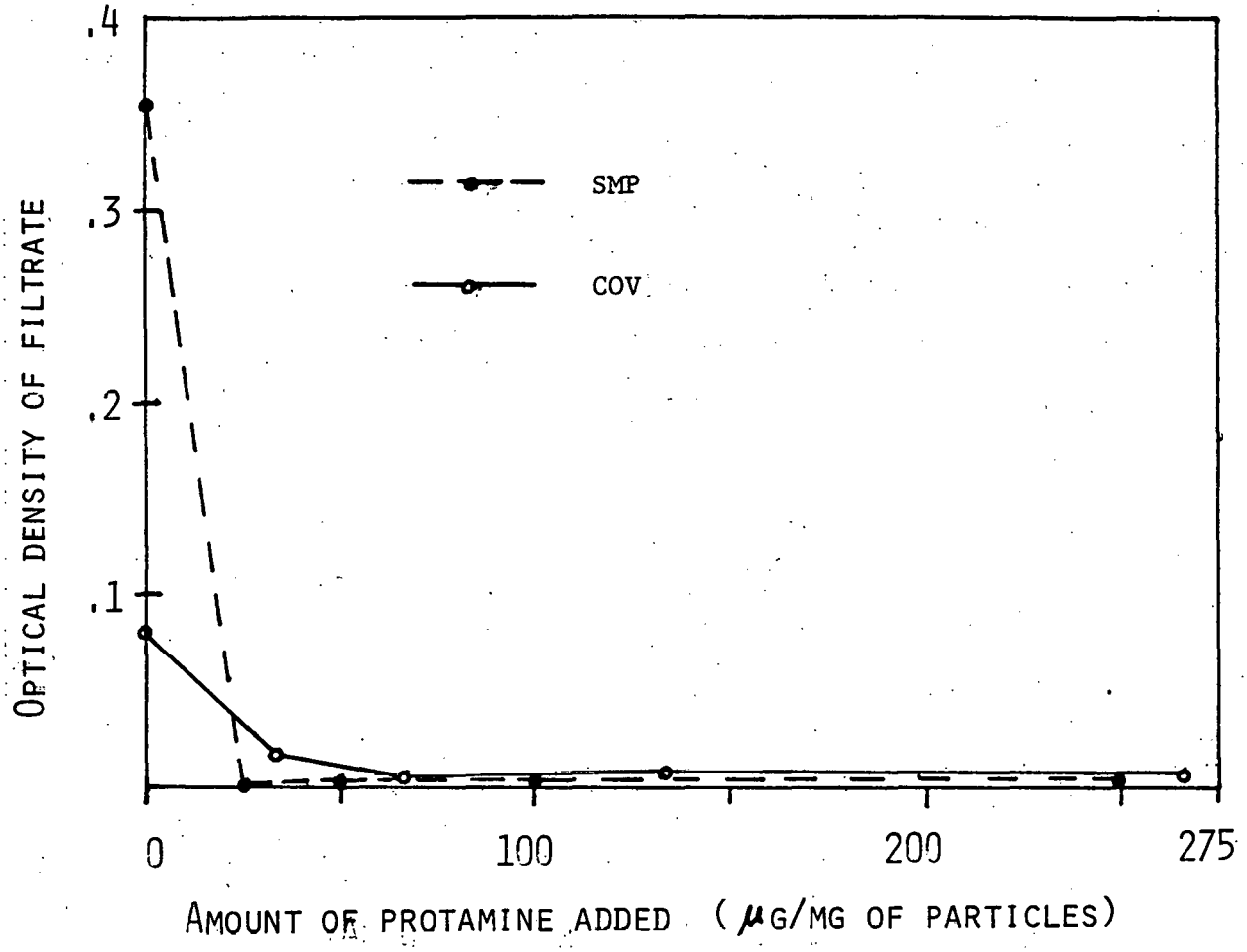

0.20
0.15
0.10
0.05
O.D.470 OF FILTRATE
o - COV
● - PLV
2
5
10
50
100
300
AMOUNT OF POLYLYSINE ADDED (μG/MG PHOSPHOLIPID)

the filtrate begins to increase again, implying that the passage of particles through the filters occurs. It is possible that the excess of polylysine results in more binding of the highly charged polylysine molecules to individual vesicles with less bridging of polylysine between binding sites on different vesicles. This would explain the decrease in effectiveness of aggregation with larger amounts of polylysine. Similarly, too little polylysine would not bind enough of the vesicles together and result in incomplete aggregation. The optimum amount of polylysine for a given suspension of vesicles can thus readily be determined from light scattering. It can be seen from Figure 18 that vesicles such as COV and liposomes which contain a greater proportion of phospholipid and less protein require relatively more polylysine to attain maximum aggregation. Protamine was also found to be effective as an aggregating polypeptide for vesicle filtration. The filtration of protamine-aggregated vesicles as a function of protamine concentration is shown in Figure 19. Excess protamine in contrast to polylysine does not appear to impair aggregation of the vesicles.

The aggregation of the vesicles begins immediately upon addition of the precipitating polypeptide and proceeds rapidly so that within seconds macroscopically visible aggregates are present. Table 21 shows the O.D. of a suspension of COV at various time points after addition of an aggregating amount of polylysine. It can be seen that the aggregation is instaneous and is complete within 30 seconds.

Table 22 shows the results of filtration of SMP with varying amounts of polylysine of high and low molecular weight and protamine. In addition to monitoring the optical density of the filtrates the actual protein

Figure 19. OPTICAL DENSITY OF FILTERED SMP AND COV SUSPENSIONS WITH VARYING AMOUNTS OF PROTAMINE



SMP and COV were made by the previously described techniques. SMP were suspended in MSH (pH 7.2) and COV were made by the dialysis method and suspended in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0). 6 mg of COV or 4 mg of SMP was diluted in the appropriate buffer to a final volume of 2 ml. Each sample contained protamine in the amount shown above. After incubation at room temperature for several minutes the suspensions were filtered through a .45μ EH type Millipore filter with a glass prefilter and the O.D. of the filtrate was read. The SMP filtrates were read at 500 nm and the COV filtrates at 470 nm.

Table 21. POLYLYSINE AGGREGATION OF COV AS A FUNCTION OF TIME

Sample	Incubation time	O.D.$_{450}$ of filtrate
Control (No polylysine)	--	.047
Control (10 μg/mg polylysine; no additional polylysine before filtration)	-	.012
90 μg/mg polylysine	t = 0	.008
90 μg/mg polylysine	t = 5"	.008
90 μg/mg polylysine	t = 15"	.006
90 μg/mg polylysine	t = 30"	.000

COV were prepared by the cholate dialysis technique in the usual manner. .1 ml of the COV suspension (4 mg of phospholipid) was added to 2.3 ml of 40 mM K_2SO_4, 10 mM HEPES, 4 mM $MgSO_4$ (pH 7.0) containing (except in the control above with no polylysine) 10 μg of polylysine (MW 70,000) per mg of vesicles. After 30 minutes of incubation at room temperature, 90 μg of polylysine was added per mg of vesicles (in a volume of .09 ml of water) to each sample (except the control above with no additional polylysine) and after an incubation for the indicated length of time the sample was filtered through a .45 μ EH type Millipore filter with a glass prefilter. The O.D.$_{450}$ of the filtrates was read on a spectrophotometer.

Table 22. OPTICAL DENSITY AND PROTEIN MEASUREMENTS ON FILTRATES OF SMP SUSPENSIONS WITH VARYING AMOUNTS OF PROTAMINE AND POLYLYSINE

	Protamine or polylysine concentration μg/mg SMP	Protamine		Polylysine (MW 3400)		Polylysine (MW 70,000)	
		$O.D._{450}$	[protein] μg/ml	$O.D._{450}$	[protein] μg/ml	$O.D._{450}$	[protein] μg/ml
(A)	0	1.790	945	1.790	945	1.790	945
	10	.940	403	.019	54	.005	52
	25	.007	39	.005	33	.005	28
	50	.008	35	.010	37	.195	138
	100	.008	36	.012	58	1.070	383
(B)	6.7	1.630	1090	.304	175	.128	126
	10	.125	103	.016	51	.016	61
	20	.008	25	.008	18	.000	16
	40	.003	10	.004	8	.007	10
	80	.004	10	.007	12	.017	14

Table 22.

SMP were made as described and suspended in LSS. In part (A) SMP were suspended at a final concentration of 2 mg/ml in 2 ml final volume of MSH containing the indicated amounts of protamine, 3400 MW polylysine or 70,000 MW polylysine. After several minutes of incubation at room temperature the suspensions were filtered through two glass prefilters and the O.D. of the filtrates were read at 450 nm. An aliquot of SMP was diluted to concentrations between 1 and 100 μg/ml in 25 mM KP$_i$ containing .1% SDS. The fluorescence of the suspensions (excitation 280 nm, emission 330 nm) was measured and a standard linear plot of SMP protein versus fluorescence obtained. Aliquots of the filtrates were then assayed for fluorescence to determine the protein concentration.

In part (B) the procedure was similar except that 40 μg of protamine or the appropriate MW polylysine was added to each of a series of samples. SMP were then added in varying amounts to give the polypeptide/SMP protein ratios shown. After filtration the O.D. and protein concentrations of the filtrates were determined as above.

content was measured using calibrated fluorescence as described in the legend of the table. It can be seen that the O.D. of the filtrates correlates well with the actual amount of SMP protein passing through the filters. Again the existence of an optimum amount of polylysine is evident above which the filtration is less complete. These data show the lack of this effect with protamine over the concentration range employed. Furthermore the second part of this table illustrates that the amount of polypeptide needed to bring about effective aggregation does not depend on the concentrations, but rather the relative amounts of particles and polypeptides. Approximately the same minimum amount of polypeptide is required per mg of particles regardless of whether the amount of polypeptide is held constant and the amount of particle concentration varied or the particle concentration held constant and the polypeptide concentration varied. The absolute particle and polypeptide concentrations in part (A) and part (B) of Table 22 at maximum filter retention of SMP differ by a factor of about 2-4 while the relative amounts of polypeptide and particles (μg/mg) at which optimum filtration occurs are about the same.

Table 23 shows the removal of oxidative activity of SMP by Millipore filtration with and without added polylysine. It can be seen that removal of the particles by the filter as judged from the presence of oxidation is only partial without polylysine, but that with added polylysine (at an amount which was judged to be optimal from the O.D. measurements) filtration is complete.

The presence of such strongly charged polypeptides in membrane

Table 23. OXIDATIVE TEST OF POLYLYSINE FILTRATION OF SMP

Sample	Respiration rate nmole O_2/minute
Control	51
Control + polylysine	54
Filtrate (no polylysine)	37
Filtrate (+ polylysine)	0

SMP were made from rat liver mitochondria as detailed in the METHODS section. After removal of external cytochrome c by the centrifugation method 100 μg of SMP protein was placed in an oxygen electrode containing 3.1 ml of MSH with 24 mM Tris-ascorbate, 65 μM CCCP, 3.9 μM antimycin A, 2 μg PMS, pH 7.2. After obtaining the respiration rate ± 130 μg of polylysine, parallel aliquots were filtered through a .45μ Millipore filter with a glass prefilter and the filtrates analyzed for respiration rate.

aggregation brings up the issue of disruption of the integrity of the membranes by the polypeptides. Figure 20 shows uptake of Ca^{++} in SMP with both RSO and ISO energization. The particles were removed from the suspension to quantitate the uptake either by the use of protein-binding HATF-type Millipore filters or by protamine aggregation and filtration with nonbinding filters. The protamine technique results in retention of even more of the transported Ca^{++} than the other method, so lytic effects do not appear to be reducing the amount of retention of Ca^{++} with the aggregration technique.

Table 24 shows filtration and centrifugation of COV which were labelled with internally trapped ^{14}C-sucrose. The polypeptide was incubated with the particles for varying lengths of time prior to filtration or centrifugation of the suspension to determine whether leakage of the label was induced by aggregation. The data show that protamine produces the most effective particle aggregation without appreciable lysis with time. Polylysine appears to induce some lysis of the vesicles with retention of only 60-70% of the internal label in the centrifuged pellet or on the filter.

Flow dialysis monitoring of leakage of ^{14}C-sucrose is shown in Figure 21. There appears to be significant leakage induced by polylysine, whereas the leakage due to protamine is not much more than control until after several minutes of exposure to the polypeptide have occurred. Since aggregation is complete in 15-30 seconds the vesicles can be filtered and washed before significant lytic effects occur.

Table 25 shows filtration of vesicles of varying phospholipid composition. The data indicate that the presence of acidic phospholipids is

Figure 20. COMPARISON OF CALCIUM UPTAKE IN SMP FILTERED WITH PROTAMINE FILTRATION AND WITH PROTEIN-BINDING FILTERS.

SMP were made from rat liver mitochondria as described in the METHODS section. The particles were suspended in MSH after removal of external cytochrome c by centrifugation. Samples consisted of 3.4 mg of SMP in 2 ml final volume of MSH plus 882 nmole/mg of Ca^{++} with 1 μCi of $^{45}Ca^{++}$ (added last), 4 mM ADP, 4 mM $MgSO_4$, 50 mM Tris-ascorbate, 3 μM antimycin A, and either 5 μg PMS (for Inside-out energization) or 3 mg of cytochrome c (for Right side out energization). After incubating for an appropriate length of time with agitation to insure adequate oxygenation of the samples, the sample was either filtered through a .45μ HATF type protein-binding Millipore filter with glass prefilter, or mixed with 200μg of protamine and then filtered through a Millipore .45μ EH type Millipore filter with glass prefilter.

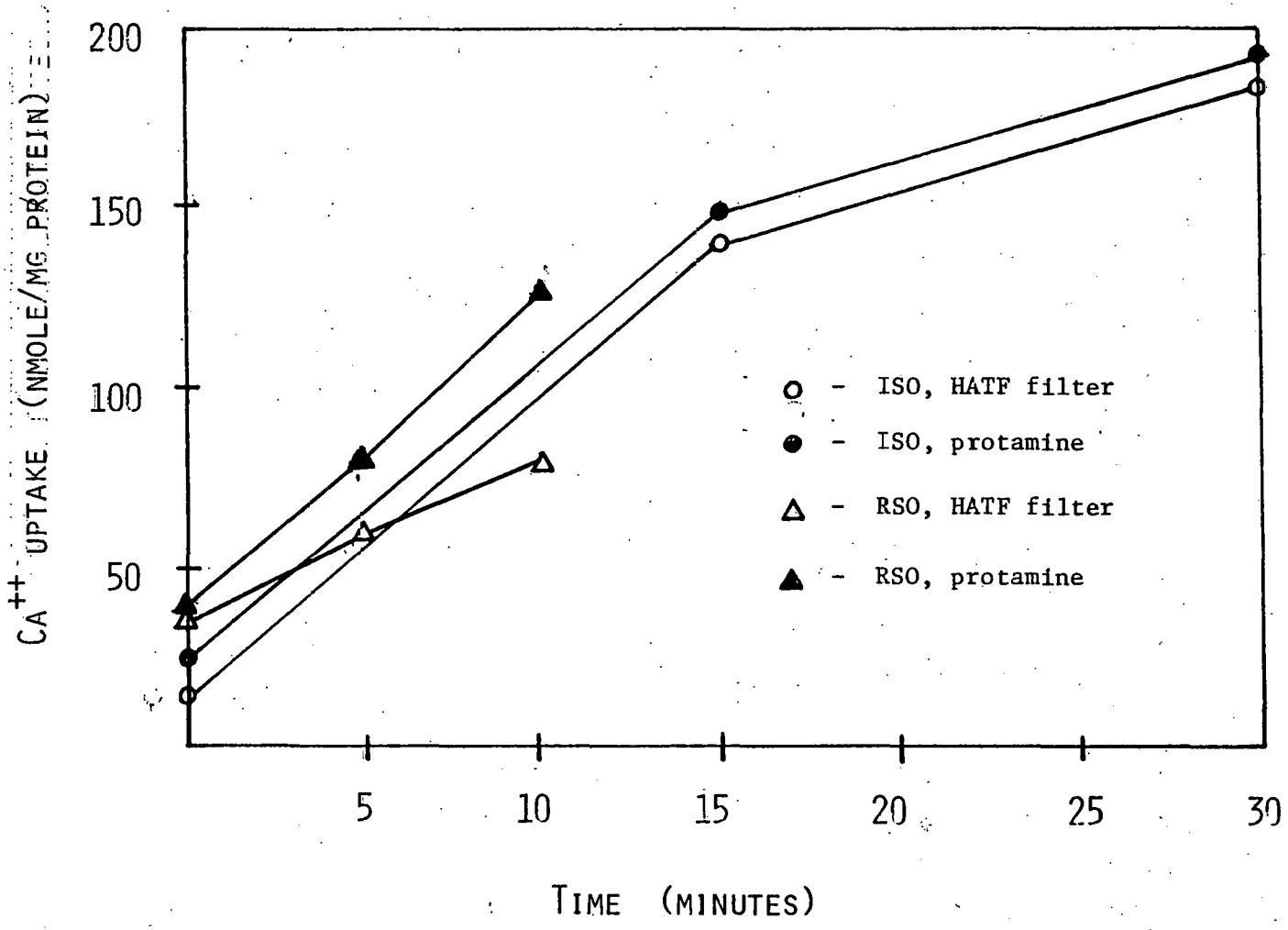

200
150
100
50
Ca^{++} UPTAKE (NMOLE/MG PROTEIN)
5
10
15
20
25
30
TIME (MINUTES)
- ISO, HATF filter
- ISO, protamine
- RSO, HATF filter
- RSO, protamine

Table 24. POLYLYSINE AND PROTAMINE FILTRATION AND CENTRIFUGATION OF ^{14}C-SUCROSE LABELLED COV

	Incubation time	% of COV's retained on filters or in pellet			
		Control	Control + Lubrol	+ Polylysine	+ Protamine
Filtration	t = 30"	1.8%	0.7%	60.0%	97.9%
	t = 5'	-	-	60.6%	96.6%
	t = 10'	-	-	61.3%	96.3%
Centrifugation	t = 30"	8.8%	6.9%	70.6%	97.0%
	t = 5'	-	-	63.8%	96.5%

^{14}C-sucrose labelled COV were prepared as described previously with 277 μg/ml of cytochrome oxidase (batch 2, unfractionated) in 40 mM K_2SO_4, 10 mM HEPES, 20 mM sucrose (pH 7.0), with 2.85 μCi/ml of ^{14}C-sucrose in the first dialysis phase (against 2.7 volumes for 5-8 hours x7). After the second phase of dialysis to remove external label (against 167 volumes of nonradioactive buffer for 6-24 hours x4) samples were made in the same buffer solution consisting of 3.2 mg/ml of vesicles, with 180 μg/ml of polylysine (MW 3400), 180 μg/ml of protamine, or .5 mg/ml of Lubrol in the appropriate samples. The samples for centrifugation were 1 ml and those for filtration were 2 ml in volume. The samples were incubated at room temperature for the indicated lengths of time after addition of the vesicles and were then either filtered through a .45 EH type Millipore filter with a glass prefilter or centrifuged in a JA-20 type rotor for 3 minutes at 15,000 rpm (the centrifugation time being started when the rotor reached a speed of 10,000 rpm). The filters were washed with 4 ml of buffer and the ^{14}C-sucrose activity in the filters, filtrates and wash was determined to obtain the % retention of the COV on the filter. The % of COV in the pellets was determined from the decrease in the radioactivity of the supernatants.

Figure 21. FLOW DIALYSIS DETERMINATION OF LEAKAGE OF ^{14}C-SUCROSE FROM LIPOSOMES BY POLYLYSINE AND PROTAMINE

Liposomes were made by sonicating asolectin in 40 mM K_2SO_4, 10 mM HEPES, 20 mM sucrose (pH 7.0) containing 3 μCi/ml of ^{14}C-sucrose at a phospholipid concentration of 20 mg/ml until the suspension clarified. The suspension was then dialyzed against nonradioactive buffer solution using the flow dialysis technique at an average flow rate of about 40 ml/hr for about 30 hours (until no detectable radioactivity was present in the dialysis solution).

Samples were made consisting of .8 ml of vesicle suspension (16 mg of vesicles) in a final volume of 1.2 ml of 40 mM K_2SO_4, 10 mM HEPES, 20 mM sucrose (pH 7.0) containing 75 μg of polylysine (MW 3400) or protamine per mg of phospholipid in the appropriate samples, or .3% sodium cholate + 4.1% (final concentration) of trichloroacetic acid in the lysis sample. Each sample was placed in the flow dialysis chamber of Colowick and Womack, 1969, immediately after addition of the liposomes and buffer solution pumped through the other part of the chamber at a rate of .36 ml/minute. The radioactivity of the effluent solution was measured at the time points indicated and the total cumulative efflux quantitated.

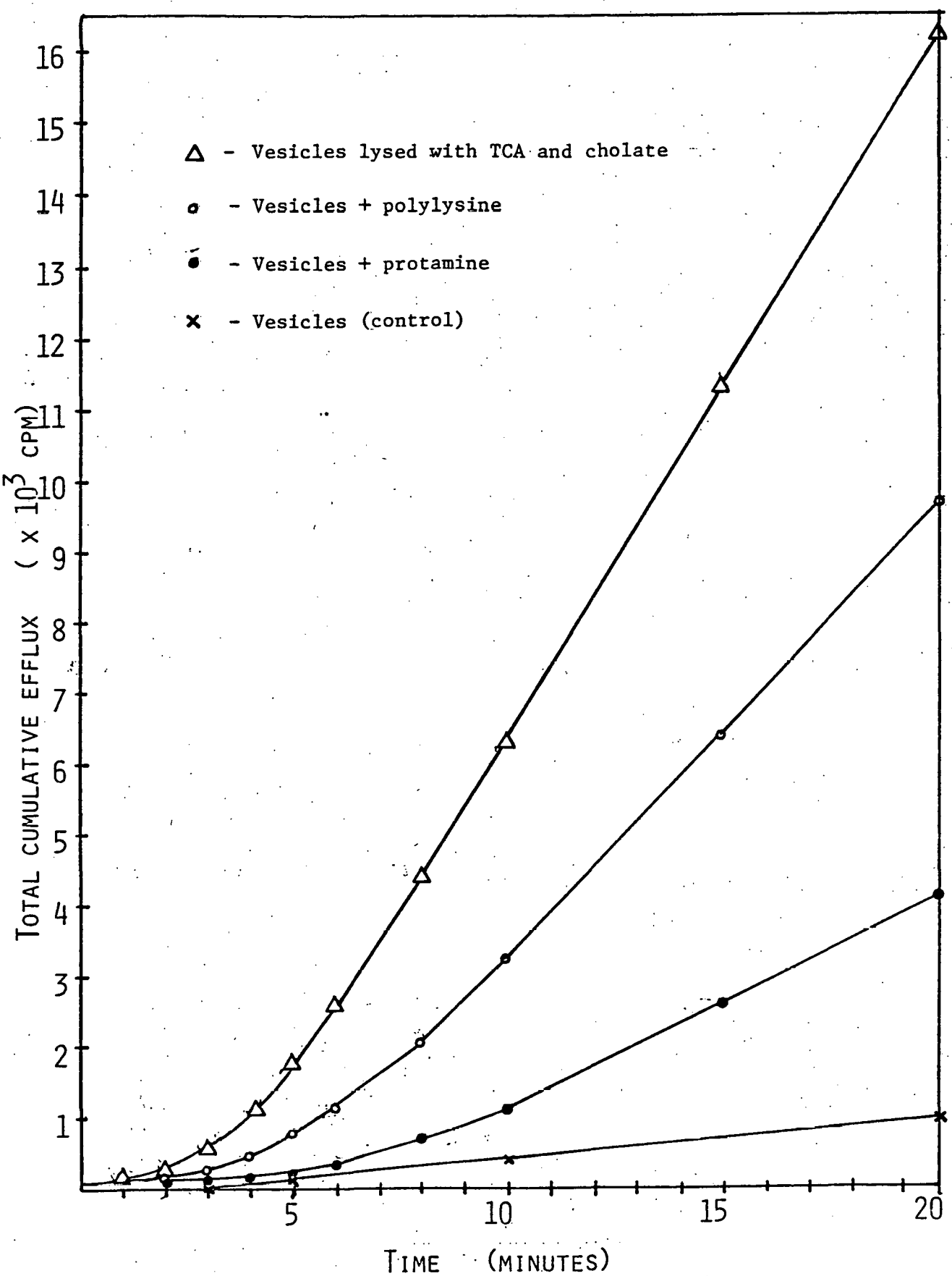

Δ - Vesicles lysed with TCA and cholate
o - Vesicles + polylysine
é - Vesicles + protamine
× - Vesicles (control)
TOTAL CUMULATIVE EFFLUX (x 10³ CPM)
TIME (MINUTES)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
5
10
15
20

Table 25. PROTAMINE FILTRATION OF VARIOUS TYPES OF PHOSPHOLIPID VESICLES

Phospholipid composition	Without protamine		With protamine
	$O.D._{450}$ suspension	$O.D._{450}$ filtrate	$O.D._{450}$ filtrate
PC	.144	.114	.111
1:1 PC:PE	.101	.077	.099
5:5:2 PC:PE:CL	.086	.076	.003
1:1:1 PC:PE:PS	.110	.068	.002
Asolectin	.098	.083	.001

PC= phosphatidyl choline (egg yolk, Sigma type IX E), PE = phosphatidyl ethanolamine (ovine brain, type II-S), PS = phosphatidyl serine (brain extract, type III), CL = cardiolipin (beef heart).

The different types of phospholipid vesicles shown above were made by drying the appropriate phospholipids under nitrogen and suspending them at a concentration of 18 mg/ml in 40 mM K_2SO_4, 10 mM HEPES (pH 7.0) by sonicating to clarity. A 2 ml sample of each aliquot was prepared by adding 5.5 mg of vesicles (.3 ml) to the appropriate amount of buffer ± 600 μg of protamine. After reading the $O.D._{450}$ of the suspension containing no protamine it was filtered through a .45μ Millipore filter with a glass prefilter and the $O.D._{450}$ of the filtrate was read. The aliquots with protamine were filtered similarly and the $O.D._{450}$ read to determine the amount of vesicle retention due to the protamine. The ratios of all phospholipids are based on weight.

necessary for aggregation by protamine to occur. It is likely that the aggregation process works by binding of the positively charged protamine molecule to acidic phospholipid groups in the membrane of the vesicles and thereby binds the vesicles together. Since acidic phospholipids are present in virtually all natural membranes, this process can be used for separation of a wide variety of small vesicles from suspending media to allow quantitation of transported substances.

It has also been noted that strongly charged substances change the optimum amount of polylysine or protamine necessary for complete filtration. Negatively charged substances such as adenine nucleotides greatly increase the amount of polypeptide needed for filtration, presumably by binding to some of the positively charged polypeptide. Addition of divalent cations in the presence of adenine nucleotides negates this effect. Since ionic interaction is the basis of the aggregation technique the ionic species present in the suspension will obviously affect the aggregation. For this reason it is important to test the filtration using the simple optical density measurement to determine the necessary amount of protamine for any given system of vesicles, transported substances, and substrates.

Discussion

The major impediment to the study of ion translocation in reconstituted proteoliposomes is the lack of adequate separatory technology. Turbidity noted when polylysine was added to suspensions of PCOV to displace external cytochrome c led to the development of the aggregation-filtration technique for vesicle separation from suspension media. The use of light scattering as a monitor of completeness of vesicle filtration is a simple and sensitive method of optimizing the aggregation- filtration procedure for a given system. Since the polypeptide addition causes aggregation of the vesicles, aggregates which are not filtered out will be present in the filtrate and will be larger sized particles than the original vesicles. The square law dependence of light scattering on particle size will accentuate the apparent amount of unfiltered material. The filtration is seen with both O.D. and protein measurements of the filtrate to be quite complete for both natural and reconstituted particles. The dependence of the aggregation on the relative amounts of the vesicles and polypeptides rather than on the concentrations supports the idea that it is binding of the polypeptides to the vesicles which causes the aggregation. This is not unreasonable since protamine and polylysine are both highly positively charged molecules (Kurt, 1960; Harmon, Hall, Crane, 1974; Smith, Conrad, 1956) , and the Results section shows that the presence of acidic phospholipids (negatively charged) in the membranes of vesicles is necessary for aggregration to occur. One of the main differences in the use of polylysine or protamine for the aggregation-filtration process is the response to amounts of polypeptide which are larger than the optimum amounts for aggregation (which is usually in the

range of 50-100 μg/mg particles for most types of particles tested). With larger amounts of polylysine aggregation is inhibited and increasing amounts of the particles pass through the filters. Polylysine is a highly charged random coil which probably occupies a larger volume than a less charged protamine of similar molecular weight. At low amounts of polypeptide relative to a given amount of vesicles both polylysine and protamine may bind to ionic sites on different vesicles, causing aggregation. As the amount of polylysine is increased more vesicles will have polylysine associated with them, which will tend to repel other polylysine molecules seeking to bind to the vesicles. There may then be an increased tendency for polylysine to bind to multiple binding sites on the same vesicle and less bridging between vesicles would occur. Protamine would not be expected to be as effective in covering the surface of a vesicle with positively charged areas and would not be as effective as polylysine in repelling the binding of other protamine molecules to a given vesicle. The situation with polylysine would be analagous to antigen-antibody interaction, which produces aggregation only in a certain optimum range of antigen and antibody.

Another difference in the behavior of polylysine and protamine is the amount of leakiness induced in the vesicles by the aggregation In the first 1-2 minutes of exposure protamine produced no more leakage of internal label from vesicles than control. This is more than enough time for filtration. Polylysine on the other hand causes much more leakage. This is probably due to the more highly charged nature of the polylysine molecule, which would tend to interact with more of the phospholipid groups of the vesicle membrane and may tend to pull some of the

acidic phospholipids from the membrane or disrupt the packing of the phospholipids in the membrane. For this reason protamine was felt to be a better aggregating agent and was used routinely in uptake experiments. The leakage was less of a problem in membranes such as SMP which contained higher proportions of protein, and polylysine was found to aggregate these types of vesicles with minimal evidence of lytic effects.

The convenience, low cost, and complete filtration afforded by the protamine filtration method made it the method of choice for separation of all types of vesicles from suspending media. Ion exchange columns are harder to use and suffer from variable vesicle retention in the columns (Gasko et al, 1976). They cannot be used in the presence of polylysine or protamine because of this problem, and this is important in the selective energization technique for COV and SMP. The protamine filtration technique is also a more general technique as it can be used for the quantitation of uptake in vesicles of uncharged substances such as sugars as well as ions. This technique should be invaluable in the reconstitution of sugar transporting systems.