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Permeability of the mitochondrial outer membrane to organic cations

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Inhibition of mitochondrial respiration by hydrophobic fluorescent dyes (Rhodamine 6G, Safranine O, Pyronine B) is much less potentiated by digitonin-lysis of the outer membrane than that by polyamines or adriamycin. This situation may be explained by impermeability of the anion-selective channels in the outer mitochondrial membrane to large cations and by the ability of hydrophobic (but not polar or amphipathic) ions to directly permeate lipid bilayers.

Until recently, it has been widely assumed that the outer membrane of the mitochondrion is freely permeable to all small (mol. wt. < 5000) molecules and ions. The only indications to the contrary have come from reports that metabolite diffusion through this membrane may be rate-limiting for reactions taking place in the intermembrane compartment [1,2]. However, recent experiments have suggested that the mitochondrial outer membrane might be an actual barrier to the diffusion of certain organic cations. Rat liver mitochondria display a marked increase in susceptibility to cationic respiratory inhibitors, adriamycin and polyamines, after lysis of their outer membranes by digitonin or osmotic shock [3,4]. In the case of spermidine, this effect was shown to correlate with increased uptake of the radiolabeled compound by mitochondria, with no concomitant significant increase in permeability of the inner membrane to sucrose [4,5]. It was concluded that the observed potentiation of respiratory inhibition was most easily explained by an inability of the cationic inhibitors to penetrate the intact outer mitochondrial membrane *.

Impermeability of the mitochondrial outer membrane to organic cations would not be inconsistent with what is known about the channel in these membranes. Although its lumen diameter is large, 2.5 nm [7], the reconstituted channel is selective for anions over cations. (Hence, its name, VDAC, for voltage-dependent, anion-selective channel.) This selectivity for anions has been linked to the presence of clusters of exposed basic amino acid residues at or near the channel opening [8,9]. Note that reconstituted VDAC is not impermeable to small cations (like K^+) and neither is the mitochondrial outer membrane [10,11]. However, relative channel permeability clearly decreases with both increased solute molecular weight and net positive charge [10].

Total impermeability of the mitochondrial outer membrane to all organic cations would, on the other hand, be at odds with the apparent ability of mitochondria to accumulate hydrophobic cationic dyes. For example, rhodamines have found widespread use as vital stains of mitochondria and so-called potentiometric dyes like Safranine O are commonly used as biophysical probes of mitochondrial function [12,13].

To resolve this possible discrepancy, we have tested the accessibility of three cationic hydrophobic dyes (Table I) to the inner mitochondrial membrane respiratory chain as a function of outer membrane intactness.

Materials and Methods. Rat liver mitochondria were isolated and succinate oxidation measured polarographically as described in Ref. 3. The outer membranes of freshly isolated rat-liver mitochondria are typically 65–85% intact, based on cytochrome *c* integrity tests [3]. Outer membrane lysis was achieved by preincubation of the mitochondria with digitonin. Concentrations of digitonin used in each case were those determined to be just sufficient to make spermidine (25 mM) fully

* Nicolay and De Kruijff [6] have reported no difference in the inhibitory effects of adriamycin on mitochondria and on mitoplasts, which are mitochondria devoid of outer membranes, in apparent disagreement with our results [3,4]. However, the experimental results in Ref. 6 indicate that the outer membranes of the mitochondria used were very leaky towards exogenous cytochrome *c*. Membrane damage may have occurred during the freezing and thawing to which the mitochondria were subjected prior to the experiments. In any event, no conclusions about the permeability of intact outer mitochondrial membranes to adriamycin may be drawn from the experiments in Ref. 6.

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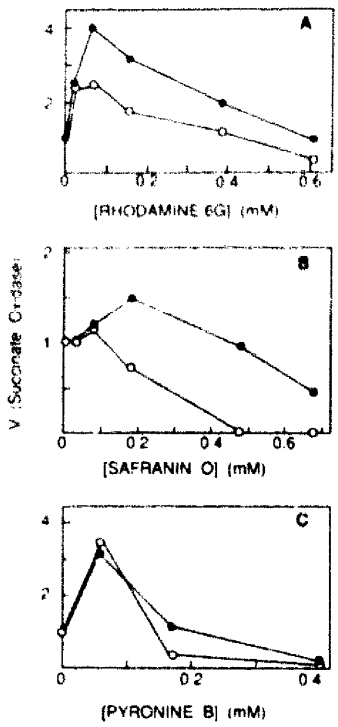

Fig. 1. Influence of digitonin treatment of mitochondria on respiratory effects of the fluorescent dyes Rhodamine 6G (A), Safranine O (B), and Pyronine B (C). V is the steady-state rate of O_2 consumption reached after addition of the indicated amount of dye relative to the rate prior to dye addition. Closed circles: unpretreated rat liver mitochondria (0.9, 0.8, 0.8 mg protein/ml, respectively, for A, B and C). Open circles: mitochondrial suspensions pre-incubated for 2 min at 27°C with 0.025% digitonin (lysed mitochondria).

accessible to the inner membrane, based on 100% inhibition of succinate oxidation. (This level of digitonin was always somewhat lower than that needed to make the outer membranes totally permeable to cytochrome *c*, which is a considerably larger molecule than the dyes being studied.)

Safranine O and Pyronine B was purchased from Aldrich Chemical Co. (Milwaukee, WI); Rhodamine 6G was purchased from Eastman Kodak Company (Rochester, NY). Concentrated stock solutions (1–5 mM) were made in 10 mM potassium phosphate buffer (pH 7.0) and stored at 4°C in the dark prior to use. Concentrations of the dyes in the stock solutions were determined spectrophotometrically.

Results and Discussion. Inhibition of mitochondrial succinate oxidation is shown in Fig. 1 for Rhodamine 6G (A), Safranine O (B) and Pyronine B (C). In each case, the effects of the dyes on isolated rat-liver mitochondria are biphasic: they stimulate respiration at low concentrations (below 0.1–0.2 mM) and become inhibitory at higher concentrations. Outer-membrane lysis by digitonin was found to have only moderate effect on these dose-effect curves. Stimulation of respiration by the rhodamine and safranine dyes is reduced by about one-half. Subsequent respiratory inhibition by the same dyes is somewhat potentiated, more so in the case of Safranine O than Rhodamine 6G. In the case of Pyronine B, there is no significant change in effectiveness of the dye after outer-membrane lysis.

These results indicate that the fluorescent dyes have ready access to the inner membrane respiratory chain in fractions of predominantly intact mitochondria and that this accessibility is only slightly increased, if at all, by outer membrane lysis. This is in stark contrast with the results obtained previously with other cationic respiratory inhibitors, namely adriamycin and polyamines [3,4]. The inhibitory effects of the latter organic cations were found to be markedly potentiated by osmotic or digitonin lysis of the outer membranes of rat liver mitochondria (see, for example, Fig. 1 in Ref. 3). The absence of similar potentiation with the fluorescent dyes used in the present study is consistent with numerous microscopic and spectroscopic experiments suggesting unimpeded uptake of these and similar dyes by mitochondria [12,13].

Table I summarizes the molecular weight and magnitude of the charge of the organic cations included in this study. There is no correlation between either physical characteristic and the ability of a compound to cross the mitochondrial outer membrane, as inferred from the potentiating effect of outer membrane lysis on inhibition by the compound. For example, both adriamycin (large potentiation: inferred impermeant) and Rhodamine 6G (slight potentiation: inferred permeant) have a charge of +1 and a molecular weight of approx. 500. The largest cation, Pyronine B, is inferred permeant while the smallest, spermidine, is inferred impermeant. Both are polyvalent cations.

However, there appears to be a correlation between the apparent ability of the organic cations to permeate the mitochondrial outer membrane and their relative hydrophobicity. Linear polyamines are very water solu-

TABLE I

Size and charge of organic cations tested for respiratory effects

Organic cation	Molecular weight	Net charge	Effect of OM lysis [a]
Inferred impermeant:			
Spermidine	145	+3	Large
Adriamycin	544	+1	Large
Inferred permeant:			
Rhodamine 6G	479	+1	Slight
Safranine O	351	+1	Moderate
Pyronine B	1042	+4	None

[a] Potentiation of the effects of the organic cation on rat liver mitochondrial succinate oxidation by digitonin lysis of the outer membrane (OM). Compounds showing a large potentiation are inferred to be unable to permeate across the intact mitochondrial outer membrane. Conversely, the absence of such potentiation suggests that the membrane is permeable to the compound.

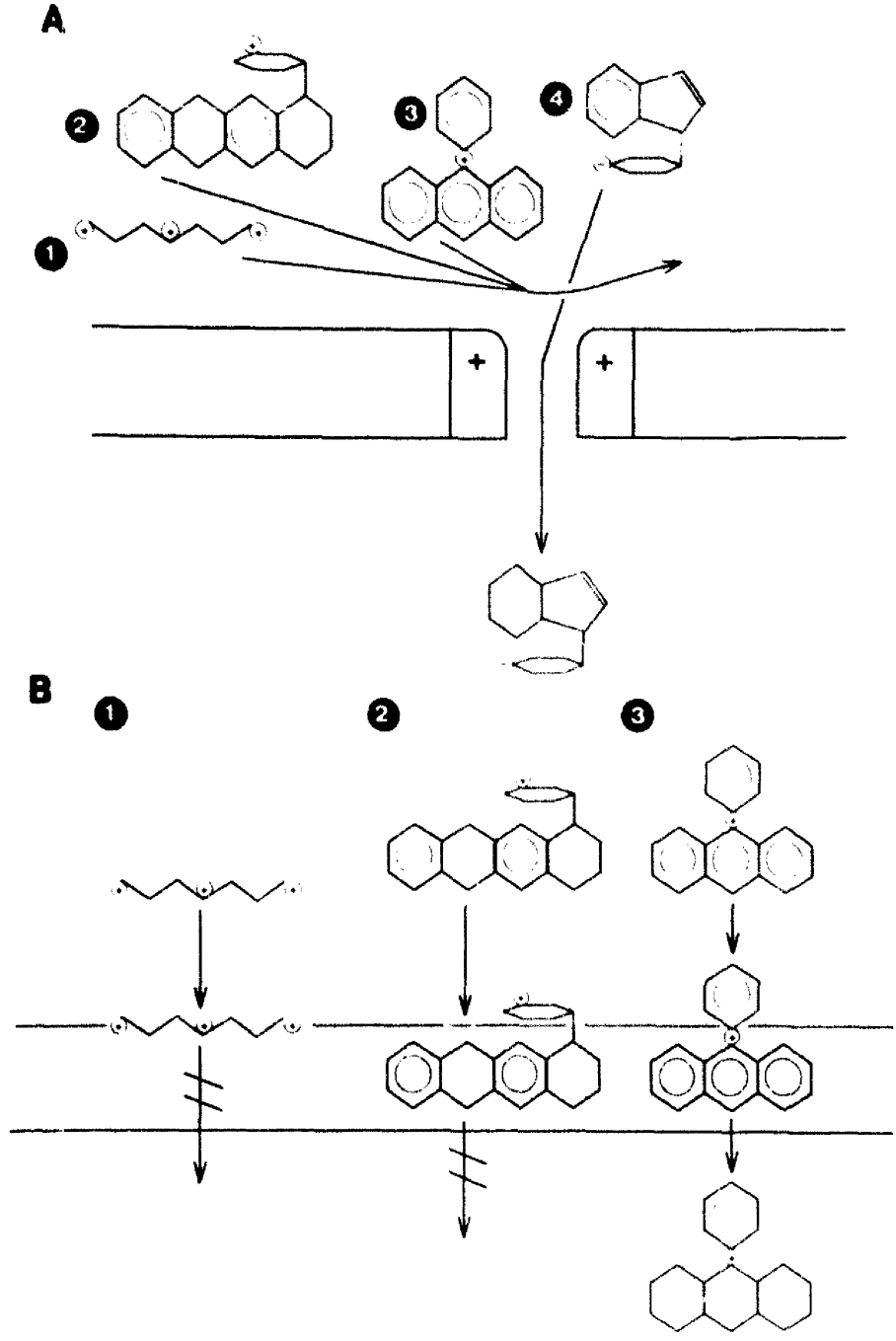

Fig. 2. The two permeability pathways through the mitochondrial outer membrane (A) The hydrophilic pathway involves the aqueous VDAC channel. Organic cations, represented schematically as aliphatic polyamines (1), adriamycin (2), and hydrophobic fluorescent dyes (3), may have restricted access to the channel because of fixed positive charges at the channel's mouth [8,9] Negatively charged metabolites, such as adenine nucleotides (4), may freely permeate the anion-selective channel (B) The hydrophobic pathway involves direct diffusion through the lipid phase of the membrane. Linear polyamines (1) and adriamycin (2) bind to the membrane but only hydrophobic compounds like the fluorescent dyes (3) may diffuse through it. (Note, organic cations and membrane channel not drawn to scale)

ble; they bind electrostatically to the polar surfaces of membranes [14,15] but there is no evidence that they directly diffuse through phospholipid bilayers to any significant extent. Adriamycin is an amphipathic molecule, composed of a large, non-polar ring system (dihydroxyanthraquinone) covalently attached to a polar, charged amino sugar (daunosamine). That this compound is moderately lipophilic is indicated by its partition coefficients in octanol/water and 1-butanol/water systems (1 and 6.4, respectively) [16,17]. There is evidence that the dihydroxyanthraquinone moiety of adriamycin imbeds into the hydrophobic interior of

bilayers [18]. Yet the drug apparently does not readily permeate across phospholipid liposome membranes [19], possibly because of its amino sugar moiety. By contrast, the fluorescent dyes tested have little or no hydrophilic character, except for their net charge. For example, Rhodamine B, which is structurally very similar to Rhodamine 6G used in these experiments, has a 1-butanol/water partition coefficient of 60.2 [20]. There is considerable evidence that such strongly hydrophobic ions easily translocate across phospholipid bilayers, due in part to charge shielding by and/or delocalization over their large, conjugated ring systems [21,22]. It has been demonstrated for example that Safranine O can be directly transported into phospholipid vesicles in response to a membrane potential [23].

We would propose, therefore, the scheme shown in Fig. 2. There are two pathways by which organic cations may permeate the mitochondrial outer membrane: a polar pathway defined by the aqueous channel, VDAC, and a hydrophobic pathway involving direct diffusion across the lipid bilayer. Because of fixed positive charges at the mouth of the VDAC pore [8,9], permeation of organic cations by the hydrophilic pathway may be limited. Thus, only very hydrophobic organic cations (such as the fluorescent dyes) may be able to translocate across the mitochondrial outer membrane. Linear polyamines or hydrophobic compounds attached to large polar residues (like adriamycin) may be unable to enter the mitochondrion by either pathway.

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