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BIOCHEMICAL CHARACTERISTICS OF THE OUTER MEMBRANES OF PLANT MITOCHONDRIA

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SUMMARY

Like the outer membranes of liver mitochondria, those of plant mitochondria are impermeable to cytochrome *c* when intact and can be ruptured by osmotic shock. Isolated plant outer mitochondrial membranes are also similar to the corresponding liver membranes in terms of phospholipid and sterol content. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis experiments indicate that a single class of proteins (apparent molecular weight 30 000) comprises the bulk of the plant outer membrane protein. There are also considerable amounts of polysaccharide associated with these membranes, which may contribute to their osmotic stability.

INTRODUCTION

Large yields of outer membranes can be isolated from plant mitochondria by controlled osmotic lysis followed by sucrose step gradient centrifugation [1, 2]. In this report, the protein and lipid compositions of these membranes are determined and shown to be similar to those of outer membranes isolated from animal mitochondria. The carbohydrate content of these membranes is also investigated and an interesting correlation is found between the uronic acid content and the osmotic stability of the outer membranes.

Preliminary results of these studies were presented at the Ninth International Congress of Biochemistry (Stockholm, 1973) [3] and at the Biochemistry/Biophysics 1974 Meeting (Minneapolis) [4].

MATERIALS AND METHODS

Membrane isolation and enzyme assays

The plant material used in these experiments were etiolated mung bean (*Phaseolus aureus*) hypocotyls, cut from seedlings grown for five days at 28 °C and 60 %

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relative humidity (unless otherwise specified), and white potato (*Solanum tuberosum*) tubers. Mitochondria from these tissues were isolated and purified by centrifugation on discontinuous sucrose gradients according to the method of Douce et al. [5]. The outer membranes were ruptured by rapidly pipetting mitochondrial pellets containing 75–100 mg protein/ml into 50 vols of a dilute sucrose solution (10 mosM final concentration) at 4 °C and stirring for 5 min. This suspension was then layered atop 0.6-0.9-1.2 M sucrose step gradients and centrifuged for 60 min at 40000 $\times g$ in a Beckman SW25.1 rotor. The purity of the outer membrane fractions obtained at the 0.6-0.9 M sucrose interface after centrifugation, in terms of enzymatic, spectroscopic and morphological criteria, has been defined in detail in previous reports [1, 2]. Similarly, the pellet after step gradient centrifugation of the swollen mitochondria has been shown to consist primarily of inner mitochondrial membranes plus matrix [1, 2]. For comparisons with outer mitochondrial membranes, light microsomal membranes were isolated by centrifuging the first high speed supernatant in the course of mitochondrial isolation at 20000 $\times g$ for 30 min, recentrifuging the membranes thus pelleted on sucrose step gradients like those used in outer membrane isolation and collecting the 0.6-0.9 M sucrose interface. In general, all membrane fractions collected from sucrose gradients were concentrated by dilution with three volumes of 10 mM potassium phosphate buffer (pH 7.2) and centrifugation at 60000 $\times g$ for 90 min.

Spectrophotometric assays of succinate : cytochrome *c* oxidoreductase, antimycin-insensitive NADH : cytochrome *c* oxidoreductase and malate dehydrogenase were performed as described by Douce et al. [2].

Protein analysis

The protein contents of membrane samples were determined by a modified Lowry method [6] or by the biuret method [7], both of which were found to give equivalent results.

Polyacrylamide gradient gel electrophoresis of outer membrane samples was performed in the laboratory of Dr. Nam Hai-Chua of The Rockefeller University. Membrane samples were prepared consisting of about 1 mg protein in 100 μl of a solution containing 0.1 M sodium carbonate and 0.1 M dithiothreitol. To each suspension were added 35 μl of 10 % sodium dodecyl sulfate, 25 μl of 60 % sucrose and 10 μl of 1 % bromphenol blue (a tracking dye), after which the suspensions were heated at 100 °C for 1 min. 5–20 μl of each sample were layered onto polyacrylamide gradient slab gels (7.5–15 % acrylamide; slab gel apparatus described by Studier [8]; discontinuous buffer system of Neville [9]). The gels were run at 15 mA for about 8 h, after which they were fixed and stained for protein with Coomassie Blue according to Fairbanks et al. [10]. The stained gels were dried and photographed and the absorbance patterns of positive transparencies measured with a Joyce Loebel microdensitometer. Each slab gel was calibrated for molecular weight by simultaneously running protein standards (cytochrome *c*, 11 700; myoglobin, 17 000; trypsin inhibitor, 21 000; DNAase, 31 000; bovine serum albumin, 68 000) in a different slot of the gel.

Lipid analyses

Total lipids were extracted from the membranes by the method of Bligh and Dyer [11].

For lipid phosphorus determinations, the methanol/chloroform phase was

evaporated to dryness under N_2, wet-ashed by the procedure of Chen et al. [12] and the phosphate present determined colorimetrically according to Johnson and Ulrich [13].

For fatty acid analyses, the methyl esterification procedure of Douce and Lance [14] was applied to the extracted lipids. The fatty acid methyl esters were subsequently suspended in petroleum ether and separated by gas-liquid chromatography (diethylene glycol succinate stationary phase column operated at 180 °C, Barber-Coleman Model 5000 Biomedical Gas Chromatograph).

For sterol determinations, neutral lipids were separated from the total lipid extract on a silicic acid column according to Parsons and Yano [15]. The sterol content of this neutral lipid fraction was determined by the Liebermann-Burchard color reaction [16] using cholesterol as the standard.

Carbohydrate analyses

Suspensions of outer membranes were dialyzed in the cold for 2×6 h against 1000 vols of 5 mM EDTA and 2×12 h against 1000 vols of distilled water to eliminate sucrose and to facilitate the detachment of polyuronides from the membranes. (The presence of polyuronides was suspected on the basis of positive reactions of outer membrane samples with both Erlich's lead subacetate and Dische's sulfuric acid/cysteine reagents [17].) The suspensions were removed from the dialysis tubing, incubated at room temperature at pH 11.5 (pH adjusted with 1 M KOH) for 45 min, followed by 90 min at 100 °C and pH 7 (pH adjusted with 5 % acetic acid). The mild alkali treatment saponifies the methyl esters of uronic acids and boiling at neutral pH causes dissociation of polyuronides by a transelimination reaction [18, 19]. The membrane suspensions were centrifuged ($25\,000 \times g$ for 60 min) and the supernatants concentrated using a Büchi rotary evaporator at 30 °C. The pellets were hydrolyzed in 2 M HCl, recentrifuged ($25\,000 \times g$ for 60 min) and the supernatants concentrated by overnight evaporation over solid NaOH in a desiccator.

Ascending paper chromatography was used to identify the sugars present in each supernatant. After evaporation, the residues were resuspended in 15 % isopropanol and run overnight on Whatman No. 4 chromatography paper, using the "pad" technique and the 80 % isopropanol solvent system described by Smith [20, 21]. After drying, the chromatograms were developed using the alkaline silver oxide, napthoresorcinol or aniline phosphate location reagents of Menzies and Seakins [22]. Sugar assignments given in the text to the resulting spots on the chromatograms were made on the bases of R_{Glc} values (i.e. the ratios of the distances travelled by the sugars from the origin to that travelled by glucose) and their color development using the different location reagents. All such assignments were confirmed by co-chromatography of the spots with the suspected sugars.

The carbohydrate contents of the different extracts were quantified by colorimetric assays. The uronic acid of the EDTA/boiling water extract was determined by the carbazole reaction of Dische as modified by Hart and Kindel [23], using galacturonic acid as the standard. The absence of significant interference by pentoses and hexoses in this reaction was confirmed by the color of the reaction mixtures (pink) and by the fact that addition of one half volume of water to the mixtures caused them to become colorless and not bright violet [24]. The phenol-sulfuric acid reaction of Dubois et al. [25] was used with glucose as the standard to assay the neutral sugar content of the acid hydrolysate.

RESULTS

Impermeability of the outer mitochondrial membrane to cytochrome c

As explained in previous reports [2, 5], unmasking of the inner membrane succinate : cytochrome *c* oxidoreductase as plant mitochondria are suspended in dilute media is a useful monitor of the extent to which the outer membranes have ruptured. The correlation between the inaccessibility of exogenous cytochrome *c* to inner membrane reducing sites and outer membrane integrity is illustrated by the results of Fig. 1. The relative yields of outer membranes from mitochondria incubated in successively more dilute sucrose solutions are low (8 % or less) until osmolarities are reached at which there is an abrupt increase in the succinate : cytochrome *c* oxidoreductase activity of the mitochondria. Thus, the outer membranes of plant mitochondria, like those of animal mitochondria, appear impermeable to cytochrome *c* when intact [26, 27] and can be ruptured by osmotic shock [28, 29]*.

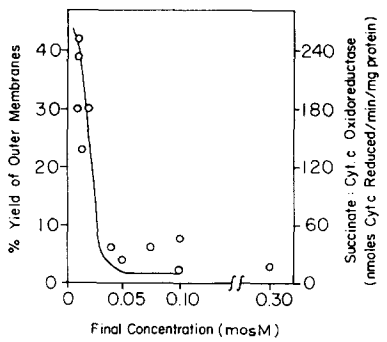

Fig. 1. Correlation between succinate : cytochrome *c* oxidoreductase activities of swollen mitochondria and outer membrane yields. Mung bean mitochondria (approx. 100 mg protein/ml) were incubated for 15 min in 50 vol of sucrose solutions of indicated final osmolarities. Percent yields of outer membranes (open circles) were calculated as the percent of total antimycin-insensitive NADH: cytochrome *c* oxidoreductase activity (an outer membrane marker activity [1, 2]) recovered at the 0.6–0.9 M sucrose interface after step gradient centrifugation of the mitochondrial suspensions (as described in Materials and Methods). The succinate: cytochrome *c* oxidoreductase profile of mung bean mitochondria (—) is redrawn from Fig. 1 of ref. 2.

Protein components of the outer membrane

Using the polyacrylamide gradient gel electrophoresis system described in Materials and Methods, 15 or more major protein bands are resolved in both inner mitochondrial membrane plus matrix and light microsomal membrane samples. Patterns of the outer mitochondrial membrane proteins, on the other hand, show but two prominent bands, centered at apparent molecular weights of 30 000 and 50 000

* These results argue against a recent suggestion [30] that the outer membranes of plant mitochondria are freely permeable to cytochrome *c* and that the increase in succinate: cytochrome *c* oxidoreductase activity seen upon hypotonic swelling of mitochondria is due solely to an inner membrane conformational change. The experimental results of the above-mentioned report can be readily explained by the presence in mitochondrial preparations of a fraction with broken or missing outer membranes, without invoking the conclusion that intact outer membranes are freely permeable to cytochrome *c*.

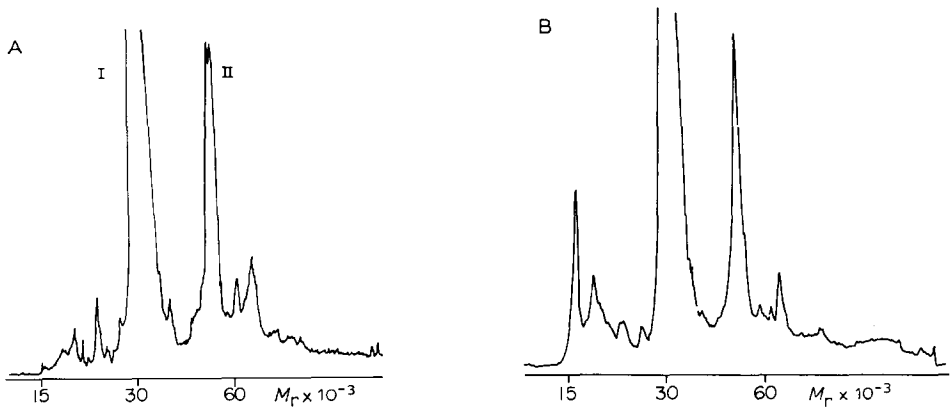

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis patterns of outer membranes of mung bean (A) and potato (B) mitochondria.

(labelled bands I and II in Fig. 2). Two components are resolvable in band II of mung bean outer mitochondrial membranes while only the lighter of the two appears in the potato outer membrane pattern. On the other hand, there is a fairly large peak near 15 000 molecular weight in the potato outer membrane pattern which is not detected in gels of mung bean outer membranes. The band I proteins of both types of outer membranes can be resolved into three principal components when the gels are underloaded. This is the case in Fig. 3 which is the gel electrophoresis pattern of the proteins of mung bean outer membranes after sonication, pelleting and resuspension. The principal effect of sonication is the selective loss of minor protein components with molecular weights greater than 50 000, indicating that the components of bands I and II are firmly attached to the outer membranes.

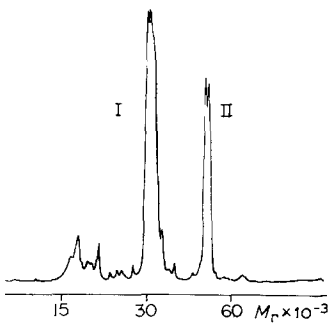

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis pattern of sonicated mung bean outer mitochondrial membranes. Isolated outer membranes (approx. 1 mg in 3 ml 10 mM potassium phosphate buffer, pH 7.2) were sonicated (2×15 s using Branson W 185 sonifier with microtip, 12 W output) and pelleted ($100\,000 \times g$ for 30 min). Subsequent sample preparation as described in text.

Lipid content and fatty acid composition of the outer membrane

The phospholipid and sterol contents of outer membranes isolated from potato and mung bean mitochondria are summarized in Table I. Also included in this table are the values reported for outer membranes isolated by osmotic lysis from liver mitochondria. The phospholipid : protein mass ratios of mung bean and potato outer mitochondrial membranes (0.49 and 0.50) fall between those determined for rat (0.35) and guinea pig liver (0.88) outer mitochondrial membranes, as does the value (0.63) recently reported for outer membranes isolated from cauliflower bud mitochondria [31]. The sterol : phospholipid molar ratio of mung bean outer mitochondrial membranes (1 : 5) on the other hand, is significantly higher than that of the corresponding liver membranes, while the value for potato outer membranes is the same as that of the guinea pig liver membranes (1 : 17).

TABLE I

LIPID COMPOSITION OF OUTER MITOCHONDRIAL MEMBRANES

Source	μmol lipid P	mg phospholipid*	μg sterol	Sterol**
	mg protein	mg protein	mg protein	Phospholipid (molar ratio)
Mung bean hypocotyl	0.67±0.10	0.49	53±10	1 : 5
Potato tuber	0.68±0.13	0.50 (0.51±0.08***)	17±6	1 : 17
Rat liver [33]	0.46±0.09	0.35	–	1 : 8
Guinea pig liver [15]	–	0.88±0.13	30±13	1 : 17

* Average phospholipid molecular weight = 730 for plants, 750 for liver.
** Molecular weight cholesterol = 390.
*** Determined by gas chromatography.

The value for the average phospholipid molecular weight (730), used to calculate the phospholipid content from the lipid phosphorus determinations of plant outer mitochondrial membranes, was arrived at from the phospholipid composition of the outer membranes of plant (potato tuber) mitochondria [32] and from the fatty acid determinations of Table II. Also presented in Table II are the fatty acid compositions of the light microsomal membranes and inner membrane plus matrix fractions of the same plant tissues. For both mung bean and potato mitochondria, the inner membrane fatty acids are considerably more desaturated than those of the outer membrane. This most likely reflects the high degree of desaturation of the cardiolipin (diphosphatidyl glycerol) of both animal [33] and plant [14] mitochondria, a phospholipid found exclusively in the inner membrane [32, 34]. Differences between outer mitochondrial and light microsomal membranes are not so consistent. The fatty acids of liver [33] and cauliflower [31] outer mitochondrial membranes have been reported to be more saturated than those of the light microsomal membranes. The opposite was found to be true for the membranes of mung bean hypocotyl, while the fatty acid compositions of the outer mitochondrial and light microsomal membranes of potato tuber are almost identical (Table II and ref. 35).

TABLE II

FATTY ACIDS OF PLANT MITOCHONDRIAL AND MICROSOMAL MEMBRANES

Percent composition of fatty acids of outer mitochondrial, inner mitochondrial and light microsomal membranes. Temperatures are those at which mung bean seedlings were grown. db/fa is the ratio of double bonds per fatty acid chain for each membrane system.

Fatty acid	Mung bean							Potato		
	Outer mitochondrial			Inner mitochondrial			Light microsomal 28 °C	Outer mitochondrial	Inner mitochondrial	Light microsomal
	15 °C	28 °C	40 °C	15 °C	28 °C	40 °C				
C_{16}	25	25	25	14.5	15.5	19.5	34	33.5	14.5	37
C_{18}	3.5	5	4	1.5	2	3	5	6	4	6
$C_{18:1}$	5.5	7	7.5	4	5	7.5	13	4	–	2
$C_{18:2}$	28.5	30	36	35	36	41	27	49	62	43
$C_{18:3}$	37	35	27.5	45	41	29	20.5	8	19.5	11
db/fa	1.74	1.72	1.62	2.09	2.00	1.77	1.29	1.26	1.83	1.21

Some disagreement exists in the literature regarding the amount of galactolipids present in the membranes of plant mitochondria. Schwertner and Biale [36], for example, contend that as much as 50 % of the lipid of potato tuber mitochondria is galactolipid and neutral lipid. Others [32, 31] find that phospholipids comprise essentially all of the acyl lipids of both membranes of plant mitochondria, suggesting that the galactolipids detected by the former are due to plastid contamination. Along these lines, a known amount of heptadecanoic acid (in ethanol) was included during the lipid extraction of potato outer membranes and the area of its gas-liquid chromatogram peak was used to calculate the absolute amounts of fatty acids present. The total amount of fatty acid determined in this way (370 μg/mg protein) was the same as that calculated on the basis of the phospholipid content (360 μg/mg protein), indicating that phospholipids are the only significant acyl lipid moiety associated with these membranes.

The affect of growth temperature on the fatty acid composition of mung bean hypocotyl mitochondrial membranes was also determined. As summarized in Table II, both the inner and outer membrane fractions contain significantly more desaturated fatty acids when grown at lower temperatures, with the largest change occurring in the ratios of linoleic ($C_{18:2}$) to linolenic ($C_{18:3}$) acids. These results are consistent with the well documented trend of the acyl lipids of poikilotherms to be less saturated at lower environmental temperatures although this may be the first such determination for particular membrane systems from higher plants.

In terms of bulk composition, fatty acids of rye seedlings show a similar shift from linoleic to linolenic acid when grown in the cold [37] while, for cells of tree bark, the ratio of oleic ($C_{18:1}$) to linoleic acid decreases [38].

Carbohydrate composition of the outer membrane

A single monosaccharide was detected in paper chromatograms of the EDTA/boiling water extract of both mung bean and potato outer mitochondrial membranes. This spot co-chromatographed with both galacturonic and glucuronic acid, but the

TABLE III

CARBOHYDRATE COMPOSITION OF OUTER MEMBRANES OF PLANT MITOCHONDRIA

Sugars present	EDTA/boiling water extract	Acid hydrolysate
	Galacturonic acid	Glucose Galactose Mannose
μg/g protein		
Mung bean	38±11	39±15
Potato	30–95	35±15

absence of lactone formation in acid solvent (pH adjusted to 1 with 1 M HCl) implies that it is galacturonic acid [39]. Chromatograms of the acid hydrolysate of the outer membranes revealed three sugar spots, corresponding to glucose and smaller amounts of galactose and mannose.

Table III summarizes the results of the colorimetric sugar assays of the extracts of mung bean and potato outer mitochondrial membranes. Both membranes contain about 40 μg neutral sugar per mg protein, with the experimental spread over three determinations as indicated. Mung bean outer mitochondrial membranes contain an equal amount of uronic acid but the outer membranes from potato mitochondria display a rather wide spread in values, ranging from 30 to 95 μg uronic acid per mg membrane protein.

Osmotic stability of the outer membrane

Polyuronides are primary constituents of plant cell walls and so are present in large amounts in plant tissue homogenates. Thus it is possible that the uronic acid detected in plant outer mitochondrial membrane fractions became attached to these membranes in the course of tissue disruption. There is evidence, on the other hand,

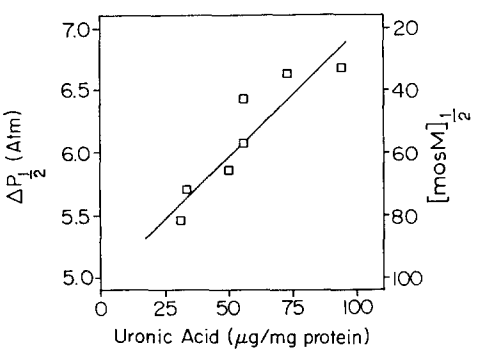

Fig. 4. Dependence of outer mitochondrial membrane osmotic stability on endogenous uronic acid content. $\Delta P_{\frac{1}{2}}$ is the osmotic shock at which the succinate: cytochrome *c* oxidoreductase activities of the different potato mitochondrial preparations are half maximum. $[mosM]_{\frac{1}{2}}$ is the corresponding final sucrose concentration. The straight line is least-squares fit to the data.

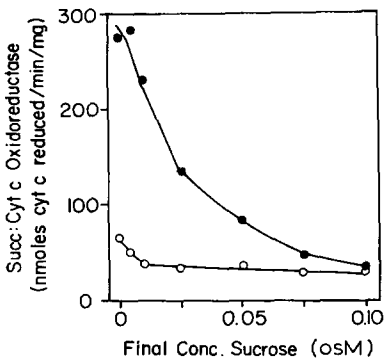

Fig. 5. Effect of mitochondrial incubation in presence of polyuronide on outer membrane rupture. Potato mitochondria (20 mg protein) stirred at 4 °C for 1 h in 5 ml of solution containing 0.3 M mannitol, 10 mM phosphate buffer (pH 7.2) and 5 mg (○) or 0 mg (●) polygalacturonic acid (Pectin, Sigma). After incubation, the two mitochondrial fractions were pelleted (10 000 × *g* for 15 min) and aliquots of each (containing approximately 2 mg protein) were added to 5 ml of sucrose solutions of the indicated final osmolarity. After 15 min at 4 °C, the succinate: cytochrome *c* oxidoreductase activities of the suspensions were monitored.

which suggests that this polysaccharide plays an important structural role in this membrane. Fig. 4 is a plot of the magnitude of the osmotic shock needed to rupture half of the outer membranes of different potato mitochondrial preparations against the uronic acid contents of the outer membranes isolated from the preparations. There is an empirical correlation between osmotic stability and polyuronide content, suggesting that the polysaccharide contributes to the mechanical strength of this organelle membrane. Subsequent observations that preincubation of potato tuber mitochondria in polygalacturonic acid prevents the osmotic rupture of outer membranes (Fig. 5) while pre-incubation in galacturonanase weakens the outer membranes (Fig. 6) support this hypothesis.

Endogenous polyuronide was found to dissociate from these membranes in the presence of EDTA. In the experiments summarized in Table IV, dialysis against 5 mM

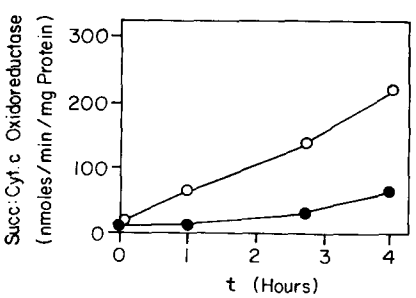

Fig. 6. Effect of polygalacturonase on osmotic fragility of outer mitochondrial membranes. Potato mitochondria (20 mg protein) were stirred at 20 °C in 20 ml of solution containing 0.3 M sucrose, 10 mM phosphate buffer (pH 7.2) and 25 mg (○) or 0 mg (●) of polygalacturonase (Sigma), an enzyme which hydrolyzes polygalacturonic acid. At indicated times, 1 ml was removed from each mitochondrial suspension and added to 2 ml distilled water. After 10 min, the succinate: cytochrome *c* oxidoreductase activity of each aliquot was assayed spectrophotometrically.

TABLE IV

EFFECTS OF DIALYSIS ON OUTER MITOCHONDRIAL MEMBRANE COMPOSITION

Marker enzyme specific activities and protein and polyuronide contents of isolated potato outer membranes after overnight dialysis against 200 vol of 10 mM potassium phosphate buffer, pH 7.2, containing indicated concentrations of EDTA or $CaCl_2$ and centrifugation (100 000 × *g*, 30 min).

	Protein content (mg)	NADH: Cytochrome *c* oxidoreductase (nmol/min per mg protein)	Uronic acid content (μg)
Initial values	0.76	127	31
After dialysis against			
5 mM EDTA	0.68	94	10
5 μM $CaCl_2$	0.73	102	32

EDTA followed by centrifugation of potato outer mitochondrial membranes resulted in a two-thirds decrease in the uronic acid content of the membranes. When, on the other hand, the dialysis was against 5 μM $CaCl_2$, the polyuronide was retained by the outer membranes. These results implicate divalent cations in the mode of binding of the negatively charged polysaccharides to these membranes and may explain the increase in outer membrane fragility observed for plant mitochondria in the presence of EDTA (Fig. 7).

DISCUSSION

While the basic similarities in gross lipid composition of animal and plant mitochondrial membranes are noteworthy, the similarities in terms of outer membrane protein components are even more striking. The outer membranes of beef heart mitochondria, isolated by freezing and thawing the mitochondria in hypotonic media, also display two principal protein bands in sodium dodecyl sulfate-polyacrylamide

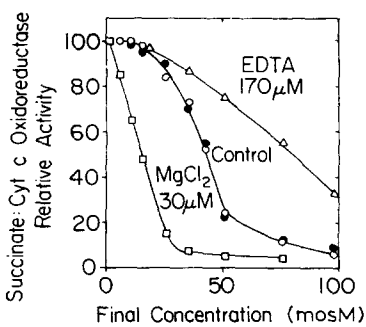

Fig. 7. Effects of Mg^{2+} and EDTA on osmotic stability of outer membranes of potato mitochondria. Succinate: cytochrome *c* oxidoreductase activities of potato mitochondrial preparations assayed as described in legend of Fig. 5 except for inclusion of indicated amounts of $MgCl_2$ or EDTA in the osmotic swelling media.

gel electrophoretograms, with molecular weights centered near 30 000 and 50 000 [40]. These bands were reported to contain about 50 and 20 % respectively of the total membrane protein, the same approximate proportions as are contained in bands I and II of plant outer membrane gels (estimated from the relative areas under the peaks of the microdensitometer traces of Fig. 3). Sodium dodecyl sulfate-polyacrylamide gel patterns of the proteins of rat liver outer mitochondrial membranes (isolated by hypotonic swelling) also display prominent bands in the neighborhood of 30 000 and 50 000 molecular weight, along with two other bands near 15 000 and 70 000 [41]. (Interestingly, potato tuber outer mitochondrial membranes contain a significant 15 000 molecular weight protein component.) The outer membranes of *Neurospora crassa* mitochondria have also been isolated and found to contain a single major protein component of molecular weight 30 000 [42, 43]. The possibility that this last protein moiety, common to all outer mitochondrial membranes studied to date, may comprise a detectable subunit structure in the plane of these membranes, is brought out in the following report [44].

In contrast, the relative amount of polysaccharide associated with outer mitochondrial membranes may represent a significant distinction between plant and animal mitochondrial membranes. Parsons et al. [34] have reported a negligible carbohydrate content for liver outer mitochondrial membranes while we find carbohydrates to make up as much as 10 % of the total membrane mass. This may, in fact, represent an even higher proportion in vivo, in light of the use of divalent metal ion chelators in the course of mitochondrial isolation.

The weakening effects which EDTA exerts on the outer membranes of plant mitochondria might be thought to involve interactions of divalent cations with the lipid and/or protein moieties of these membranes, instead of with the polyuronide components as proposed above. Calcium is known, for example, to have a condensing or rigidifying effect on lipid monolayers and bilayers. Such effects are most pronounced, however, with net negatively charged lipids such as fatty acids, cardiolipin, phosphatidylserine and phosphatidic acid, while the two phospholipids which constitute the bulk of plant outer mitochondrial membrane lipids, phosphatidylcholine and phosphatidylethanolamine [31], are essentially unaffected by Ca^{2+} [45–48]. It is also unlikely that EDTA effects the binding of outer membrane protein since overnight dialysis of outer membranes against 5 mM EDTA did not significantly decrease the protein content of these membranes (Table IV).

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