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Pore Size and Properties of Channels from Mitochondria Isolated from *Neurospora crassa*

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Summary. A triton X100 extract of mitochondria, isolated from a wall-less mutant of *Neurospora crassa*, can be used to insert channels into planar lipid bilayers. These channels have the same properties as the VDAC channels previously reported (Colombini, 1979, *Nature (London)* **279**:643) in the outer membrane of rat liver mitochondria. When large multiwalled liposomes are produced from mixtures of phospholipids and *N. crassa* mitochondrial membrane material, these liposomes are now permeable to nonelectrolytes up to the size of polyethylene glycol of 3400 mol wt. This yields an estimated radius for the channels inserted into the liposomes of 20 Å.

It is proposed that VDAC is the channel which allows the outer mitochondrial membrane to be permeable to small molecules and that this channel has a pore size of 20 Å in radius.

The outer mitochondrial membrane is known to be permeable to small molecules while being impermeable to large polymers (Werkheiser & Bartley, 1957; O'Brien & Brierley, 1965; Pfaff et al., 1968; Wojtczak & Zuluska, 1969). This sieving property is characteristic of channels. Evidence for channels in the outer membrane of plants has been reported from electron microscopic studies (Parsons, Bonner & Verboon, 1965; Parsons, Williams & Chance, 1966) and X-ray diffraction (Mannella & Bonner, 1975).

Channels have been extracted (Schein, Colombini & Finkelstein, 1976; Colombini, 1979) from the mitochondria of a variety of organisms ranging from protozoans to mammals. These channels, after insertion into a planar lipid bilayer, have the following properties in common which characterize them: (i) the permeability to ions which they induce in the lipid bilayer is quantized into units of uniform size; (ii) the permeability is greater for anions than cations of comparable size and charge; (iii) the permeability decreases as the transmembrane voltage is increased in both the positive and negative direction. Thus this permeability is produced by voltage dependent anion-selective channels (VDAC). These properties which characterize VDAC distinguish it from all other conducting pathways that have been observed in lipid bilayers. Since similar molecules have been found in mitochondria from a wide variety of species, the term VDAC is being used to refer to all these molecules in much the same way that the same enzyme in different species is given the same name. VDAC channels have been shown to be located in the outer membrane of rat liver mitochondria (Colombini, 1979) and are a good candidate for the major permeability pathway of the outer mitochondrial membrane.

This paper describes the properties of VDAC from *Neurospora crassa* mitochondria and reports an estimate for the pore size of this channel.

Materials and Methods

Preparation of Mitochondria

The culture of *sl Neurospora crassa* was a generous gift from Dr. Gene A. Scarborough. The cells were grown and maintained as he described (Scarborough, 1978).

Mitochondria were isolated by differential centrifugation. Cells grown in 250 ml of suspension culture were centrifuged at $1{,}000 \times g$ for 10 min and gently suspended in 0.15 M sucrose, 10 mM Tris Cl pH 7.6, 0.1 mM Na_2 EDTA (final volume of 30 ml). The cells were broken by means of a glass-Teflon tissue homogenizer (breakage was monitored microscopically). The lysate was centrifuged at $1{,}000 \times g$ for 10 min, and the supernatant thus obtained was centrifuged at $12{,}000 \times g$ for 30 min. This pellet was resuspended in 0.25 M sucrose, 10 mM Tris Cl, pH 7.6, 0.1 mM Na_2 EDTA. This suspension was centrifuged at $12{,}000 \times g$ for 30 min, and the resulting pellet was suspended in 1 mM KCl, 1 mM HEPES (Na^+ salt, pH 7.0), 15% (vol/vol) DMSO (the final protein concentration was 1 to 5 mg per ml). This material was stored at −20 °C. In this form the activity is stable for at least 5 months.

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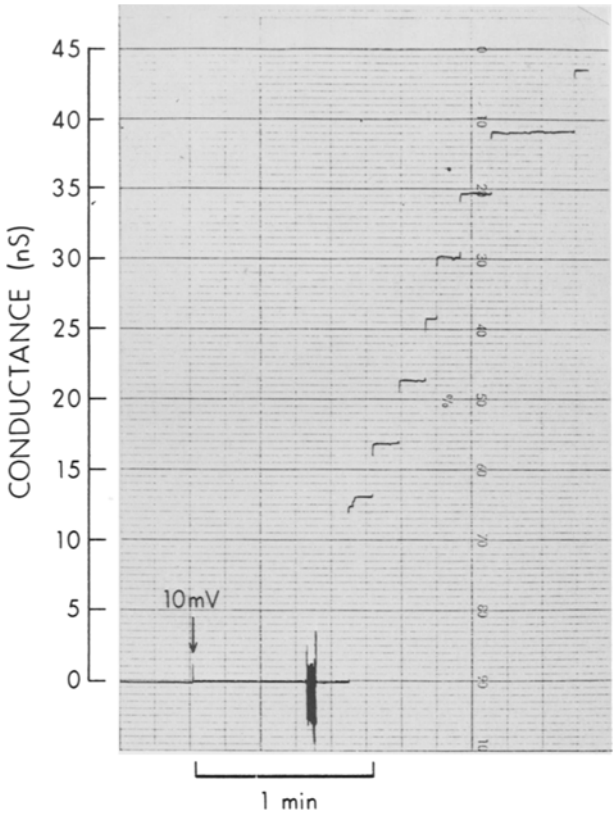

Fig. 1. Insertion of VDAC into a planar lipid bilayer. A planar lipid bilayer was produced, using asolectin (soybean phospholipids), by the monolayer technique. (The aqueous phase was 1.0 M KCl, 5 mM $CaCl_2$.) The transmembrane voltage was clamped on 0 mV until the point indicated by the arrow; after that point the voltage was 10 mV. At the point indicated by the noisy record, 5 µl of a triton X100 extract of *sl* mitochondria (*see* Materials and Methods) was added to the aqueous phase on one side of the membrane

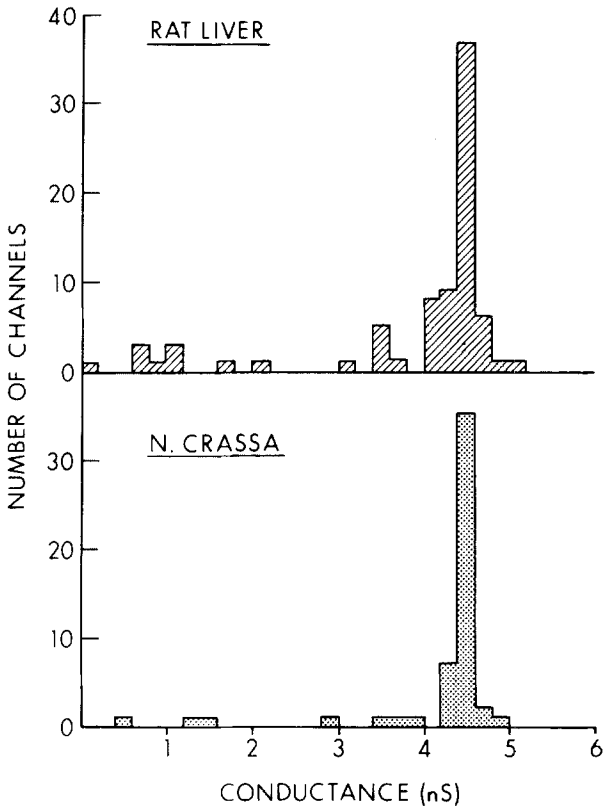

Fig. 2. Histogram of abrupt conductance changes observed in a planar bilayer treated as described in Fig. 1. The data was obtained in the presence of 1.0 M KCl, 5 mM $CaCl_2$ in the aqueous phases. The triton extract of rat liver mitochondria was obtained in a similar way to that described for *sl* mitochondria in the Materials and Methods section

Preparation of Liposomes

Control liposomes were made by the method previously described (Bangham, DeGier & Grenville, 1967). Forty-six milligrams of egg phosphatidyl choline and 4 mg of egg phosphatidic acid were dried down, (*in vacuo*) by means of a rotary evaporator, in a round bottom flask. Liposomes were produced upon addition of 2.5 ml of 20 mM KCl, 1 mM EDTA (Na^+ salt, pH 7.0) to the dried lipid followed by swirling.

Liposomes containing mitochondrial protein were produced by adding, to the lipid which was dried down as described above, mitochondrial membranes (1 mg protein per 20 mg dried lipid) in 1 mM KCl, 1 mM HEPES, (Na^+ salt, pH 7.0) such that the final lipid concentration was 20 mg per ml. In order to obtain the mitochondrial membrane suspension, mitochondria produced and stored as described above were diluted 15-fold with 1 mM KCl 1 mM HEPES (Na^+ salt, pH 7.0), and centrifuged at 27,000 × *g* for 10 min at ~4 °C. The pellet was suspended in the KCl-HEPES solution to a final concentration of 1 mg protein per ml.

The lipid-mitochondrial membrane suspension was sonicated at ~0 °C in the Cup Horn attachment to the Branson Sonifier (model W185) at setting #10 (140 W) for a few minutes (until clarified). The material was lyophilized in aliquots and stored at −20 °C. Liposomes were produced by suspending this material with 20 mM KCl, 1 mM EDTA (Na^+ salt, pH 7.0), to a final lipid concentration of 20 mg per ml.

Preparation of Triton Extract of Mitochondria

Mitochondria were isolated from *sl* cells and stored as described above. To the mitochondrial suspension (< 10 mg protein/ml) was added sufficient 20% (vol/vol)triton X100 to yield a final detergent concentration of 2% (vol/vol). This extract was allowed to stand at room temperature for at least 1 hr prior to assay. For extended periods of time it was stored at 4 °C. When the extract was again used after being stored at 4 °C it was allowed to stand at room temperature for at least one hour prior to use. Extracts which were more than one week old were discarded. Sodium azide (0.05% (wt/vol) final concentration) may be added to the extract to prevent bacterial growth.

Miscellaneous

Protein was assayed by the method of Lowry et al., 1951. Egg phosphatidyl choline (P5763) was obtained from Sigma Chemical Co. (St. Louis, Mo.); egg phosphatidic acid (860101) was obtained from Avanti Biochemicals, Inc. (Birmingham, Ala.); asolectin was prepared as described previously (Schein et al., 1976). All other chemicals were reagent grade.

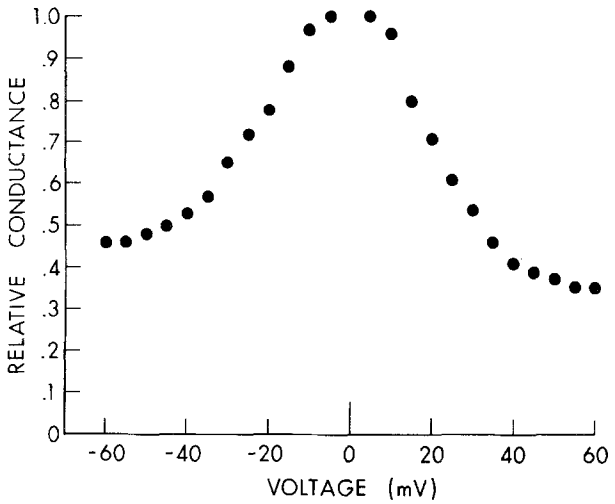

Fig. 3. The variation of conductance with voltage of a VDAC-containing membrane. A membrane treated as described in Fig. 1 was observed until the rate of conductance increase due to VDAC entry was small compared to the amount of conductance already present in the membrane. A triangular voltage waveform was applied to the membrane spanning the voltage range from −60 to +60 mV at a frequency of 5 mHz (*see* Schein et al., 1976) and the current monitored. After verifying that, as described previously for VDAC from *P. aurelia* mitochondria (Schien et al., 1976), the rate of channel turn-on was fast (a few msec), the part of the response to the triangular wave in which the channels were turning on (high field to low field) was used to construct the figure shown. At 5 mV intervals the current was read and the conductance calculated. The sign of the voltage refers to the side to which the mitochondrial extract was added to the aqueous phase

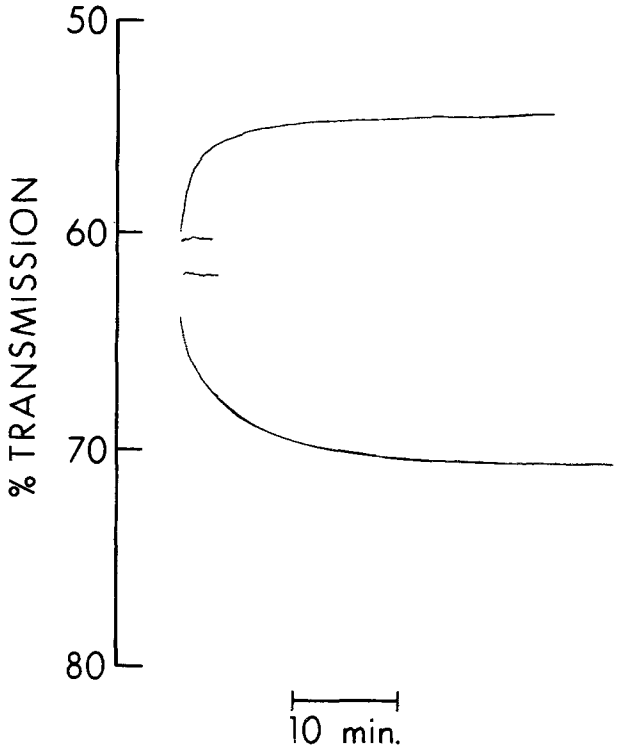

Fig. 4. Response of control liposomes to changes in medium tonicity. A spectrophotometer set to read a wavelength of 400 nm was used to monitor changes in % transmission which is a measure of the amount of light scattered by the liposomes. Aliquots of 150 µl of liposomes prepared as described in Materials and Methods were suspended in 2.5 ml of 80 mM sucrose (top trace) or 20 mM KCl, 1 mM Na_2 EDTA, pH 7.0 (next lower trace), or 40 mM sucrose (next lower trace) or 16 mM sucrose (lowest trace)

Results

Properties of Channels from sl Neurospora crassa

Planar lipid bilayers were made by the monolayer technique as described previously (Schein et al., 1976). The aqueous phase used in all experiments described in this report was symmetrical 1.0 M KCl, 5 mM $CaCl_2$. In the presence of a 10-mV transmembrane potential 5 µl of the triton[1] extract of *sl* mitochondria was added to the aqueous phase. After a short lag period, conducting units entered the bilayers as shown in Fig. 1. Most of the units have a conductance of 4.5 nS as shown in the histogram (Fig. 2). The same experiment done on rat liver mitochondria yields a histogram which is remarkably similar to that for the *sl* mitochondria. One major difference, not shown on the histogram, is the presence of conducting units which are multiples of the basic 4.5-nS conductance. These multiple units are frequently observed in experiments with *sl* mitochondria but rare or nonexistent in experiments with rat liver mitochondria. They occur too frequently in *sl* experiments to be merely coincidental insertion of independent channels simultaneously. An example of one of these multiples is the first insertion in Fig. 1. This particular insertion is three times the basic unit size. It is believed that these may be aggregated channels inserting as a unit.

Another feature of the histogram is the presence of smaller units, especially in the case of rat liver. These smaller units, generally speaking, fall into two categories: units ~1 nS and units ~3.5 nS. Examples of these are the second and third insertions in Fig. 1. It is felt that these represent substates of the whole channel. Not only do their conductances add up to 4.5 nS, but also, in any one experiment, the number of 1 nS insertions is roughly equal to the number of 3.5 nS insertions. The 3.5-nS insertion is probably the substate of the channel. When the channel goes to the 4.5 nS state, one observes a transition of 1 nS.

Figure 3 shows how the conductance of a population of these channels varies with transmembrane voltage.

[1] Triton X100 is not responsible for the observations. In our hands, the addition of triton does not produce changes in membrane conductance which are reproducible or recognizable as anything other than membrane breakdown. Moreover, the original observations on VDAC (Schein et al., 1976) were done in the absence of any detergents.

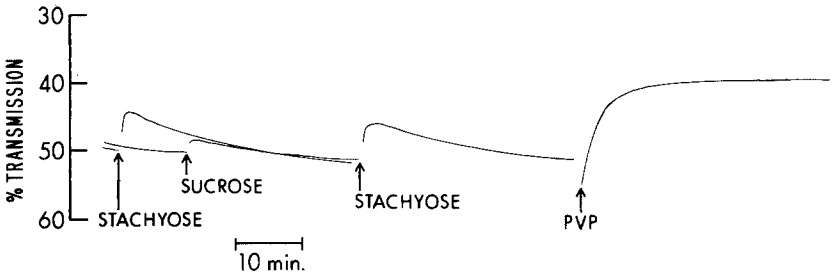

Fig. 5. Responses of mitochondrialstoffs-containing liposomes to sucrose, stachyose, and polyvinylpyrrolidone. Aliquots of 150 µl of liposomes prepared as described in Materials and Methods (containing mitochondrial protein) were added to 2.5 ml of 20 mM KCl, 1 mM Na_2 EDTA, pH 7.0. The figure shows two such experiments. In the first, 40 µl of 2.0-M sucrose was added to the cuvette after the initial transient due to the dilution of the liposomes had subsided. After the sucrose-induced transient had subsided, 100 µl of 0.73-M stachyose was added and later 100 µl of 8 g per 10 ml of polyvinylpyrolidone (40,000 average mol wt) was added. In the second experiment, fresh liposomes diluted as described above were treated with 100 µl of 0.73-M stachyose at the time shown. Note that the response to stachyose is essentially the same in both experiments

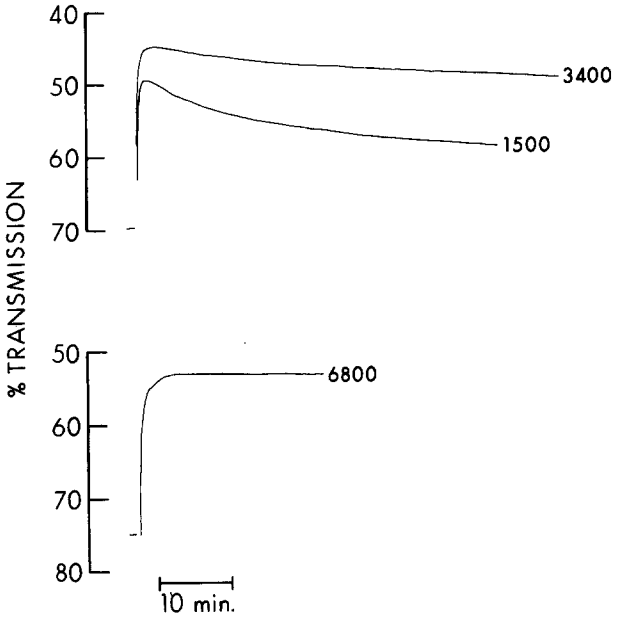

Fig. 6. Response of mitochondrialstoffs-containing liposomes to polyethylene glycols of the molecular weights indicated. To liposomes prepared and diluted as described in Fig. 5 was added 200 µl of 100 mM polyethylene glycol (PEG) of the molecular weight indicated on the figure. The concentration of the 6800 mol wt PEG was only 50 mM, due to limited solubility

Table 1. Estimation of pore size

PEG[a]	$\bar{r}_{es}$[b] (Å)	$\bar{r}_g$[b] (Å)	Permeability
1300–1600	12	12	+
3000–3700	19	18	+
6000–7500	29	26	–

[a] The mol wt range of the polyethylene glycol used was obtained from the supplier, Union Carbide.

[b] The equivalent hydrodynamic diffusion radii or Einstein-Stokes radii ($\bar{r}_{es}$) and the statistical radii of gyration ($\bar{r}_g$) are those reported previously by Scherrer and Gerhardt (1971).

Pore Size of Channels from sl Neurospora crassa

Multi-walled liposomes were prepared, as described in the Methods section, with and without *sl* mitochondria. Figure 4 shows how the control liposomes (without mitochondria) respond to osmotic pressure changes. The liposomes, prepared in 20 mM KCl, 1 mM Na_2 EDTA, pH 7, were added to solutions of varying composition. The optical density was monitored at 400 nm. Liposomes suspended in 20 mM KCl, 1 mM Na_2 EDTA, pH 7.0, or in 40 mM sucrose did not vary in size with time evidenced by no change with time of the light transmitted through the cuvette. On the other hand, an exponential decrease and increase in percent transmission was observed when liposomes were transferred to 80 and 16 mM sucrose, respectively. This is interpreted as liposome shrinkage and swelling, respectively, as was shown by Bangham, De Gier and Greville (1967).

Similar experiments done on mitochondrial-protein containing liposomes show biphasic curves (Figs. 5 and 6). When the osmotic pressure of the medium outside the liposomes is increased by the addition of a solute, the percent transmission decreased transiently reaching a peak followed by an increase toward the initial value. This is interpreted as a transient shrinkage followed by a reswelling due to the fact that the water moves out of the liposomes faster than the solute can move in. As the size of the solute increases, the amount of shrinkage increases and the reswelling rate decreases (Figs. 5 and 6). Stachyose, a tetrose of mol wt 666, causes a much greater transient swelling than sucrose does. The transient swelling can be evoked several times on the same liposomes by adding more solute. When a series

of polyethylene glycols[2] (PEG) of the average molecular weights indicated in Fig. 6 are used, the pore size of the channels produced in these liposomes can be estimated. PEG-3400 causes shrinkage followed by slow reswelling, while PEG-6800 causes shrinkage but no detectable reswelling was observed.

Discussion

Multilamellar lipid vesicles can be made permeable to a variety of small nonelectrolytes by doping them with material from mitochondria of *N. crassa*. This permeability is manifested by the observation that the liposomes are only transiently affected by increasing the medium osmotic pressure with small nonelectrolytes (<3400 mol wt). The permeability cut-off between 3400 and 6800 mol wt is a characteristic property of a large channel and not of a damaged membrane. It is proposed that these channels are the same as those observed in the planar bilayer.

Although the polyethylene glycols used were not monodisperse (Table 1), their molecular weight range was rather narrow. The radii of these polymers are those reported by Scherrer and Gerhart (1971) showing good agreement between the two methods of estimating the radius. The results indicate that the pore size of the channels is approximately 40 Å in diameter.

The heterogeneity of the polyethylene glycol might be expected to make this estimate an upper limit. The initial liposome contraction phase must be due to water movement out of the liposome in response to the osmotic pressure difference while the subsequent expansion is due to the movement of nonelectrolyte and water back into the liposome. In a heterogeneous sample, the smaller molecular weight components may be permeable causing only partial reswelling. In the experiment involving the 3400 mol wt polyethylene glycol, the liposomes continue to reswell at a slow rate for an hour without detectable saturation. Hence a large fraction of this material must be able to enter the liposome. On the other hand, the 6800 mol wt material shows no sign of reswelling, indicating that very little of this material can enter the liposome.

The channel's estimated radius (20 Å) is consistent with the known permeability properties of the outer mitochondrial membrane. Inulin (Stokes-Einstein radius of 14 Å – Scherrer & Gerhart, 1971) was shown to be capable of penetrating the outer membrane by Werkheiser & Bartley (1957). On the other hand, Wojtczak & Zaluska (1969) were able to demonstrate that cytochrome *c* ($30\times34\times34$ Å – Dickerson et al., 1971) added to intact mitochondria did not have access to the intramembrane space. Although, at first glance, the cytochrome *c* results seem to argue for a smaller pore size, it must be remembered that cytochrome *c* is highly positively charged. On *a priori* grounds, charged molecules would be expected to be less permeable than uncharged molecules because they must meet both the steric requirements and electrical constraints. For example, work by Gunnar Wallenius (1954) showed that the glomerular permeability to hemoglobin (radius 32 Å) and albumin (radius 35 Å) was very low despite a measured effective radius of the permeability barrier of 50 Å using nonelectrolytes. Clearly VDAC's preference for anions would compound the situation. The estimate of 25 to 30 Å made by Parsons et al. (1965) for the pores observed in negatively stained outer membranes might be somewhat low or reflect a specie difference.

It is interesting to compare these findings to those done on porin, a channel in the outer membrane of *E. coli*. The size cut-off for porin is about 600 mol wt (Nakae, 1976). This smaller pore size is consistent with the lower conductance of porin as compared to VDAC (2 nS (Benz et al., 1978) *vs.* 4.5 nS for VDAC in 1.0 M KCl). On the other hand, the outer membranes of *Pseudomonas aeruginosa* contain channels with an exclusion limit which is very similar to that described in this paper (Hancock & Nikaido, 1978).

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Note added in proof. Carmen A. Mannella and Anthony J. Ratkowski have submitted an abstract to the 19th annual meeting of the American Society of Cell Biology (Nov. 4 to 8, Toronto, Canada) in which they report that the X-ray diffraction patterns of outer mitochondrial membranes of plant and fungal origin fit well with a model of hollow cylinders 40 Å internal diameter and 55 Å external diameter. In these organisms the major protein in the outer mitochondrial membrane appears to be the channel.

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[2] Although low molecular weight polyethylene glycols are permeable through lipid bilayers, molecules with molecular weights greater than 1,000 are sufficiently impermeable to be useful in these experiments. Control experiments done with liposomes (without protein) showed no significant permeability for PEG-1500 and PEG-3400.

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