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On the Mechanism of Spermine Transport in Liver Mitochondria*

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Spermine penetrates the mitochondrial matrix at significant rates which increase sharply and non-ohmically with membrane potential. In this respect, spermine uptake is qualitatively similar to that of other cations whose electrophoretic transport has been studied in mitochondria. At 200 mV and 1 mM spermine, the observed rate of spermine uptake was about 7 nmol × mg^{-1} × min^{-1}, and the rate constant was about 8 times greater than that of tetraethylammonium cation. These rates are remarkably rapid considering that spermine is largely tetravalent at the pH of the experiment. The fluxes of spermine and tetraethylammonium are log-linear with membrane potential. The slope of the tetraethylammonium plot is consistent with leakage of this ion across a sharp Eyring barrier located in the middle of the membrane. The slope of the spermine plot is half that predicted by such a leak pathway, raising the possibility that spermine may cross the inner membrane by means of a channel. Whatever its mechanism of penetration, if comparable rates of uptake obtain *in vivo* and if spermine is not metabolized within the mitochondrial matrix, then a separate efflux mechanism would appear to be required to prevent unlimited spermine loading.

Among its various actions on cellular metabolism (1, 2), spermine exerts a protective and restorative effect on mitochondrial processes (3), counteracting the damaging effects of aging. This action has been referred to the ability of polyamines to damp Ca^{2+} and phosphate cycling and the concomitant efflux of endogenous Mg^{2+} and adenine nucleotides (4).

The stabilizing effect of spermine on mitochondria has generally been attributed to the binding of this polyamine to anionic sites on the cytosolic side of the inner membrane. Recently, however, we have shown (5) that spermine at physiological concentrations may also be transported into the matrix of rat liver mitochondria, provided that mitochondria are energized and inorganic phosphate is simultaneously transported.

In this paper, it is shown that spermine uptake is sharply dependent on the membrane potential in a manner which indicates transport of cationic spermine. A significant permeability to spermine was unexpected in view of the fact that this polyamine is tetravalent at physiological pH (6). From a comparison of the potential-dependent transport of spermine and tetraethylammonium cation and using a new theoretical approach to non-ohmic ion fluxes in mitochondria (7),[1] we reach the surprising conclusion that spermine may be transported across the inner membrane with two positive charges.

EXPERIMENTAL PROCEDURES

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes[2] (pH 7.4) by conventional differential centrifugation. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as standard. Incubations were carried out at 20 °C with 1 mg of mitochondrial protein/ml in the following standard medium: 200 mM sucrose, 10 mM Hepes-Cl (pH 7.4), 5 mM succinate, and 1.25 μM rotenone. Sodium salts were used. Other additions are indicated in the descriptions of specific experiments.

Uptake of [^{14}C]spermine and [^{14}C]tetraethylammonium was determined by a centrifugal filtration method as previously described (5) using a 3:7 mixture of dinonyl phthalate (bis(3,5,5-trimethylhexyl) phthalate; Fluka AG Chemische Fabrik, Buchs, Switzerland) and Siliconol DC 550 (Serva Feinbiochemica, Heidelberg, West Germany).

Membrane potential ($\Delta\Psi$) was measured by monitoring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode prepared in our laboratory according to published procedures (8, 9) and an Ag/AgCl reference electrode. TPP^+ was included at concentrations of 2 μM in order to achieve high sensitivity in measurements and to avoid toxic effects on the proton ATPase (10) and on calcium movements (11). The membrane potential measured with the TPP^+ selective electrode was calibrated using the equation: $\Delta\Psi = (\Delta\Psi_{electrode} - 66.16\ mV)/0.92$ as proposed by Jensen *et al.* (12).

Mitochondrial matrix volume was calculated from the distributions of [^{14}C]sucrose and ^3H_2O according to Palmieri and Klingenberg (13).

RESULTS

As shown in Fig. 1, rat liver mitochondria respiring on succinate in the presence of rotenone take up spermine slowly and linearly with time when 1 mM P_i is present in the incubation medium. The slow spermine uptake ended after 20–30 min of incubation, when approximately 50–60 nmol of spermine/mg of mitochondrial protein were taken up. Spermine uptake was also observed in the presence of 20 mM acetate, but this occurred at a slower rate and to a lesser extent than in the presence of 1 mM P_i (see Fig. 1). The small uptake of spermine observed in the absence of any added permeant anion might be due to the presence of endogenous P_i. Under all conditions, the slow phase of spermine accumulation was preceded by a phase of very rapid uptake of approximately 10–12 nmol/mg. This instantaneous uptake is very likely due to electrostatic binding of spermine to the accessible surfaces of mitochondrial membranes since it occurred in the absence of added P_i and in the presence of FCCP.

As shown in Fig. 1 (*inset*), 1 mM P_i induced an increase in $\Delta\Psi$ above the maximum values obtained with succinate ener-

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[1] K. D. Garlid, manuscript in preparation.

[2] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $\Delta\Psi$, membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, TPP^+, tetraphenylphosphonium ion.

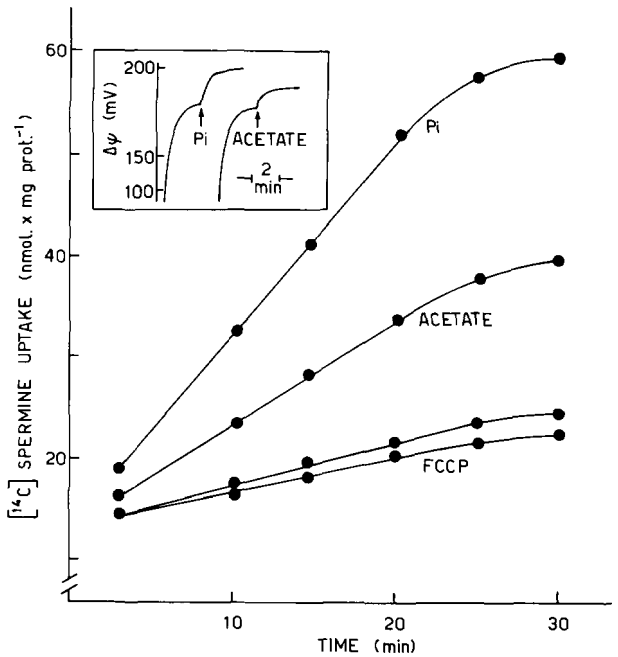

FIG. 1. **Effects of medium anion composition on spermine uptake and ΔΨ.** Rat liver mitochondria were incubated in the standard medium as described under "Experimental Procedures" containing 1 mM [^{14}C]spermine (50 μCi/mmol). When present, 1 mM sodium P_i, 20 mM sodium acetate, and FCCP (0.1 μg/mg of protein) were added as indicated.

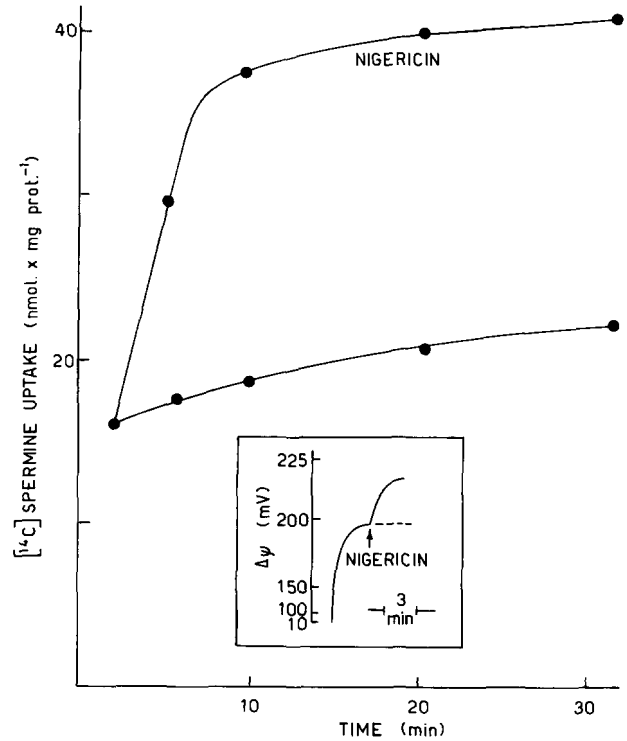

FIG. 2. **Effect of nigericin on spermine uptake and ΔΨ.** Mitochondria were incubated in the standard medium containing 1 mM [^{14}C]spermine. When present, nigericin (0.33 μg/mg) was added.

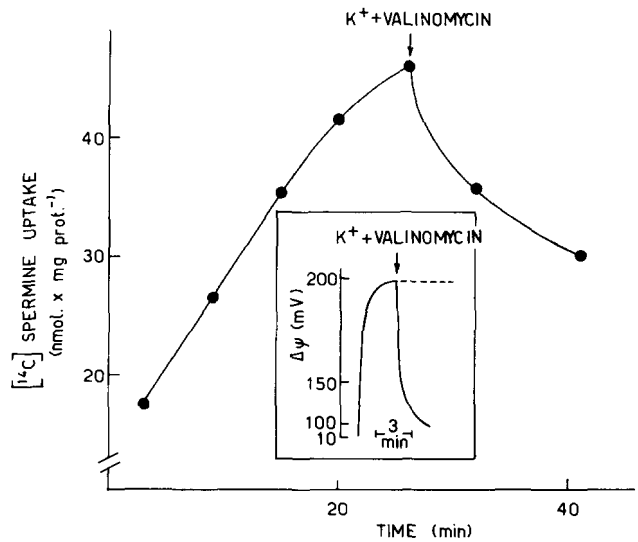

FIG. 3. **Effect of K^+ + valinomycin on spermine uptake and ΔΨ.** Mitochondria were incubated in the standard medium containing 1 mM [^{14}C]spermine and 1 mM P_i. At 25 min, 10 mM KCl and valinomycin (0.33 μg/mg) were added.

gization alone (from 180 to 200 mV). Acetate, although present at higher concentrations, induced a significantly lower increase in ΔΨ. This difference correlates quite well with the difference in observed rates of spermine uptake associated with these two permeant anions.

The conclusion that spermine uptake is dependent on a relatively small increment in ΔΨ is further supported by the experiment reported in Fig. 2, where the ΔΨ of mitochondria energized with succinate was increased by addition of nigericin (Fig. 2, *inset*) (14). It may be observed that spermine uptake in the presence of nigericin was more rapid but less extensive than that observed in the presence of added P_i (compare spermine uptake in Figs. 1 and 2). The faster rate of spermine uptake most likely reflects the higher potential achieved in the presence of nigericin. The lower extent of spermine uptake is probably due in part to the absence of P_i, which coprecipitates with spermine within the matrix space (5), and to a shutoff of respiration resulting from the intense acidification attendant on the addition of nigericin to mitochondria suspended in K^+-free medium.

In contrast to the effect of nigericin, the addition of valinomycin plus K^+, which causes a reduction in the membrane potential (15), interrupted the spermine uptake induced by P_i in energized mitochondria (see Fig. 3). Following cessation of uptake, accumulated spermine was slowly released from mitochondria, as shown in Fig. 3.

The dependence of the rate of spermine uptake on ΔΨ is shown in Fig. 4. The high ΔΨ (approximately 200 mV) obtained by incubating liver mitochondria in the presence of both succinate and P_i was gradually decreased by successive additions of minute amounts of FCCP (see Brown and Brand (16)). A dramatic decrease of spermine uptake was observed when ΔΨ was decreased from 200 to approximately 180 mV, suggesting that spermine uptake by rat liver mitochondria is governed by a very narrow range of ΔΨ. For comparison, the relationship between ΔΨ and the rate of tetraethylammonium uptake is also reported in Fig. 4. It was surprising to find that the potential-dependent uptake of tetravalent spermine was considerably faster than that of monovalent tetraethylammonium over the entire range studied.

When mitochondria preloaded with [^{14}C]spermine were resuspended in a new medium, a rapid, partial release of spermine was observed (Fig. 5). Since this release is enhanced by external spermine and since it is also observed in uncoupled mitochondria, it probably represents loss of [^{14}C]spermine bound to the surfaces of mitochondrial membranes.

A significant amount of accumulated spermine was retained through several washes by energized, but not by de-energized,

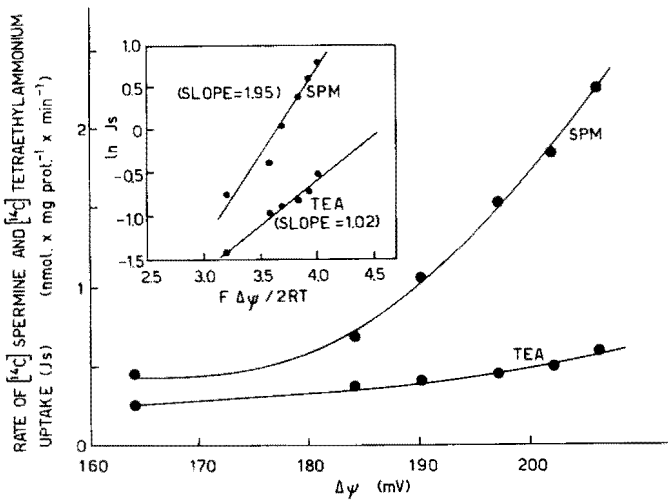

FIG. 4. **Rates of spermine and tetraethylammonium uptake *versus* ΔΨ.** Mitochondria were incubated for 10 min in the standard medium containing 1 mM P_i, 1 mM [^{14}C]spermine (*SPM*) or 2 mM [^{14}C]tetraethylammonium (*TEA*) (25 μCi/mmol) was added as indicated. ΔΨ was manipulated by including limiting amounts of FCCP (0–60 nM). The highest values of ΔΨ were obtained by adding nigericin (0.33 μg/mg). The values of cation uptake were corrected for instantaneous electrostatic cation binding to mitochondria. *Inset*, J_s is the rate of cation uptake. The *lines* in the *inset* are drawn according to the linear regression of the data (ln J_s *versus* $F\,\Delta\Psi/2RT$).

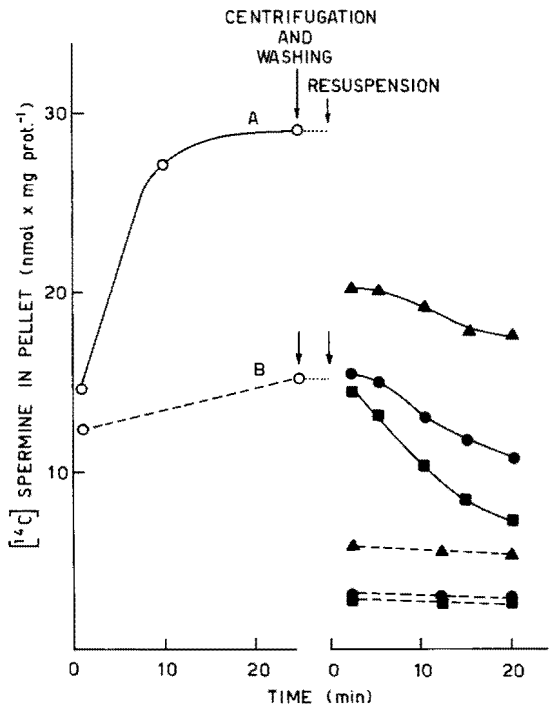

FIG. 5. **Exchange between accumulated and external spermine.** Mitochondria were incubated in the standard medium containing 1 mM [^{14}C]spermine and 1 mM P_i in the absence (*A, solid lines*) and in the presence (*B, dashed lines*) of FCCP (0.1 μg/mg). After 25 min of incubation, mitochondrial suspensions were centrifuged at 3°C for 2 min at 17,3000 × *g*. The supernatants were discarded, and the pellets were washed three times with cold standard medium and resuspended at 25°C in standard medium plus 1 mM P_i (▲---▲), in standard medium plus 1 mM P_i and 1 mM spermine (●---●), and in standard medium plus 1 mM spermine (■---■). In *A*, (*solid lines*), ΔΨ was maintained at high values following the washes. In *B*, (*dashed lines*), FCCP (0.1 μg/mg) was also present in the resuspension medium.

mitochondria (Fig. 5). In energized mitochondria, the nearly instantaneous release was followed by a slow, long-lasting release which was also enhanced by the presence of spermine in the incubation medium. The dependence on external spermine raises the possibility that this secondary energy-dependent efflux may reflect an exchange between accumulated and external spermine.

DISCUSSION

The results reported in this paper provide further evidence that spermine not only binds to mitochondrial membranes but also undergoes transport into the matrix (5). The binding, probably electrostatic in nature, takes place very rapidly and is unaffected by both the functional state of the mitochondria and the composition of the suspending medium. Spermine binding is followed by a slow, long-lasting uptake which is, in contrast, highly dependent on mitochondrial energization and on the presence of P_i in the medium (5).

A number of observations led us to propose earlier (5) that spermine uptake depends on P_i cotransport. Spermine is transported into respiring mitochondria concurrently with P_i; spermine facilitates the transport and accumulation of P_i; and spermine transport is inhibited by mersalyl, a known inhibitor of phosphate transport. Present results show that cotransport with P_i is not an absolute requirement for spermine uptake. Thus, acetate also induces spermine uptake (Fig. 1), and nigericin promotes spermine uptake in the absence of any permeant anion (Fig. 2). These findings appear to rule out a direct effect of P_i on spermine transport.

The common effect of P_i, acetate, and nigericin on respiring mitochondria is the reduction of the pH gradient and consequent shift of the protonmotive energy into a higher electrical potential gradient. This dependence of spermine uptake on ΔΨ implies that spermine is crossing the membrane electrophoretically rather than electroneutrally. This conclusion is further supported by the observation that valinomycin, which induces a rapid decrease of ΔΨ in mitochondria respiring in K^+ salts, causes cessation of spermine uptake, followed by efflux of accumulated spermine (Fig. 3).

The finding that significant spermine transport occurs only at high ΔΨ is reminiscent of the behavior of inorganic cations in liver (16), heart (17), and blowfly (18) mitochondria. From our results with spermine and tetraethylammonium (Fig. 4), together with those of Brown and Brand (16) with K^+, tetramethylammonium, and choline, we may conclude that all cations exhibit a nonlinear current-voltage relationship. This behavior falls under the rubric of non-ohmic conductance, first described in mitochondria by Nicholls (19).

At the pH used in our experiments and using the pK_a values provided by Tabor and Tabor (6), free spermine is about 97% tetravalent, 3% trivalent, and 0.01% divalent. The finding that mitochondria are permeable to this polycation is unexpected and raises the question of how spermine is transported across the inner membrane. At first glance, this question seems unanswerable since mechanisms of electrophoretic transport of simple cations, such as K^+, H^+, or quaternary ammoniums, across the inner membrane are unknown. Nevertheless, an analysis of ion leaks through energy-transducing membranes (7),[1] based on the properties of an insulating inner membrane (20) operating under high transmembrane forces, provides a practical means to address such questions. The main virtue of this procedure, aside from its inherent simplicity, is that it permits quantitative analysis of force-flux data such as those obtained in our studies.

At high values of ΔΨ, the equation for influx, *J*, of a cation

of valence, z, can be written (7) as follows:

$$\ln J = \ln(PC) + zF\,\Delta\Psi/2RT \quad (1)$$

where P is the permeability to the cation, C is its concentration in the medium, and RT/F is 25.2 mV at 25°C. The factor ½ is equivalent to assuming that a sharp energy barrier for ion translocation is located at the center of the membrane (21, 22). For simple ion leaks, data plotted according to Equation 1 should be log-linear with slopes equal to the valences of the transported species.

The logarithms of spermine and tetraethylammonium uptake rates are plotted *versus* $F\,\Delta\Psi/2RT$ in Fig. 4 (*inset*). The linearity of the curve for univalent tetraethylammonium and its unitary slope supports the validity of this theoretical approach. We have observed similar results for passive proton flux in mitochondria (7). The spermine data also yield log-linear force-flux plots (see Fig. 4, *inset*). This confirms the conclusion that spermine crosses the inner membrane electrophoretically, but the constants obtained from these plots raise new and important questions about the underlying mechanisms of spermine influx and efflux in mitochondria.

From the intercepts of the semilog plots, the exchange fluxes at $\Delta\Psi = 0$ are 7.4×10^{-4} and 9.5×10^{-3} nmol/(mg·min) for spermine and tetraethylammonium, respectively. By using 400 cm^2/mg for inner membrane surface area (23), the apparent permeability coefficients for spermine and tetraethylammonium are 3×10^{-11} and 2×10^{-10} cm/s, respectively. The difference in permeabilities for these two ions becomes even smaller when account is taken of the fact that much of the spermine will be complexed with the P_i used in these experiments. The energy barrier to the leak of polyvalent spermine must be very much higher than that for monovalent tetraethylammonium, and it is remarkable that their permeabilities should be of the same order.

It is also remarkable that the slope of the spermine plot is 2, rather than 4, as expected for tetravalent spermine at pH 7.4. This finding can be rationalized in terms of the leak model by considering the fact that the energy barrier to transport will necessarily become much lower as the number of charges is reduced. This will, of course, be offset by the decrease in the concentration of each species with lower charge. According to this model, the optimum value for these two factors is achieved with divalently charged spermine. But then the permeability of divalent spermine must be about 1000 times higher than that of monovalent tetraethylammonium, even assuming all spermine to be free. It is difficult to imagine a divalent cation having a higher leak conductance than a monovalent cation, but the possibility cannot be ruled out by our studies.

A second possibility is that the energy barrier for spermine is specifically lowered by the availability of an ion channel. Thus, the slope of 2 and the high permeability are both consistent with tetravalent spermine conductance through a channel with two energy barriers of equal height (21), created, for example, by placement of an energy well in the center of the membrane.[1] The previous finding that spermine uptake shows saturation kinetics (5) is consistent with a specific pathway, but far from conclusive on this point. This hypothetical pathway may be specific for spermine, or it may be a known channel or carrier for which spermine is a previously unrecognized substrate. The insensitivity of spermine transport to ruthenium red (5) would appear to rule out the Ca^{2+} uniporter. Other pathways which may be utilized by spermine include the ornithine carrier (24) and the pathway utilized by the polybasic signal sequence of imported mitochondrial proteins (25). The inference that mitochondria may contain a spermine transporter is highly tentative; however, the availability of a quantitative model for nonlinear ion fluxes provides a means of evaluating this question experimentally. The existence of such a transport pathway is sufficiently interesting to warrant such studies.

Whichever mechanism is valid for spermine *influx*, our analysis carries important implications for spermine *efflux* under physiological conditions. Spermine is present in the cytosol at reasonably high concentrations; and its inner membrane permeability, while higher than expected, is nevertheless exceedingly low. As far as we know, mitochondria never develop high *positive* potentials *in vivo*. Thus, they will be unable to eject this cation via the same leak pathway by which it entered because the high energy barrier, augmented by the high negative potential, will prevent significant outward diffusion of spermine. In order to avoid limitless accumulation, a *separate* mechanism for release of spermine from the matrix would appear to be required, placing spermine in the context of other cations which undergo uniport/antiport cycling by mitochondria (26). The observed slow release of previously accumulated spermine (Fig. 5) may reflect operation of such a mechanism. Spermine efflux is a complex process. It is inhibited by mersalyl when respiration is blocked (5) but stimulated by mersalyl in respiring mitochondria (27). The properties of spermine release and the possible existence of specific uptake and release mechanisms are under active investigation in our laboratories.

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