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Large Cation-Selective Pores from Rat Liver Peroxisomal Membranes Incorporated to Planar Lipid Bilayers

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Summary. Fusion of a highly purified fraction of rat liver peroxisomal membranes to planar lipid bilayers incorporates large, cation-selective voltage-dependent pores. The P_K/P_{Cl} ratio of these pores, estimated in KCl gradients, is close to 4. The pores display several conductance states and spend most of the time open at voltages near 0 mV, closing at more positive and negative voltages. At voltages near 0 mV the most frequent open state has a conductance of 2.4 nS in 0.3 M KCl. At voltages more positive and more negative than 10 mV the most frequent open state displays a conductance of 1.2 nS in 0.3 M KCl. With these results pore diameters of 3 and 1.5 nm, respectively, can be estimated. We suggest that these pores might account for the unusually high permeability of peroxisomes to low molecular weight solutes. Fusion also incorporates a perfectly anion-selective, two-open states channel with conductances of 50 and 100 pS in 0.1 M KCl.

Key Words peroxisome · fusion · planar bilayer · pore

Introduction

The properties and specific differentiation of the membranes in cell organelles are largely responsible for their structural and functional properties. Experimental evidence shows that similar to plasma membranes, organelle membranes have sites of recognition, enzymes, cytochromes and specific proteins. They also have special mechanisms involved in ionic and neutral solute translocation, such as the large anion-selective pores present in the outer membrane of mitochondria and chloroplasts [2] and the calcium and potassium channels present in the sarcoplasmic reticulum from vertebrate skeletal muscle [9, 17, 23]. A better knowledge of the distribution and properties of these channels or pores is helpful in understanding the control of molecular traffic inside the cell.

Peroxisomes constitute a family of subcellular organelles characteristic of eucaryotic cells. Their function is only partially understood, particularly for animal peroxisomes [6, 8, 25]. Their involvement in various aspects of lipid metabolism has been stressed, particularly after they were shown to oxidize fatty acids via a β-oxidative pathway [10], and more recently, because the metabolic errors recognized in a genetic disorder found in humans have been associated to the complete or almost complete absence of peroxisomes [8, 22].

On the basis of indirect evidences it is known that several different molecules pass across the peroxisomal membrane, but the molecular basis of this translocation is not understood for any of them. It is well known that the peroxisome membrane is unusually permeable to low molecular weight solutes [6], but CoA-thioesters fatty acid do not cross it freely [12, 24]. Proteins incorporate post-translationally into peroxisomes from the cytoplasm by mechanisms not well established [11]. In addition, proteins easily leak in vitro from peroxisomes [13], a phenomenon responsible for at least part of the enzymes present in high-speed supernatants from tissue homogenates and for the presence of peroxisomal ghosts in particular fractions observed by electron microscopy [6, 13].

To gain insight into the mechanisms responsible for the peculiar permeability properties of peroxisomes, studies were performed taking advantage of methods now available for the isolation of these organelles [27, 28] and their membranes [7, 16] and also the development of techniques that allow to incorporate membrane fragments to planar lipid bilayers, rendering their permeability properties susceptible to electrophysiological analysis [18].

We report here some results which constitute initial findings in the study of peroxisomal membrane permeability using the fusion method developed by Miller [19]. The fusion of peroxisomal membranes to planar lipid bilayers results in the incorporation of pores, slightly cation selective. The presence of these channels could well account for some of the permeability properties of peroxisomes.

Materials and Methods

Membrane Preparation

Peroxisomes were isolated from normal male rat liver by isopycnic equilibrium in metrizamide gradients loaded with a peroxisome-rich fraction obtained by differential centrifugation [5, 12, 27]. Peroxisomes were more than 95% pure as estimated from catalase specific activity. The remaining 5% protein was equally distributed among endoplasmic reticulum and mitochondria as estimated from measurements of NADPH cytochrome *c* reductase and glutamate dehydrogenase, respectively [5, 15]. Peroxisomal membranes were prepared treating the peroxisomal fraction with 10 mM sodium pyrophosphate, pH 9.0 [16] or 0.1 M sodium carbonate, pH 11.5 [7]. The peroxisomal fraction from the metrizamide gradient was diluted 10-fold in 0.25 M sucrose and centrifuged for 20 min at 28,000 rpm in a #65 Beckman rotor. The pellet was taken to the same volume of the sucrose dilution with either pyrophosphate or carbonate buffer and after 30 min in ice with gentle stirring the membranes were sedimented 60 min at 50,000 rpm in the same rotor. The pellet surface was gently rinsed with distilled water and the membranes were suspended in 0.4 M sucrose by sonication.

Bilayer Setup and Electrical Measurements

Bilayers were formed according to Mueller et al. [20] in a hole (300 μm diameter) made on a Teflon® partition separating two compartments. Both compartments were filled with 100 mM KCl buffered with 5 mM MOPS-K, pH 7.0. Lipids consisted of a 10 mg/ml solution of brain phosphatidylserine-brain phosphatidylethanolamine 1 : 1 (Avanti Polar, Birmingham, Ala.) in *n*-decane. After bilayer formation the KCl concentration on the *cis* chamber was increased by adding an aliquot of 3 M KCl, to favor fusion. Peroxisomal membranes (3 to 10 μg/ml final protein concentration) were added into the *cis* side. Channel incorporation was detected as the appearance of square fluctuations in current. Current was measured with a two-electrode voltage clamp [1]. The *cis* chamber was connected to a voltage pulse generator and the opposite *trans* chamber to a current-voltage converter, through Ag/AgCl electrodes. Electrodes were connected to the solution via agar bridges made in 1 M KCl and the *trans* side is virtual ground. The current was low-pass filtered, amplified and stored in tape.

Single-channel currents were estimated from the amplitude of discrete current jumps. Data were analyzed by hand.

Results

Fusion of Peroxisomal Membrane Incorporates Channels in Planar Bilayers

The addition of peroxisomal membrane vesicles to the *cis* chamber, in the presence of an osmotic gradient, induces stepwise increases in bilayer conductance, as illustrated in Fig. 1*A*. The Figure shows a record of membrane current obtained in the presence of a KCl gradient at zero applied voltage. At the time labeled with an arrow an aliquot of peroxisomal membrane vesicles was added to the *cis* chamber, under stirring. As shown, soon after the addition, an upward jump in the current is observed, followed by current fluctuations. The appearance of a positive current at zero applied voltage indicates the insertion of a cation-selective conductance pathway. With better time resolution, it can be shown that the current fluctuations seen in Fig. 1*A* are actually due to the opening and closing of channels, as illustrated in Fig. 1*B*. Channel insertion was observed both with the PP_i and the carbonate isolation procedures. In what follows, we describe the most salient features of these channels.

Large, Cation-Selective Channels

The records of current fluctuations shown in Fig. 1*A* and *B*, indicate that the channels inserted into planar bilayers upon fusing peroxisomal membranes

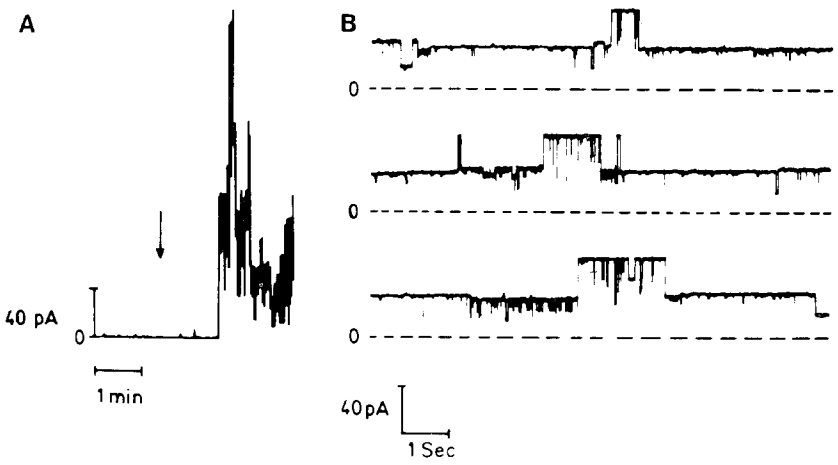

Figure 1. Fusion of peroxisomal membrane vesicles with planar lipid bilayers. Membranes were formed in symmetrical 0.1 M KCl. Then the KCl concentration in the *cis* side was raised to 0.4 M; vesicles were added to the *cis* chamber, under stirring. (*A*) Experimental record of current increase in a bilayer. The applied voltage was 0 mV. (*B*) Experimental record of channel current fluctuations from an experiment similar to that shown in *A*, with better time resolution. The dotted line indicates zero-current level

are cation selective. The study presented in Fig. 2*A–D* confirms it. This Figure shows fluctuations in membrane current in the presence of 0.4 M KCl *cis* and 0.1 M KCl *trans* at different applied voltages. The occurrence of discrete fluctuations in membrane current at +20 and 0 mV are shown in *A* and *B*. At −20 mV (*C*) the fluctuations are no longer observed. The trace in 2*D* shows the occurrence of discrete fluctuations at −30 mV, but reversed in respect to those seen at 20 and 0 mV. Thus, the reversal potential, under these experimental conditions, is close to −20 mV, confirming their cation selectivity. This value is about 12 mV more positive than the expected reversal potential for a strictly cation-selective channel. The magnitude of the measured reversal potential corresponds to a permeability ratio (P_K/P_{Cl}) of about 4.

Voltage Dependence

It was found that the conductance of bilayers containing cation-selective channels depends on the applied voltage as illustrated in Fig. 3*A–C*, and in the inset to this Figure. Figure 3*A* displays a record of the current flow through a membrane containing several channels after the applied voltage was changed from zero to −30 mV. The current relaxes to a new value in discrete jumps. Figure 3*B* and *C* shows the membrane current after changing the voltage from zero to +20 and +40 mV, respectively. As seen, the current decreases in discrete

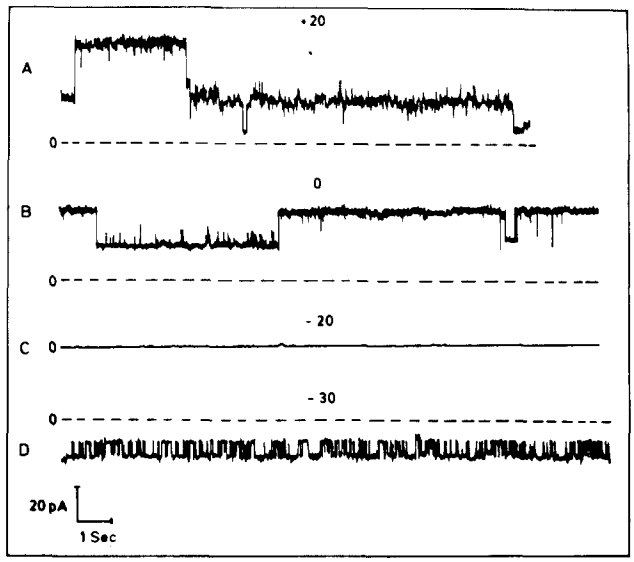

Fig. 2. Channel current fluctuations in a KCl gradient at different applied voltages. Experimental conditions were the same as in Fig. 1.

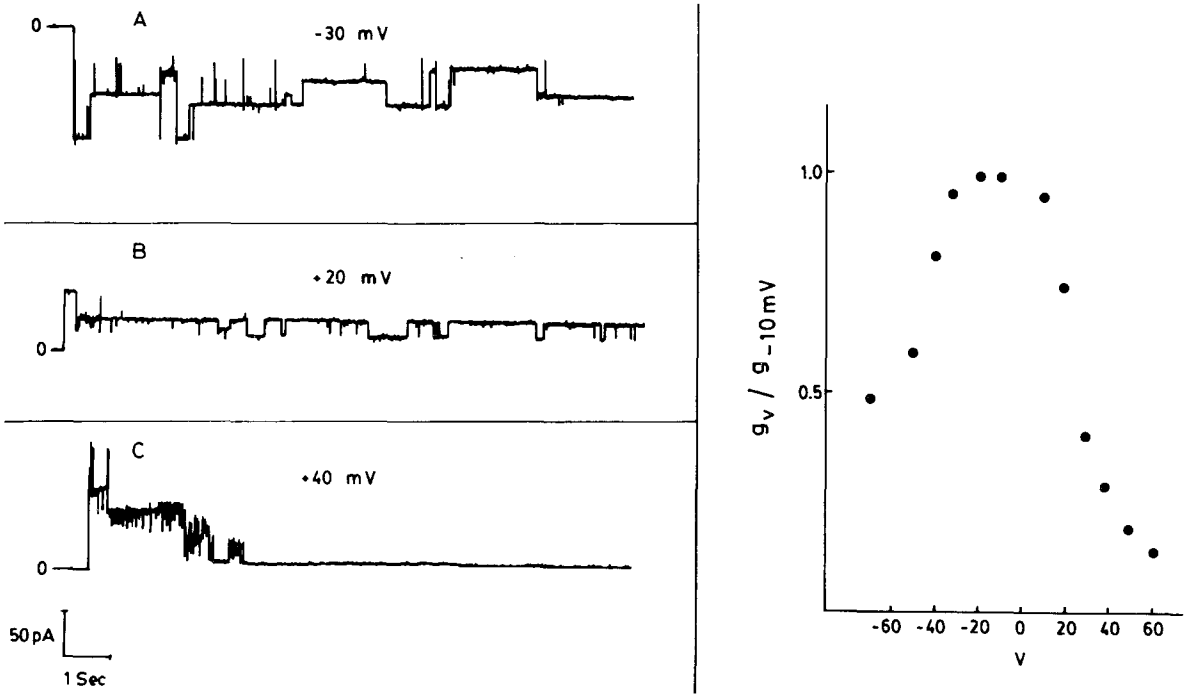

Fig. 3. Channel voltage dependence. Current was measured in symmetrical 0.3 M KCl, at the indicated voltages. The zero at the left of the records indicate zero-current level. Notice that the discrete current transitions seen at −30 mV display several levels. Inset: Relative conductance of a bilayer as as a function of the applied voltage in symmetrical 0.3 M KCl

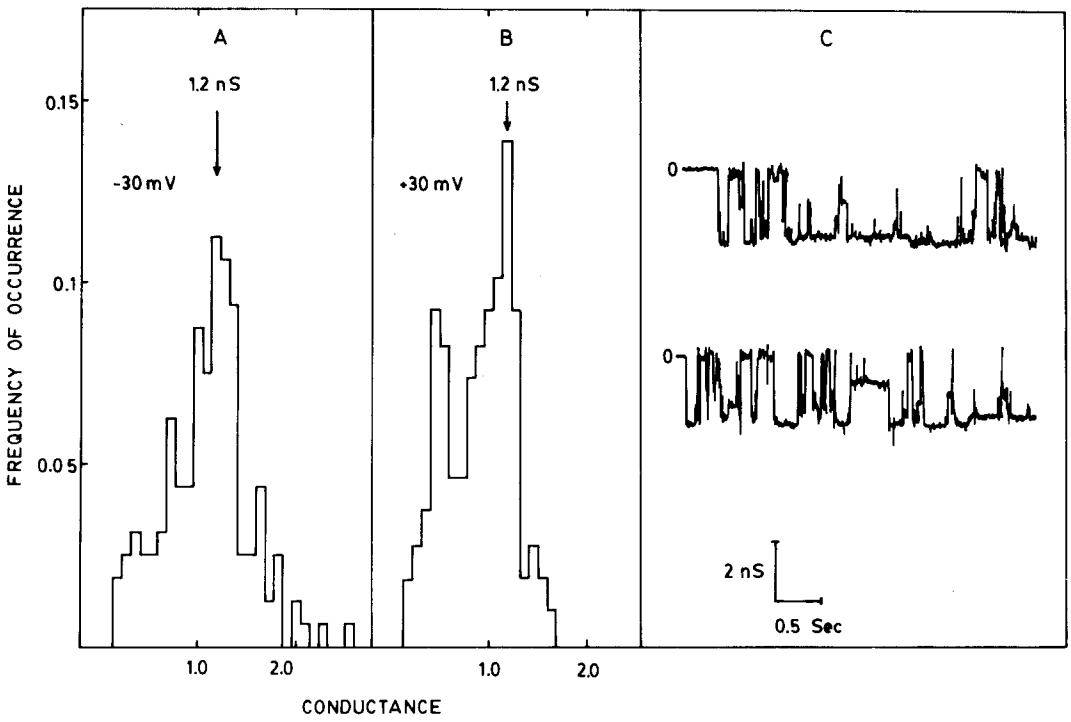

Fig. 4. Conductance histogram. Channel current transitions were measured under conditions identical to those of Fig. 3. Each histogram was built from 160 discrete transitions. *C* displays single-channel currents at −10 mV applied voltage to document the occurrence of open-close transitions of 2.4 nS

jumps at more positive voltages. Furthermore, at any voltage the current is always larger immediately after the voltage jump than in steady state suggesting that the open-state configurations are more probable at zero applied voltage. The insert to Fig. 3 provides evidence in favor of this idea. It shows a plot of the relative conductance ($g_v/g_{-10\,mV}$) obtained from a membrane containing several channels. As shown, the relative conductance decreases both at voltages more positive and negative than −10 mV. However, this decrease is more pronounced at positive voltages. In the positive quadrant, the conductance changes *e*-fold for each 30 mV.

The Large Cation-Selective Channel Displays Multiple Open States

The inspection of experimental records, as those shown in Fig. 3, reveals that the size of the discrete current transitions observed after fusing peroxisomal membranes with planar bilayers are not defined by a single value. In fact, multiple transition amplitudes are discernible suggesting the presence of several open configurations. Figure 4*A* and *B* show conductance histograms derived from the same membrane, at two different applied voltages. Clearly, the histograms do not represent a simple Gaussian distribution. However, both histograms, show a major peak at 1.2 nS, although other maxima are apparent. The 1.2 nS peak occurs at applied voltages more positive and more negative than −10 mV. At −10 mV the occurrence of conductance transitions having a value of 2.4 nS is often observed, as demonstrated in Fig. 4*C*. Thus, the potential difference across the membrane not only controls the probability of these channels being open but also which open configuration the channel acquires.

Fusion also Incorporates a Smaller, Anion-Selective Channel

We have often observed the insertion of a channel whose selectivity, conductance and kinetic properties differ completely from those of the large cation-selective pore described above. Figure 5*A* shows a record of the activity of this channel obtained in symmetrical, 0.1 M KCl. It is apparent that the channel can reach two open states. Figure 5*B* displays a conductance histogram derived from the record shown in Fig. 5*A*. It shows two peaks at 50 and 100 pS, which correspond to the open states of the channel. The probability of these open states

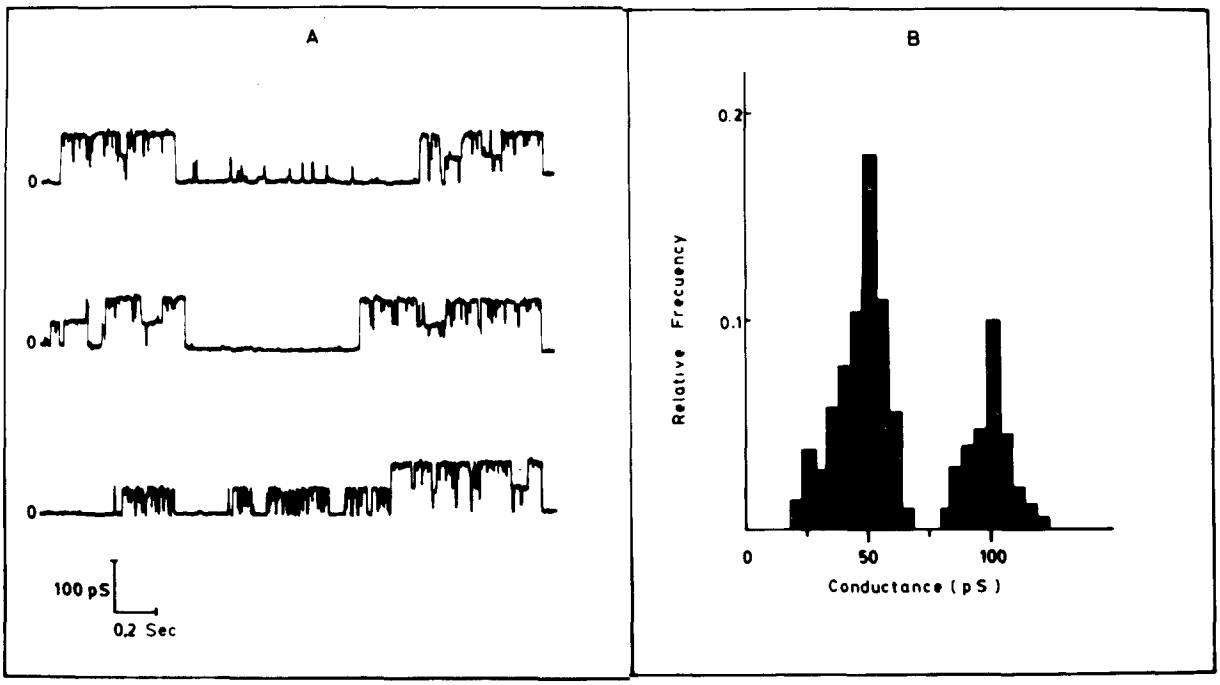

Fig. 5. Conductance histogram of the anion-selective channel. Experiments were made in symmetrical 0.1 M KCl. (*A*) Record of single-channel currents at −100 mV. Zero-current level is indicated at the left of each trace. (*B*) Conductance histogram. Single-channel transitions were measured at −100 mV. The histogram was built from 190 transitions

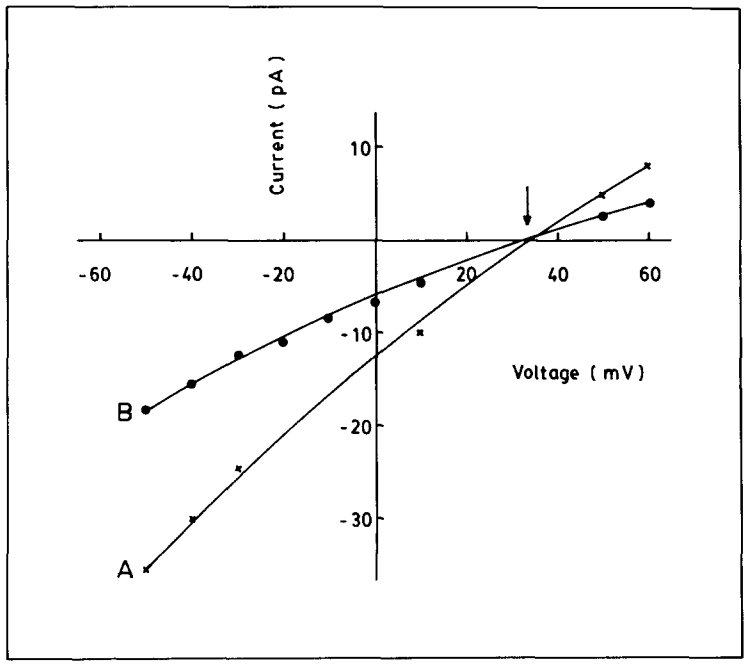

Fig. 6. Current-voltage relationship in KCl gradient. The current through the open channel was determined by measuring the height of the current jumps at different potentials. The *cis* chamber contained 0.4 M KCl, the opposite chamber 0.1 M KCl. Curve *A* shows the *I/V* relationship for the high conductance state and *B*, for the low conductance state. The arrow indicates the reversal potential for a perfectly anion-selective channel. Experimental points were joined by eye

is modulated by the applied voltage (*not shown*). These features, namely the occurrence of two open states whose probabilities depend on the applied voltage resemble the behavior of the anion-selective channel described by White and Miller [29]. Furthermore, Fig. 6 shows that this channel is perfectly anion selective.

It was noticed that the incorporation of the anion-selective channel to the planar bilayer differs with the insertion of the large cation-selective pore.

First, insertion of the anionic channel is less frequent. Second, this channel usually incorporates previous to and independent from the cationic pore. Third, the anion-selective channel always incorporates as singlets while the large, cationic pore usually inserts in multiples.

Discussion

The incorporation of peroxisomal membrane fragments into planar lipid bilayers allows the study of the ionic permeability characteristics of these organelles with electrophysiological procedures. The membranes employed in this study were obtained by two different methods already applied to biochemical studies: carbonate extraction, which solubilizes the matrix proteins and also the nucleoid, a proteic paracrystalline aggregate of urate oxidase [7] and pyrophosphate extraction, a procedure that does not solubilize the nucleoid [16]. For both isolation procedures, it has been estimated that the membrane accounts for ten percent of the peroxisomal protein, approximately 0.4 mg per gram wet weight of liver. Both preparations gave the same results when fused with planar lipid bilayers.

The isolation of peroxisomes in isopycnic metrizamide gradients consistently yields highly purified organelles [5, 27]. The degree of purification was estimated from the specific activity of catalase, a marker enzyme for peroxisomes and from the specific activity of markers for contaminants such as endoplasmic reticulum mitochondria and lysosomes.

The most frequent result obtained was the incorporation of the large conductance cation-selective channels. Channels were detected in all the peroxisomal membrane preparations studied. At applied voltages more positive and more negative than −10 mV, in 0.3 M KCl the most frequent discrete conductance transition has a value of 1.2 nS. By assuming that the channel is a water-filled pore 7.5-nm long, a pore diameter of some 1.5 nm can be estimated [4]. On the other hand, near zero-applied voltage, the most frequent conductance transition corresponds to a value of 2.4 nS. In this case the pore diameter would be of some 3.0 nm. The pore displays a voltage dependence such that it dwells most of the time in the open state at potentials near zero and closes at more positive voltages. At present, there is not enough information to speculate on the possible functional role of this voltage dependence.

The slight cation-selectivity of these channels and their large conductance are properties similar to those of the porins present in the outer membrane of gram-negative bacteria. Solute permeation through porins is mainly determined by solute size [3].

In conclusion, our observations support the hypothesis that the high permeability observed in peroxisomes might, at least in part, be due to the presence of large conductance pores in their membranes. Our estimates suggest that these pores could accommodate solutes of up to 3.0 nm in diameter.

As pointed out, the most frequent observation made in our studies is the insertion of large cation-selective pores. However, the incorporation of a perfectly anion-selective channel, with a smaller conductance was also noticed. An anion-selective channel with somewhat similar properties has been detected in membranes derived from the electric organ of *Torpedo* [29]. This channel incorporates less frequently, but independently from the large one, a fact that allows the study of its behavior separately. The occurrence of this channel in our experimental records might reflect the presence of small amounts of contaminant membranes. Indeed, whether or not the experimental results are due to the presence of nonperoxisomal contaminant material is a question for which a definitive answer might come only from new data obtained from other experimental approaches such as fractionation aiming at the purification of other organelles or from hypothetical functional evaluation of the channel with peroxisomal substrates or products. The main potential source of contaminating membranes in the peroxisomal fraction, which is over 95% pure, are mitochondria and endoplasmic reticulum. These membranes contribute little protein to the fraction [5, 27, 28]. Furthermore, the pore found in mitochondria outer membrane is anion selective [2]. The possibility of selective enrichment of the peroxisomal fraction in plasma membrane differentiations is also unlikely since negligible activity of alkaline phosphodiesterase, a plasma membrane marker, is detected in the peroxisomal fraction [28] measurements that we have confirmed (Leighton, *unpublished results*).

The finding of large channels in the peroxisomal membrane is supported by complementary evidence which illustrate their unusually high permeability [6, 26]. Biochemically, the latency shown by the fatty acyl-CoA oxidase [12, 24] suggests that the pores might actually discriminate among fatty acids esterified with either CoA or carnitine, residues with large difference in size and charge [14].

Morphologically, freeze-fracture studies of the peroxisomal membrane reveal the presence of abundant 7- to 8-nm particles that probably correspond to integral membrane proteins [21] and

some of which might structure the pores described in this report.

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