Phospholipid, Enzymatic, and Polypeptide Analyses of the Mitochondrial Membranes from *Saccharomyces carlsbergensis*†

A. Joanne Bednarz-Prashad‡ and Charles E. Mize*,§

ABSTRACT: *Saccharomyces carlsbergensis* grown in the absence of inositol undergoes "inositolless death" and shows marked changes in phospholipid composition in the mitochondrial membranes. Mitochondria from cells grown in the presence of inositol contain 0.203 mg of total phospholipid/mg of protein; mitochondria from cells grown in its absence contain only 0.085 mg of phospholipid/mg of protein. The mitochondrial phospholipids from inositol-deficient cells have significantly decreased amounts of cardiolipin and phosphatidylinositol and increased amounts of phosphatidylcholine. Phospholipid alterations are also apparent in the separated mitochondrial outer and inner membranes. In both cases, the ratio of phospholipid to protein in the inositol-deficient membranes is only 12 to 14% of that ratio in mitochondrial membranes from supplemented cells. The mitochondrial outer membranes demonstrate a significant loss of cardiolipin and phosphatidylinositol, as well as the loss of detectable monoamine oxidase activity. The whole mitochondria and separated mitochondrial membranes show distinct polypeptide composition when analyzed by polyacrylamide gel electrophoresis, and inositol deprivation results in major alterations in the amounts of several of these polypeptides. Whole mitochondria from inositol-deficient cells have reduced amounts of polypeptides with molecular weights of 55 000–65 000 and 45 000–50 000. The inner membrane also shows the loss or reduction of a polypeptide of molecular weight 62 000, as well as 85 000 and 33 000. The outer membrane, like the inner membrane, has reduced amounts of a polypeptide of molecular weight near 30 000.

Membranes from prokaryotes and eukaryotes are remarkable for their great variety of lipid components (Hunter and Rose, 1971; Law and Snyder, 1972). Alteration of this lipid network can influence the organization and properties of the membranes (Van Deenen, 1965). For example, the unsaturated fatty acids of *Saccharomyces cerevisiae* may be reduced or depleted by anaerobic growth conditions or by mutations affecting fatty acid desaturase (Linnane et al., 1972). This results in drastic conformational changes in the mitochondrial membrane and, in the case of the fatty acid desaturase mutant, the loss of respiration and phosphorylation. Similar results have been reported for *S. cerevisiae* mutants requiring oleic acid or ergosterol (Resnick and Mortimer, 1966; Bard, 1972; Karst and Lacroute, 1973; Gollub et al., 1974).

Phospholipids also appear to play a particularly indispensable role in yeast cell membranes. The availability of a phospholipid component to the cell can affect mitochondrial composition and overall cell function in wild-type strains and in yeast mutants requiring that component (Waechter and Lester, 1971; Becker and Lester, 1976; Henry and Keith, 1971; Ratcliff et al., 1973; Shafai and Lewin, 1968). We have undertaken studies of such a mutant of *S. carlsbergensis*, which requires inositol for optimum growth. Almost 90% of the inositol in yeast is found in phospholipid, and in the absence of this membrane phospholipid precursor growth of *S. carlsbergensis* is significantly retarded. Respiration and cytochrome content decline (Ridgway and Douglas, 1958a,b), and the cells accumulate acetoin (Lewin and Smith, 1964), acetaldehyde and glycerol (Lewin, 1965), and triglycerides (Shafai and Lewin, 1968), suggesting abnormal mitochondrial function (Ridgway and Douglas, 1958b), including malfunction of the citric acid cycle (Lewin, 1965). Fatty acids from endogenous triglycerides are utilized for phospholipid synthesis in the mutant supplemented with inositol; in unsupplemented cells, neutral lipids and triglycerides accumulate, fatty acid synthetase activity increases, and phosphatidylinositol content declines (Shafai and Lewin, 1968; Johnston and Paltauf, 1970).

A method was reported previously (Bednarz-Prashad and Mize, 1974) detailing a procedure for obtaining mitochondria from inositol-supplemented and -unsupplemented *S. carlsbergensis* and for fractionating these mitochondria to yield well-separated mitochondrial inner and outer membranes. Sonication, as opposed to detergents (such as digitonin) or bile salts (such as deoxycholate), was used to lyse the mitochondria before membrane separation in an effort to avoid chemical modifications of the lipids of the mitochondrial membranes. Further data are reported in this paper demonstrating the separation of the mitochondrial membranes. Alterations in phospholipid composition in the mitochondria due to inositol deprivation during cell growth are presented. Furthermore, phospholipid data are reported for separated mitochondrial membranes. These data have been unavailable previously in yeast systems. Finally, alterations are shown in polypeptide composition in the mitochondrial membranes due to inositol-deficient cell growth.

Experimental Procedures

Cell Growth, Fractionation, and Membrane Separation. The procedures for cell growth and harvesting have been published (Bednarz-Prashad and Mize, 1974). Briefly, *S. carlsbergensis* strain 4228 (ATCC 9080) was grown on a chemically defined medium using 2% galactose as carbon

† From the Departments of Biochemistry and Pediatrics, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235. *Received November 29, 1977; revised manuscript received June 7, 1978.* Supported by Robert A. Welch Foundation Grant I-454 and United States Public Health Service Grant RR-05426. A portion of this work was presented at the American Society of Biological Chemists, 65th Annual Meeting, Minneapolis, Minn., June 2–7, 1974. The previous paper in this series is Bednarz-Prashad and Mize (1974).

‡ Robert A. Welch Foundation Postdoctoral Fellow. Present address: Department of Microbiology, University of Texas Health Science Center at Houston, Houston, Texas.

§ A United States Public Health Service Career Development Awardee during the period of this investigation.

0006-2960/78/0417-4178$01.00/0

source. Inositol (0.5 mg/mL) was added to the medium for culturing inocula for subsequent experiments and to the growth medium for experiments involving inositol-supplemented cells. Cultures were grown to a maximum OD_{620} of 0.5 (representing midlog phase of growth for inositol-supplemented cells) and 0.35 (midlog growth phase for inositol-deficient cells). Inoculating at OD_{620} 0.03–0.04, the cells reach midlog phase of growth in 8–12 h. Cell number, as determined by colony count, indicates that the culture is actively growing at midlog phase. Where indicated, cells were grown in the presence of [^{14}C]-stearate as described previously (Bednarz-Prashad and Mize, 1974). The procedures for fractionation and mitochondrial membrane separation also have been published (Bednarz-Prashad and Mize, 1974). Once prepared, the mitochondrial membranes were analyzed immediately for enzyme activity and/or phospholipid content.

Enzyme Assays. Enzymatic activities were determined with Beckman Model 25 and Cary Model 15 recording spectrophotometers at ambient temperature, the reference cuvettes containing all the reaction components except the substrate. ATPase activity was measured according to Post and Sen (1967). Kynurenine hydroxylase (EC 1.14.1.2) was assayed as described by Bandlow (1972); an extinction coefficient of 7.3×10^6 mol^{-1} cm^2 was used. Monoamine oxidase (EC 1.4.3.4) was assayed according to the Schnaitman et al. modification (1967) of the system described by Tabor et al. (1955). One unit of monoamine oxidase activity is that amount of enzyme producing a change in optical density at 250 nm of 0.001/min at 27 °C. When lipid micelles were present in these assays, they were added to the membrane fractions in ratios of 1 and 2 mg of phospholipid/mg of protein. The phospholipid–protein mixtures were incubated 3–5 min before taking aliquots for assay. Adenylate kinase (EC 2.7.4.3) was assayed according to Schnaitman and Greenawalt (1968). Malate dehydrogenase (EC 1.1.1.37) was assayed according to Ochoa (1955). Cytochrome *c* oxidase (EC 1.9.3.1) was assayed according to Wharton and Tzagoloff (1967). Units of enzyme activity are expressed as change in optical density at the appropriate wavelength per minute. Reduced cytochrome *c* was prepared as described (Bednarz-Prashad and Mize, 1974). Whole mitochondria were broken by sonication or freeze-thaw cycles before assaying. Protein was determined colorimetrically (Lowry et al., 1951).

Lipid Extraction and Analyses. Total lipids were extracted from mitochondria and submitochondrial fractions with chloroform–methanol (2:1, 25 volumes/volume membrane fraction). Chloroform and aqueous phases were separated after the addition of 0.2 volume of HCl–H_2O (1:2000). The chloroform phase was evaporated just to dryness under a stream of N_2 at 24 °C. The total lipids were resuspended in methanol, and separation into major subclasses was done by one-dimensional thin-layer chromatography (TLC) on silica gel G (Baxter et al., 1967). The developing solvent contained hexane–diethyl ether–glacial acetic acid (80:20:2, v/v). The phospholipids remained at the origin and were scraped from the plate and eluted from the silica gel G with methanol. The volume of the phospholipid samples was reduced under N_2 as described above. Individual phospholipids were separated by two-dimensional TLC on silica gel H (Renkonen and Varo, 1967). The developing solvents contained the following: system 1 (first dimension) chloroform–methanol–ammonia (97.5:52.5:7.5, v/v) and system 2 (second dimension) chloroform–acetone–methanol–glacial acetic acid–water (45:18:9:9:4.5). They were identified by their respective R_f values determined from simultaneous chromatograms of commercially available reference standards, which were repurified by TLC in this laboratory. Controls using these reference standards were included in every chromatographic procedure. Visualization and identification of the phospholipids were achieved by exposure to I_2 vapor and by staining properties with ninhydrin, bismuth nitrate (Dragendorff reagent), and molybdic acid (Skidmore and Entenman, 1962). Thin-layer plates were prepared and analyzed in duplicate.

The results of this lipid-extraction method were compared among three slightly different extraction procedures. In some cases, the membrane sample was treated with ethanol before extraction in order to inactivate yeast phospholipase present in the mitochondrial fractions which could break down phospholipids during the extraction procedure (Van Deenen, 1965; Bjerve et al., 1974). After $CHCl_3$–CH_3OH treatment, the extraction mixture was acidified with H_2SO_4 (a nonvolatile acid) instead of HCl (a more volatile acid). H_2SO_4 residues in the lipid extract might cause the breakdown of some lipids or phospholipids during processing of the sample. In addition, in other cases, lipid extracts were stored for periods of up to 2 months at −70 °C prior to phospholipid thin-layer chromatography and analyses. Only in the case of the samples treated with H_2SO_4 and stored for 2 months before analysis were increased amounts of lysophospholipid derivatives detected. Treatment of the samples with EtOH resulted in data indistinguishable from that obtained using the standard $CHCl_3$–CH_3OH + HCl method. Using this standard method, phospholipids were not selectively lost during the analysis nor were lysophosphatide artifacts introduced. The standard method used in this study gave results consistent from experiment to experiment.

Lipid phosphorus was determined colorimetrically by the method of Bartlett (1959) and the micro method of Parker and Peterson (1965). Duplicate controls were run for each chromatographic procedure and consisted of blank TLC plates, developed, handled, and sprayed exactly as were the sample plates. Areas equivalent to each phospholipid spot were scraped from the control, blank plates and served as background controls in the colorimetric assays.

Polyacrylamide Gel Electrophoresis. The whole mitochondria and mitochondrial fractions were subjected to NaDodSO$_4$[1]–polyacrylamide gel electrophoresis. Electrophoresis was carried out on 10-cm-high gel slabs $^1/_{16}$ in. thick in a slab gel apparatus (Studier, 1973). The gel was a continuous, 5–20% polyacrylamide gradient gel, containing 0.4% bisacrylamide, 0.41 M Tris-HCl, pH 8.8, 0.1% NaDodSO$_4$, 0.1% *N,N,N′,N′*-tetramethylethylenediamine, and 0.02% ammonium sulfate; a 2-cm-high stacking gel was used which contained 4% acrylamide, 0.1% bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% NaDodSO$_4$, 0.05% *N,N,N′,N′*-tetramethylethylenediamine, and 1% ammonium persulfate; the electrophoresis buffer contained 0.025 M Tris, 0.192 M glycine, and 0.1% NaDodSO$_4$, pH 8.2 (Laemmli, 1970).

The mitochondria and mitochondrial fractions were boiled for 1 min in the presence of 0.065 M Tris-HCl, pH 6.8, 10% glycerol, 2.5% NaDodSO$_4$, and 1% β-mercaptoethanol. Appropriate samples (80–90 μg) were placed in alternate sample wells in the stacking gel with sample buffer being added to unoccupied sample wells. The two outermost wells contained known molecular weight protein standards (lysozyme 14 300;

[1] Abbreviations used are: NaDodSO$_4$, sodium dodecyl sulfate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; CL, cardiolipin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

TABLE I: Distribution of Mitochondrial Enzyme Activities after Varied Amounts of Sonication.[a]

enzymatic marker	sonication time (min)	total units on gradient	act. units recov in peak 1	2	3
malate dehydrogenase	0	7.82	0.16	0	7.51
	0.25	2.41	0.15	0.09	2.1
	1.0	5.80	2.16	0.48	2.85
	2.0	5.99	3.79	0.46	1.49
kynurenine hydroxylase	0	0.037	0	0	0.036
	0.25	0.140	0.006	0.039	0.095
	1.0	0.180	0.069	0.07	0.02
	2.0	0.410	0.14	0.16	0.03
cytochrome *c* oxidase	0	139	0	0	136.8
	0.25	94	2.1	3.2	86.6
	1.0	216	7.1	17.3	162.2
	2.0	133	18.2	30.5	61.3

[a] Enzyme assays are described in Experimental Procedures. Cells were grown in the presence of 0.5 mg/mL inositol. The three peaks (1, 2, and 3) are curves defined by plotting activity of the enzymatic marker vs. fraction number from the collected sucrose gradient. Peak 1 covers an area on the gradient from 10 to 22% sucrose; peak 2 from 22 to 35% sucrose; peak 3 from 48 to 64% sucrose. In the case of kynurenine hydroxylase, there is one broad peak (from 10 to 35% sucrose) defined by plotting activity vs. fraction number and encompassing peaks 1 and 2. Peak 1, and the fractions composing it, were separated from peak 2 on the basis of the presence of (soluble) malate dehydrogenase activity.

trypsin inhibitor 22 700; ovalbumin 43 000; pyruvate kinase 57 000; bovine serum albumin 68 000; and β-galactosidase 130 000). Electrophoresis occurred for 22 h at 30 V, constant voltage, and 7.5 mA (at the start). At the end of 22 h, the gel was fixed in 50% Cl_3AcOH for 50 min at 4 °C, stained at 37 °C for 30 min in 0.1% Coomassie brilliant blue in 40% Cl_3-AcOH, and destained in 7.5% acetic acid.

The sample and standard channels were sliced from the slab gel and scanned at 600 nm in a Gilford recording spectrophotometer.

Standard Materials. Silica gel G and H were obtained from E. M. Laboratories (Merck, Darmstadt, Germany); individual phospholipids used as standards were purchased from Supelco, Inc. (Bellfonte, Pa.) and Applied Science Laboratories, Inc. (Inglewood, Calif.). Sources for other chemicals have been reported (Bednarz-Prashad and Mize, 1974).

Polyacrylamide, *N,N'*-methylenebisacrylamide, Trizma base, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman Kodak, Rochester, N.Y. Ultrapure $NaDodSO_4$ was obtained from BDH Chemicals Ltd., Poole, England. Coomassie brilliant blue was obtained from Bio-Rad, Richmond, Calif. Ultrapure molecular weight standards for gel electrophoresis were obtained from Sigma Chemical Co., St. Louis, Mo.

Results

Enzyme Activities in the Separated Membranes. Separation of mitochondrial membranes in this study involves isolating mitochondria from yeast spheroplasts and subjecting them to intermittent sonication in a salt water–ice bath. The sonication must be carefully controlled so that the temperature of the mitochondrial suspension never exceeds 6 °C. Sonication intervals of 2–3 s, followed by 50 s of cooling and stirring, are optimum. The total time of sonication affects the degree of separation of the outer from the inner membrane. When sonicated mitochondria are centrifuged on a 10–70– continuous sucrose gradient (in 0.02 M Tris, 0.001 M EDTA, pH 7.4) at 4 °C, the activities of three mitochondrial enzymes separate and localize in three regions of the gradient. Peak 1, from 10–22% sucrose, contains predominantly malate dehydrogenase activity. Peak 2, from 22–35% sucrose, contains predominantly cytochrome *c* oxidase activity. Peak 3, from 48–64% sucrose, contains predominantly cytochrome *c* oxidase activity.

The data in Table I show the distribution of three marker enzymes for the mitochondrial matrix, outer membrane and inner membrane among peaks 1–3, respectively. For these data, a total mitochondrial suspension was divided into four equal parts. Each part was then subjected to the intervals of sonication shown in Table I before density gradient centrifugation (Bednarz-Prashad and Mize, 1974). At 0 min of sonication, there is essentially no mitochondrial breakage and, after gradient centrifugation, at least 96% of all activities appear in one major peak (peak 3). After 15 s of sonication, 0.15 unit of the malate dehydrogenase activity appears as a small peak at 10–22% sucrose (peak 1), 0.09 unit at 22–35% sucrose (peak 2), and 2.1 units (87%) as a major peak at 48–64% sucrose (peak 3). This distribution of malate dehydrogenase activity indicates some damage to the mitochondrial inner membrane and release of matrix material. In peak 2, 0.039 unit (28%) of the kynurenine hydroxylase activity appears, suggesting some release of outer membrane from the mitochondrion. However, 0.095 unit (68%) of the activity units still remains with the mitochondria and inner membrane in peak 3. Of the cytochrome *c* oxidase activity, only 2–3% appear in peaks 1 and 2; 86.6 units (92%) of these activity units remain in peak 3, at the main mitochondria and inner-membrane peak.

After 1 min of sonication, a significant amount of both malate dehydrogenase and kynurenine hydroxylase activity units is released from the mitochondria. Thirty-six percent (2.2 units) of the malate dehydrogenase activity units appear in peak 1. Other soluble mitochondrial enzymes, such as adenylate kinase, also appear in this peak. A small amount of malate dehydrogenase activity appears in peak 2, where the outer-membrane vesicles concentrate in this gradient. However, 49% (2.85 units) of the malate dehydrogenase activity units are still detected in the, now, mainly inner-membrane vesicle fractions in peak 3. This suggests that some intact mitochondria probably still are present or that some inner membrane still encloses matrix material. Seventy-seven percent (0.139 unit) of the kynurenine hydroxylase activity units, and hence outer-membrane function, appears in peaks 1 and 2 as a major broad peak which were collected as two fractions based on malate dehydrogenase activity distribution. This

TABLE II: Specific Activity of Marker Enzymes in Mitochondria and Separated Mitochondrial Membranes from Cells Grown in the Presence or Absence of Inositol.[a]

fraction	enzyme assay	specific activity[b] −inositol	+inositol
sonicat Mt[a]	monoamine oxidase	0	0.0256
	kynurenine hydroxylase	0.14	0.17
	cytochrome *c* oxidase	11.05	8.8
Mt outer membr	monoamine oxidase	0	0.0324
	kynurenine hydroxylase	0.377	0.37
	cytochrome *c* oxidase	0.97	1.525
Mt inner membr	monoamine oxidase	0	0
	kynurenine hydroxylase	0	0
	cytochrome *c* oxidase	15.1	15.73

[a] Cell growth, fractionation, and enzyme assay methods are presented under Experimental Section; sonication time 1 min. [b] Specific activity is expressed as change in OD min^{-1} (mg of Lowry protein)$^{-1}$.

TABLE III: Phospholipid Composition of Whole Mitochondria.[a]

phospholipid	+inositol[b] mg of phospholipid/mg of protein	+inositol[b] %	−inositol[b] mg of phospholipid/mg of protein	−inositol[b] %
CL	0.027	13.3 ± 1.4	0.006	7.3 ± 0.6
PI	0.014	6.9 ± 3.1	0.003	3.7 ± 2.3
PC	0.094	46.5 ± 2.1	0.052	60.5 ± 2.3
LPC	0.004	1.8 ± 1.2	ND	ND
PE	0.041	20.3 ± 5.1	0.015	17.2 ± 2.2
LPE	0.001	0.5 ± 0.5	0.006	7.4 ± 1.1
PS	0.005	2.3 ± 1.1	ND	ND
LPS	ND	ND	0.001	1.1 ± 1.1
unknown	0.017	8.4 ± 1.4	0.002	2.8 ± 2.0
total	0.203		0.085	

[a] Phospholipid extraction and analyses are described in Experimental Procedures. The reported values are percentages of the total phospholipids recovered from the plate; 85–95% of the phospholipid phosphorus applied was routinely recovered. Unknown phospholipids include unidentified phospholipids on the chromatogram (see text). ND is not detectable. Data are included for mitochondria from inositol-supplemented and unsupplemented cells. Each data point is the average of the results from six separate experiments with standard error of the mean. [b] *S. carlsbergensis* mitochondria.

length of sonication yields the optimum release of outer-membrane function without excessive contamination of these fractions by inner-membrane function. A similar pattern to kynurenine hydroxylase distributions can be shown using another outer-membrane marker (monoamine oxidase). However, this enzymatic marker can be monitored only in mitochondria from cells grown in the presence of inositol (Bednarz-Prashad and Mize, 1974) and was not monitored routinely in these experiments. Only 11% (0.02 unit) of the kynurenine hydroxylase activity units is still present in peak 3, with the inner membrane. This may indicate the presence of a few remaining intact mitochondria and/or the presence of points of adhesion of the outer with the inner membrane (Hackenbrock, 1968) which remain in peak 3. Only 11% (24.4 units) of the cytochrome *c* oxidase units appear in peaks 1 and 2. This suggests the vesicularization of some of the inner membrane. As seen in Table I, prolonged sonication (2 min) increases this apparent vesicularization and produces an increasing amount of cytochrome *c* oxidase activity in peaks 1 and 2. It can also be seen that increased time of sonication, up to 2 min, does not result in significant loss of enzyme activity units. In fact, in the case of kynurenine hydroxylase the amount of measurable activity units is significantly increased by sonication. For all further experiments, the mitochondria were handled in a manner which gave inner- and outer-membrane separation equivalent to the results of 1 min of sonication. That is, at least 75% of the outer-membrane marker activities (kynurenine hydroxylase and monoamine oxidase) was present in peaks 1 and 2 which comprised the outer-membrane fraction. No more than 8–11% of the inner-membrane marker activity (cytochrome *c* oxidase) was present in these regions. In some cases, no cytochrome *c* oxidase activity was detectable in the outer-membrane fraction of peaks 1 and 2.

The data in Table II show the specific activities of mitochondrial markers associated with the outer (monoamine oxidase and kynurenine hydroxylase) and inner membranes (cytochrome *c* oxidase). As stated earlier, monoamine oxidase cannot be demonstrated in cells grown in the absence of inositol. However, the activities of the other two marker enzymes (cytochrome *c* oxidase and kynurenine hydroxylase) are essentially the same in mitochondrial membranes from cells grown in the presence or absence of inositol. Cytochrome oxidase activity appears to localize in the inner-membrane fractions, although some activity is measurable in the outer-membrane fraction. Kynurenine hydroxylase and monamine oxidase activities are measurable only in the mitochondrial preparation or in the outer-membrane fractions. It can be seen from these data that the degree of membrane separation measured by the localization of enzyme activity in the gradient is similar in material obtained from cells grown in the presence or absence of inositol.

Monitoring for Na^+,K^+-dependent, ouabain-sensitive ATPase activity as a monitor for nonmitochondrial membranes demonstrated that 21–23% of the total units present in the lysed spheroplast preparation was present in the mitochondrial fraction. Essentially all of these units were present in the outer-membrane fraction after sucrose gradient centrifugation of the disrupted mitochondria.

Electron microscopy of the pelleted material from combined peaks 1 and 2 (outer-membrane material) revealed vesicularized membranes all of approximately the same size and appearance (data not shown). The pelleted material from peak 3 contained large membrane vesicles and an occasional intact mitochondrion or inner-membrane structure with the outer membrane still attached. This correlates with the observation that there is still 11% of the total outer-membrane kynurenine hydroxylase activity in the inner-membrane fraction (Table I). Previous data (Bednarz-Prashad and Mize, 1974) also indicate that about 11% of a tritium label introduced covalently onto the outer membrane of an intact mitochondrion appears with the inner-membrane fraction after mitochondrial disruption and centrifugation.

The membrane fractions thus separated on the sucrose gradient are not absolutely free of contaminating membrane material, as discussed above. The morphologic, radioactive label, and enzymatic studies define, nonetheless, a high degree of outer- and inner-membrane purification.

Phospholipid Analyses of Mitochondria and Mitochondrial Fractions. The data in Tables III and IV are the results of phospholipid analyses of whole mitochondria and of mitochondrial outer (peaks 1 and 2, Table I) and inner membranes (peak 3, Table I), respectively. These fractions were taken from cells grown to midlog phase in the presence and absence of inositol.

TABLE IV: Phospholipid Composition of Mitochondrial Inner and Outer Membranes.[a]

phospholipid	+inositol[b] mg of phospholipid/mg of protein	+inositol[b] %	−inositol[b] mg of phospholipid/mg of protein	−inositol[b] %
inner membrane				
CL	0.036	9.2 ± 2.2	0.007	12.8 ± 1.6
PI	0.041	10.3 ± 1.7	0.002	3.8 ± 1.5
PC	0.096	24.4 ± 1.3	0.013	22.8 ± 6.5
LPC	0.006	1.6 ± 1.8	0.003	5.5 ± 1.6
PE	0.109	27.6 ± 3.3	0.008	14.7 ± 4.4
LPE	0.014	3.7 ± 2.3	0.001	1.9 ± 1.8
PS	0.031	7.9 ± 1.6	ND	ND
LPS	ND	ND	0.002	3.7 ± 1.7
Unk	0.060	15.2 ± 3.9	0.020	34.9 ± 5.2
total	0.393		0.056	
outer membrane				
CL	0.060	6.1 ± 1.8	0.001	0.7 ± 0.5
PI	0.108	10.9 ± 1.6	0.004	3.4 ± 1.3
PC	0.434	43.7 ± 3.7	0.093	78.5 ± 6.0
LPC	0.123	12.4 ± 2.8	0.001	1.1 ± 0.9
PE	0.160	16.1 ± 1.9	0.010	8.7 ± 5.1
LPE	0.027	2.7 ± 0.8	ND	ND
PS	0.028	2.8 ± 1.8	0.002	1.8 ± 1.0
LPS	0.004	0.4 ± 0.5	0.003	2.1 ± 0.5
Unk	0.048	4.8 ± 2.7	0.004	3.6 ± 0.9
total	0.992		0.118	

[a] Phospholipid extraction and analyses are described under Experimental Procedures. Data are included for mitochondrial inner and outer membranes from inositol-supplemented and -unsupplemented cells. Each data point is the average of the results from five separate experiments with standard error of the mean. [b] *S. carlsbergensis*.

S. carlsbergensis mitochondria taken from viable cells grown in the absence of inositol contain a lower ratio of phospholipid–protein (mg/mg) than those mitochondria taken from supplemented cells (Table III). This pattern is consistent throughout the growth of the culture. That is, the phospholipid–protein ratio of the outer membranes increases with age from early-log [0.352 mg of phospholipid/mg of protein, inositol-supplemented (I+); 0.051, inositol-deprived (I−)] through midlog (Table IV), to late-log phase (0.866, I+; 0.169, I−). The inner membranes show a similar pattern from early-log phase of growth (0.149, I+; 0.049, I−), through midlog phase (Table IV), to late log phase (0.301, I+; 0.175, I−). Also, as would be expected in inositol-deficient mitochondria, PI comprises less of the total phospholipid. The content of cardiolipin is lower and the trace amounts of PS and LPC in inositol-supplemented mitochondria were not detectable in the inositol-deficient mitochondria. LPE and PC were in greater concentration in the inositol-deficient mitochondria, and the trace amount of LPS found was not detectable in the inositol-supplemented state.

The data in Table IV show the phospholipid composition of the separated mitochondrial membranes from *S. carlsbergensis* at midlog growth in the presence or absence of inositol. It is evident that inositol deficiency results in a lowered ratio of phospholipid–protein (mg/mg) in the mitochondrial membranes. In the inner membrane, a decrease in PI, PE, and PS content and an increase in the content of CL, LPC, and LPS are associated with inositol deficiency. Also, there was an increase in unidentifiable phospholipid; 35% of the total phosphorus migrated as three to four molecular species near or at the origin on the two-dimensional thin-layer plate (see Experimental Procedures). This material reacted with ninhydrin and did not correspond to any of the standard phospholipids, such as LPS, LPC, or LPI, which are known to remain near the origin. A tentative identification of these species as phosphosphingolipids or their derivatives is compatible with these characteristics.

Preliminary experiments have been conducted in which cells were grown in the presence of [^{14}C]stearate (Experimental Procedures). This results in the incorporation of ^{14}C label into the lipids of all cellular membranes (Johnston and Paltauf, 1970). Lipid analyses of mitochondrial outer membranes from such labeled, actively growing cells grown in the presence or absence of inositol revealed that greater than 90% of the ^{14}C label recovered from the thin-layer chromatogram was present in identifiable phospholipid species shown in Table IV.

In the case of the mitochondrial inner membranes from inositol-supplemented cells, 5 to 8% of the recoverable ^{14}C label was present at the origin and at the confluences of the two solvent fronts (diagonally opposite the origin). This latter ^{14}C-labeled material was not associated with lipid phosphorus. In the mitochondrial inner membranes from inositol-deficient cells, the ^{14}C-labeled material recoverable at or near the origin increased to 22–25% of the radioactivity on the chromatogram; it appeared to be associated with the three to four molecular species mentioned above.

A comparison of the supplemented outer- and inner-membrane phospholipid composition shows that the outer membrane has a lower percent of cardiolipin, PE, and PS than the inner membrane but more PC and LPC. The mitochondrial outer membrane from inositol-supplemented cells repeatedly showed substantial LPC content. The phospholipid/protein ratio in the outer membrane is three times that in the inner membrane. This same ratio is found in the separated mitochondrial membranes from guinea pig liver (Parsons et al., 1966). Inositol deprivation in the *S. carlsbergensis* mitochondrial outer membrane causes decreases in the ratio of phospholipid/protein and in the content of cardiolipin, PI,

LPC, PE, LPE, and PS. The percent content of PC is dramatically increased. In whole mitochondria from inositol-deficient mitochondria, LPE comprises 7.4% of the total phospholipid. The LPE in the separated mitochondrial membranes totals only 1.9% of the phospholipid in the outer plus inner membranes. The same lipid-extraction and analysis procedures were used for the whole and separated mitochondria. As stated earlier (see Experimental Procedures), the lipid-extraction procedure used gave results which were comparable to those obtained with other procedures and were consistent from experiment to experiment. Therefore, this apparent difference in LPE content could not be explained by a differential loss due to the lipid extraction. It could be that LPE in the mitochondria from inositol-deficient yeast cells may be subject to dissociation from the mitochondrial membranes upon sonication. In such a case, the LPE would contribute to the total phospholipid content in an analysis of the whole mitochondrion. In contrast, when the mitochondria were separated by sucrose gradient into their inner and outer membranes, such loosely associated LPE would not be isolated with either membrane.

These observed distributions of phospholipid classes occur at both early- (5–6.5 h) and late-log harvests (15–17 h) within both the inositol-supplemented or -unsupplemented series of experiments (data not shown). Nonspecific effects of culture aging do not therefore account for the phospholipid changes observed as a function of inositol deficiency.

Phospholipid Supplementation of Outer-Membrane Fractions. In addition to phospholipid alterations, inositol deprivation results in an apparent loss of monoamine oxidase activity (Bednarz-Prashad and Mize, 1974). There is no detectable monoamine oxidase activity in broken mitochondria or mitochondrial outer membranes from inositol-deficient cells. Another mitochondrial outer-membrane enzyme, kynurenine hydroxylase, is unaffected by inositol deprivation. Attempts were made to restore monoamine oxidase activity in deficient outer membranes by the addition of phospholipid micelles made from mitochondria taken from cells grown in the presence of inositol.

The monoamine oxidase activity in mitochondrial outer membranes from supplemented cells was stimulated slightly in the presence of phospholipid micelles compared to the activity in their absence (4.3 and 1.6 units/mg of protein, respectively). However, addition of the micelles did not promote any monoamine oxidase activity in inositol-deficient outer membranes or broken mitochondria.

Another mitochondrial outer-membrane activity was assayed to detect possible detergent effects of the phospholipid micelles. Kynurenine hydroxylase was equally active in membranes from supplemented and unsupplemented cells and was neither stimulated nor inhibited by the addition of the micelles. Thus, if monoamine oxidase is present in the unsupplemented cells, it cannot be activated by phospholipid micelles under these conditions.

Polyacrylamide Gel Electrophoresis Studies of Supplemented and Unsupplemented Mitochondria and Mitochondrial Membranes. Mitochondria taken from cells grown in the presence or absence of inositol were treated with naDodSO$_4$ and subjected to one-dimensional polyacrylamide slab-gel electrophoresis. Inspection of the scans of these gels (Figure 1) revealed that whole mitochondria from inositol-deficient cells had major decreases in three polypeptides, two with molecular weights near 55 000–60 000 and one with a molecular weight of 45 000–50 000, and an increase in one polypeptide of approximately 25 000–30 000 molecular weight. The polypeptides of 55 000–60 000 and 45 000–50 000 molecular

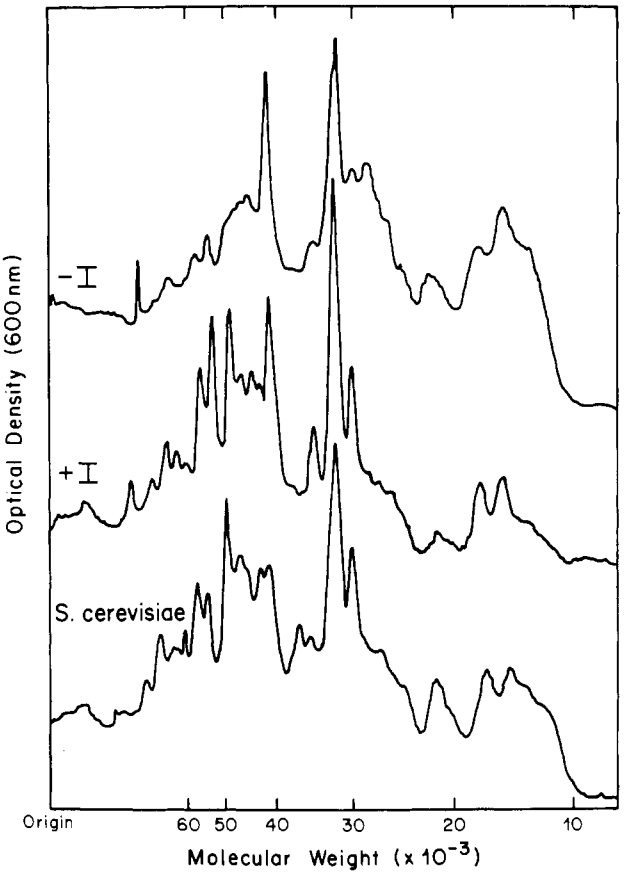

FIGURE 1: Effect of the absence of inositol from the growth medium on the polypeptide species of mitochondrial membranes of *Saccharomyces carlsbergensis*. Mitochondrial membranes from inositol-supplemented (I+) and -deficient (I−) cells, as well as from *S. cerevisiae*, were prepared and subjected to polyacrylamide gel electrophoresis as described under Experimental Procedures. Channels containing the separated polypeptides were cut from the slab gels and scanned at 600 nm.

weight were present in similar amounts in both the inositol-supplemented *S. carlsbergensis* mitochondria and in a strain of *S. cerevisiae* (ATCC 12341). The polypeptide appearing at 25 000–30 000 molecular weight was unique to the mitochondria from inositol-deficient cells. Another difference in the scans is the appearance of a polypeptide of molecular weight 34 000–36 000. This polypeptide appears only in the *S. cerevisiae* mitochondria and may represent a species-specific polypeptide.

Densitometric tracings of polyacrylamide gels of the separated mitochondrial membranes are shown in Figure 2. Comparison of the inositol-supplemented (Figure 2a) and -deficient (Figure 2b) inner membranes reveals that both have major polypeptide species of 59 000, 55 000–60 000, and 35 000–37 000 molecular weight (peaks 5, 6, and 11). In both supplemented (Figure 2c) and deficient (Figure 2d) outer membranes, a major polypeptide species near 35 000 molecular weight is present (peak h). As a result of inositol deficiency, major polypeptide alterations occur in the inner membrane. There is a marked reduction or loss of polypeptides of molecular weight 85 000, 62 000, and 33 000 (Figure 2a,b, peaks 2, 4, and 12). Inositol-deficient outer membrane also has a reduction or loss of a polypeptide of molecular weight 33 000 (Figure 2c,d, peak i) compared to supplemented outer membrane.

The densitometric tracing of mitochondrial inner membranes from supplemented cells can be compared to a similar tracing of mammalian mitochondria inner membrane (Ca-

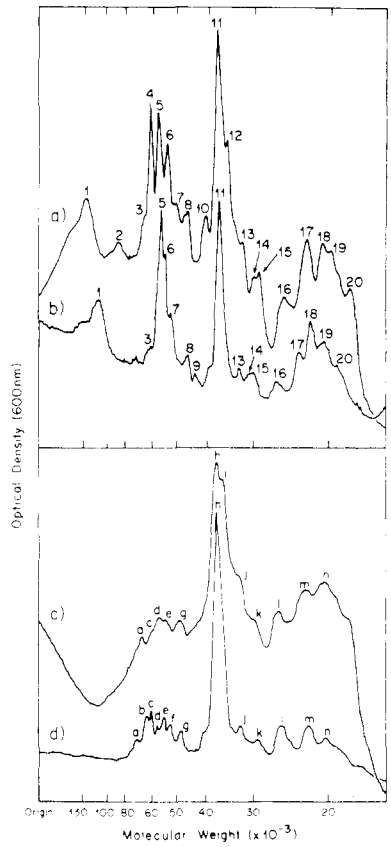

FIGURE 2: Effect of the absence of inositol from the growth medium on polypeptide species of mitochondrial inner membranes from (a) inositol-supplemented and (b) inositol-deficient cells and on mitochondrial outer membranes from (c) inositol-supplemented and (d) inositol-deficient cells. Conditions are as described for Figure 1.

paldi, 1974). Yeast inner membrane revealed a few more low-molecular-weight polypeptides (10 000–20 000) and a few less high-molecular-weight species (80 000–130 000). A striking difference occurs in the 30 000 and 33 000–35 000 molecular weight regions. In *S. carlsbergensis*, there is a major protein species at 33 000 molecular weight (Figure 2a, peak 12). In mammalian mitochondria a 30 000 molecular weight species is predominant and has been identified tentatively (Capaldi, 1974) as being hydrophobic protein in mammalian inner membrane.

Discussion

The separation of the mitochondrial membranes used in these studies was demonstrated in a previous paper by monitoring a tritium label introduced onto the surface of the mitochondrion before mitochondrial disruption (Bednarz-Prashad and Mize, 1974). The mitochondrial membranes had also been labeled with ^{14}C during cell growth by incorporation of [^{14}C]stearate from the growth medium into the membrane lipids. Most of the tritium label coincided with one of the two bands of ^{14}C-labeled membranes on a sucrose gradient which was designed to separate the mitochondrial membranes. The outer-membrane marker enzymes, monoamine oxidase and kynurenine hydroxylase, also coincided with the band of mitochondrial membranes containing both ^{14}C and ^{3}H activity. The data of Table I demonstrate this membrane separation by the criteria of the location of an inner-membrane marker (cytochrome *c* oxidase) and soluble matrix enzyme (malate dehydrogenase), as well as the above-mentioned outer-membrane marker enzymes. Although sonication of the mitochondrial preparation for a total of 1 min yielded optimum membrane separation, the inner-membrane fraction contains approximately 11.1% of the total kynurenine hydroxylase activity, and the outer-membrane fraction contains a total of 11.3% of the cytochrome *c* oxidase activity units. Since these two enzymes are membrane associated (Bandlow, 1972), we can use these data as an indication of the degree of "cross-contamination" between the membranes.

The phospholipid analyses of the mitochondrial membranes taken from *S. carlsbergensis* cells grown to midlog phase in the presence of inositol (Tables III and IV) can be compared with published results of analyses of mammalian mitochondrial membranes (Parsons et al., 1966; Stoffel and Schiefer, 1968). In both cases, the inner membrane contains a greater percentage of cardiolipin and PE than does the outer membrane. The outer membrane contains more PC. The ratios of phospholipid to protein are also similar to data from separated mammalian mitochondria (Parsons and Yano, 1967). This further supports the reliability of the separation of the membranes as judged by enzyme analysis.

In the case of the whole mitochondria phospholipids, the primary difference between *Saccharomyces* (Table III above; Paltauf and Schatz, 1969) and the mammalian mitochondrial phospholipids (Parsons et al., 1966; Stoffel and Schiefer, 1968) is the consistent presence of lyso forms of phospholipids in *Saccharomyces* mitochondria.

The data on the phospholipid composition of the mitochondrial membranes taken from cells grown to midlog phase of growth in the absence of inositol (Table IV) show that the deficient outer membranes probably have a markedly altered phospholipid structure and charge due to the striking increase of PC, a zwitterionic phospholipid. The outer membrane also has a marked reduction in acidic phospholipids such as cardiolipin, PI, and PS. The inner membrane did not show such a clear loss of acidic phospholipids. PI and PS were markedly reduced but not CL. There was a slight reduction in such phospholipids as PC, PE, and LPE. We did not detect phospholipid material with characteristic mobility of CDP-diacylglycerol in our analyses. The presence of such phospholipids in lipid extracts of whole cells of inositol-deficient *S. cerevisiae* has been reported (Becker and Lester, 1977). As suggested by these authors, the different extraction procedures may explain the difference between the data of Becker and Lester and that reported here and in the literature (Henry et al., 1977). In addition, the data reported here concern isolated mitochondria and mitochondrial membranes rather than whole cells.

Inositol deprivation in *S. carlsbergensis* is pleiotropic in effect, in many instances affecting membrane function as well as lipid metabolism (see Introduction; Angus and Lester, 1972). We have shown that inositol deprivation results in marked alteration in phospholipid and protein composition in the separated mitochondrial membranes, and in agreement with other investigators (Shafai and Lewin, 1968; Johnston and Paltauf, 1970) a marked decrease in phosphatidylinositol content, in particular, in the intact mitochondrial membranes. This alteration in membrane phospholipids, specifically of PI content, could be the initial effect of inositol deprivation in the inositol-requiring mutant and subsequently could result in some of the changes in membrane-associated functions seen in inositol-deprived cells.

For example, this change in the phospholipid composition may explain the loss of monoamine oxidase activity in mitochondria from inositol-deficient cells. It has been reported that lipid micelles of acidic phospholipids or mixtures of acidic and nonacidic phospholipids bind monoamine oxidase, but micelles of nonacidic phospholipids do not (Olivecrona and Oreland, 1971; Ekstedt et al., 1975). Inositol deprivation results in a

reduction of major acidic phospholipids in *S. carlsbergensis*. It is not known if *S. carlsbergensis* grown in the absence of inositol does not synthesize monoamine oxidase or if the enzyme is synthesized but not active. Phospholipid-reactivation studies did not demonstrate restoration of monoamine oxidase activity in the isolated outer membranes or in sonicated, unfractionated mitochondria. This result suggests that the monoamine oxidase is not present in a state that can be activated. However, a stronger support of this observation would be data from supplementation studies using micelles of individual phospholipids, particularly of PI. Studies using antibody to the monoamine oxidase protein or polypeptide(s) may resolve the question of lack of synthesis of the enzyme or synthesis of the enzyme in an inactive form. If monoamine oxidase is not synthesized due to the absence of a specific phospholipid (caused by inositol deprivation during growth), then phospholipid supplementation studies of mitochondrial preparations would not be expected to produce measurable monoamine oxidase activity. That polypeptide changes occur in inositol-deficient cells is supported by the data in Figures 1 and 2, which show that several polypeptides are significantly reduced or are absent in inositol-deficient mitochondria. however, it is not known yet whether these polypeptide changes are a result of altered protein synthesis or altered phospholipid environment and inability of the synthesized polypeptide to integrate with the membrane. If the 33 000 molecular weight species (peak 12) of *S. carlsbergensis* inner membrane is the same type of protein as its apparent counterpart in mammalian inner membrane (Capaldi, 1974), i.e., a hydrophobic protein, the loss or reduction of this hydrophobic species under conditions of inositolless growth may be related to the observed phospholipid changes.

Hydrophobic protein–phospholipid interactions are undoubtedly important to the functional integrity of the membrane. Lack of inositol in an inositol-requiring mutant results in loss of PI in the yeast mitochondrial membranes. Inositol deficiency may not affect phospholipid metabolism in the yeast cell, but the loss of PI from the mitochondrial membranes may alter the ability of other phospholipids to be incorporated into these membranes. An improper phospholipid environment could also alter the ability of certain proteins to be incorporated into the mitochondrial membranes or, once incorporated, to be functional in the membranes. In this way, inositol deficiency in an inositol-requiring yeast cell could result in gross alterations of the mitochondrial function and ultimately in the total cellular function.

Acknowledgments

The authors are especially grateful to Dr. Nagindra Prashad of the Department of Neurology. He gave generous help in the performance of gradient gel electrophoresis and advised us in its interpretation. The authors also thank Dr. Charles Hackenbrock and Mrs. Mary Tobleman, Department of Cell Biology, for initial help and interpretation with preparation of samples for electron microscopy.

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Topology of Amino Phospholipids in Bovine Retinal Rod Outer Segment Disk Membranes†

Richard C. Crain,‡ Guido V. Marinetti,* and David F. O'Brien

ABSTRACT: The topology of phosphatidylethanolamine and phosphatidylserine in bovine retinal rod disks has been studied with covalent probes. Phosphatidylethanolamine, which comprises 40% of the total membrane phospholipid, was found to be asymmetrically arranged with 18–27% located on the inner surface, 63–72% on the outer surface, and 6–14% not readily available to labeling with chemical probes. Phosphatidylserine, which comprises 10–13% of the total membrane phospholipid, was found to have an arrangement with 25–35% located on the inner surface, 25–31% on the outer surface, and 35–50% resistant to labeling with chemical probes. These results are based on the accessibility of phosphatidylethanolamine and phosphatidylserine to labeling by trinitrobenzenesulfonate at 0 °C in the presence and absence of valinomycin and to labeling by isethionyl acetimidate at 21 °C. Trinitrobenzenesulfonate very slowly penetrates the membrane at 0 °C, whereas it rapidly penetrates at either 21 or 0 °C in the presence of valinomycin. Cross-linking of retinal rod outer segment disks with the penetrating probe difluorodinitrobenzene at 21 °C is dependent on the concentration of difluorodinitrobenzene and the reaction temperature. Maximal cross-linking occurs at 75 μM difluorodinitrobenzene. At this concentration, 72% of phosphatidylethanolamine is cross-linked to phosphatidylethanolamine, 43% of phosphatidylserine is cross-linked to phosphatidylethanolamine, and 6% of the total phosphatidylethanolamine and 37% of the phosphatidylserine are cross-linked to membrane protein. Cross-linking of amino phospholipids to protein is not influenced appreciably by light exposure or temperature. Fatty acid analyses of cross-linked and non-cross-linked amino phospholipids indicate a random array of lipids with respect to their fatty acid content in the rod disk membrane.

Phospholipid asymmetry has been noted recently in a variety of biological membranes (for a review, see Gordesky, 1976; Rothman & Lenard, 1977; Bergelson & Barsukov, 1977). A number of studies used covalent chemical probes (Bretscher, 1972; Gordesky & Marinetti, 1973; Gordesky et al., 1975; Rothman & Kennedy, 1977), phospholipases (Verkleij et al., 1973; Chap et al., 1975; Nilsson & Dallner, 1977), or phospholipid-exchange proteins (Bloj & Zilversmith, 1976; Rothman et al., 1976) to measure asymmetry in both biological and artificial membranes. Furthermore, a reaction of red cell ghosts with the cross-linking agent DFDNB[1] has indicated the existence of clusters of amino phospholipids (Marinetti, 1977).

Several properties of rhodopsin, the major protein of the ROS disk membrane, are very sensitive to environmental factors. These properties of rhodopsin include its light sensitivity, its ability to be regenerated after bleaching, and the kinetics of its light-induced bleaching (Ebrey & Honig, 1975). In the native membrane these properties are dependent on lipid–protein interactions. Rhodopsin can be delipidated, purified, and reconstituted into phospholipid bilayers, digalactosyldiglyceride bilayers, and digitonin micelles. The rhodopsin in these recombinants exists in a regenerable configuration, a characteristic of native rhodopsin (Hong & Hubbell, 1973). It has also been found that the photochemical functionality of recombinants of rhodopsin in artificial phospholipid bilayers is relatively independent of the polar head-group composition of the bilayer but dependent on the lipid fluidity, which is determined by the degree of fatty acid unsaturation (O'Brien et al., 1977).

The composition of rod outer segment membranes which are primarily rod disk membranes has been reviewed (Daemen, 1973). The membrane protein is made up of 80–90% rhodopsin. The majority of the lipid of rod outer segment membranes is composed of phospholipids (80–90%). The lipids are characterized by a high content of polyunsaturated fatty acids. Thus, the rod outer segment membrane is highly unsaturated, has a low cholesterol content, and is very fluid. It has been suggested that the phospholipids may provide a fluid hydrophobic environment for the highly organized visual pigment system.

The present investigation was undertaken to elucidate the phospholipid topology in the ROS disk membrane. Preliminary studies (Raubach et al., 1974) have suggested that there is an asymmetric arrangement of PtdEtn and PtdSer in the disk

† From the Department of Biochemistry, The University of Rochester Medical Center, Rochester, New York 14642, and the Research Laboratories, Eastman Kodak Co., Rochester, New York 14650. *Received April 25, 1978.* This work was supported in part by Grant HLB-02063 from the National Institutes of Health. A portion of this work was presented at the American Society of Biological Chemists (ASBC) Meeting, Atlanta, Ga., June, 1978.

‡ Present address: Division of Nutritional Sciences, Cornell University, Ithaca, N.Y. 14853.

[1] Abbreviations used: ROS, rod outer segment; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; TNBS, 2,4,6-trinitrobenzenesulfonate; FDNB, 1-fluoro-2,4-dinitrobenzene; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMA, dimethylacetal; GC, gas chromatography; DNP, dinitrophenyl; TNP, trinitrophenyl; FDNB, fluorodinitrophenyl; Etn, ethanolamine; Ser, serine; MP, methyl picolinimidate; IAI, isethionyl acetimidate; EDTA, (ethylenedinitrilo)tetraacetic acid.

0006-2960/78/0417-4186$01.00/0