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ENERGY-DEPENDENT ACCUMULATION OF THE UNCOUPLER PICRATE AND PROTON FLUX IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY: In the presence of ATP and oxidizable substrate, submitochondrial particles accumulate up to 7 nmol of picrate/mg of protein. Half of this value is reached at 5 μM picrate in the medium, and maximal energy-dependent accumulation occurs at 25 μM picrate. Mitochondrial proton fluxes calculated under such conditions are 0.80 and 1.08 pmol $H^+/cm^2 \cdot sec$ at 10 μM and 25 μM picrate, respectively. These values are similar to those reported for state 4, and are therefore not large enough for uncoupling by picrate through proton translocation. The energy-dependent spectral response of oxonol VI is reversed to 50 % by 40 μM picrate, suggesting that abolishment of membrane potential is responsible for uncoupling of submitochondrial particles by picrate.

It has been shown previously that certain anions such as picrate (1) and tetraphenylborate (2) behave like uncouplers in inside-out oriented submitochondrial particles, but not in mitochondria, and that they do not function as efficient protonophores in substrate pulsed submitochondrial particles. These data are in apparent contradiction with the correlations reported by others between uncoupling potency and the ability of an uncoupler to induce transmembrane proton transport (3,4). In an attempt to resolve this problem, McLaughlin et al. (5,6) proposed that picrate accumulates electrophoretically in submitochondrial particles but not in mitochondria, because of the unfavorable polarity of the membrane potential in the latter. McLaughlin et al. assume that at steady state the concentration of picrate is high enough to allow effective protonophoretic uncoupling through an outward directed flux of undissociated

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picric acid. At a potential of 175 mV, a pH gradient of unity, and a concentration of 10 μM in the medium, these authors arrived at a steady state concentration of 1.1 mM picrate inside the particles, and a corresponding flux of 0.43 pmol $H^+/cm^2 \cdot sec$.

Recently, Michels and Bakker (7) studied picrate uncoupling in E. coli membrane systems. They found that picrate accumulation by inverted vesicles is driven by the membrane potential, and that uncoupling of these vesicles is accompanied by a decrease in membrane potential, which is in qualitative agreement with the predictions of the model of McLaughlin et al. (5,6).

We have experimentally determined the extent of energy-dependent picrate accumulation in submitochondrial particles. Our data confirm the calculations, but not the conclusions of McLaughlin et al. (5,6) concerning protonophoretic uncoupling by picrate. This is because picrate induced proton flux estimated by three different methods is much smaller than that required for uncoupling. In addition, our data indicate that among the components of the protonmotive force, the membrane potential rather then the pH gradient is abolished by picrate.

MATERIALS AND METHODS

For picrate binding studies, submitochondrial particles (8) were suspended in a buffer containing 250 mM sucrose, 10 mM Tris-chloride, pH 7.8, 1mM succinate, 1 mM ATP, 1 mM $MgCL_2$, and various concentrations of picrate, incubated for 7 min at room temperature, and sedimented at 23° for 45 min at 100,000 g. The amount of picrate in the pellet was determined according to (9). In the supernatant, picrate was estimated spectrophotometrically at 400 nm. Energy-dependent spectral shifts of oxonol VI (1 μM) in the presence of 0.1 mg/ml submitochondrial particles (10), 200 mM sucrose, 50 mM Tris-sulfate, pH 7.5, and 10 mM $MgCl_2$ were monitored at 630-602 nm. Protein was determined by the biuret method (11). Oxonol VI was synthesized as described (12). 5-Chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide (S-13) was a gift from Monsanto Chemical Co., St. Louis, Mo.

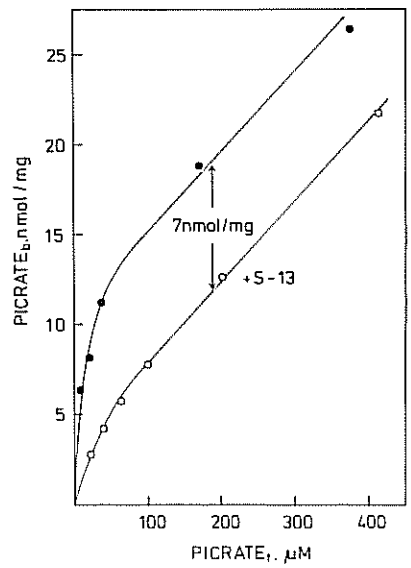

Fig. 1. Accumulation of picrate by energized submitochondrial particles in the presence and absence of the uncoupler S-13. Submitochondrial particles (3.2 mg/ml) were incubated at room temperature with increasing amounts of picrate, and in the presence and absence of S-13 (2.2 nmol/mg protein). After centrifugation, picrate was determined in pellet (picrate$_b$) and supernatant (picrate$_f$). For details, see Materials and Methods.

RESULTS

Fig. 1 shows the extent of picrate uptake by submitochondrial particles under energized and deenergized conditions. At picrate concentrations of 10 μM and 25 μM, respectively, 5.1 and 6.8 nmol picrate/mg of protein is accumulated in an uncoupler sensitive manner. With a free volume of 2.5 μl/mg submitochondrial particle protein (13), these values correspond to intravesicular concentrations of 2.0 mM and 2.7 mM picrate. With these data, a permeability coefficient of 0.4 cm/sec for picric acid (5), and an inside pH of 6.3 (5), picrate-mediated proton fluxes can be estimated directly and compared with the calculated data of others. It is seen in Table I that proton transport values estimated from the present results are similar in magnitude to those

Table I. Effect of picrate, DNP and FCCP on proton translocation through the inner mitochondrial membrane

uncoupler	proton flux (pmol $H^+/cm^2 \cdot sec$)	ref.
picrate (10 μM)	0.43	5
(10 μM)	0.80[a]	
(10 μM)	0.28[b,c]	
(25 μM)	1.08[a]	
(25 μM)	0.78[b,c]	
DNP (100 μM)	11.3[d]	14
FCCP (0.5 μM)	16.3[d]	14
no addition	1.25[d]	14
no addition	1.05[b]	

[a] calculated from data of Fig. 1, using a value of 2.5 μl/mg submitochondrial particles (13) and a permeability coefficient of 0.4 cm/sec for picric acid (5).

[b] calculated from substrate pulse data (1) using relaxation rate constants of 0.023, 0.029, and 0.04/sec at 0, 10 and 25 μM picrate, respectively, a buffering power differential of 12 μmol H^+/pH unit·g protein (14), a proton-motive force corresponding to 4.5 pH units (14), and a value of $1.18 \cdot 10^6$ cm^2/g submitochondrial protein derived from a membrane thickness of 7 nm and a membrane density of 1.21 g/ml (15).

[c] after subtraction of proton flux in the absence of picrate.

[d] data from ref. 14 were recalculated using a factor of $4 \cdot 10^5$ cm^2/g rat liver mitochondrial protein (14).

predicted by McLaughlin at al. (5,6) and those calculated from our earlier kinetic data (1). Significantly, all of the values of picrate induced proton flux shown in this Table are close to the values obtained by Mitchell and Moyle (14) under conditions of minimal mitochondrial proton flux, i.e., in state 4 (not shown) or in the absence of uncouplers. By contrast, the proton fluxes in the presence of 2,4-dinitrophenol (DNP) and carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) are 9 to 13-fold higher and of the same order of magnitude as the state 3 values (14).

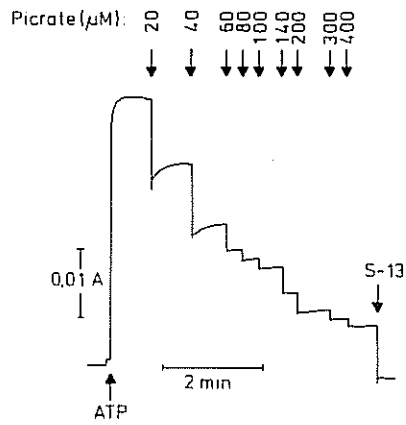

Fig. 2. Effect of picrate on the energy-dependent spectral response of oxonol VI in submitochondrial particles. Submitochondrial particles (0.1 mg/ml) were energized with ATP (5 mM) in the presence of oxonol VI (1 μM). Arrows and numbers indicate picrate additions and resulting total picrate concentrations. Complete deenergization was achieved by S-13 (1 nmol/mg protein). Temperature: 30°. For details, see Materials and Methods.

Rather than being a protonophore, picrate appears to be more effective in decreasing the membrane potential. It is seen in Fig. 2 that 50 % of the potential-sensitive oxonol response (12) is abolished by 40 μM picrate, the same concentration which inhibits the rate of ATP-dependent electron flow from succinate to NAD by 50 % (1).

DISCUSSION

Picrate concentrations accumulated under energized conditions by submitochondrial particles are of the same order of magnitude as those predicted by McLaughlin et al. (5,6). The inside concentrations of picrate and the corresponding proton flux values (Table I) calculated by these authors (1.1. mM picrate; 0.43 pmol $H^+/cm^2 \cdot sec$) agree closely with the values derived from Fig. 1 (2.0 mM picrate; 0.80 pmol $H^+/cm^2 \cdot sec$), even though the permea-

bility coefficient used by McLaughlin et al. for submitochondrial particles was determined in an artificial bilayer system (5). Taking into account that the mitochondrial membrane contains only about 30 volume % phospholipids[1], and using a correspondingly smaller permeability coefficient (0.12 cm/sec) for neutral picric acid, a proton flux of 0.24 pmol $H^+/cm^2 \cdot sec$ can be calculated at 10 μM picrate, a value which is still comparable to the one reported by McLaughlin et al. (5,6). Using either this or the uncorrected value for comparison, it is apparent from Table I that picrate induced proton fluxes (lines 1 to 5) are only about as large as those occuring without picrate addition in submitochondrial particles (line 9), in anaerobic mitochondria (line 8) or in state 4 (14). Thus, on the basis of quantitative considerations, it can be concluded that uncoupling by picrate cannot be explained by picric acid induced proton flux. This is in agreement with our earlier conclusion based on substrate pulse data, but differs from the interpretation given by McLaughlin et al. (5,6). These authors used a conductance coefficient of $4.5 \cdot 10^{-7}$ mho/cm^2 for the mitochondrial membrane (14), corresponding to line 8 in Table I. They, therefore, compared picrate induced proton flux with the situation in anaerobic mitochondria without uncoupler additions and in state 4 (14), rather than with state 3 or the uncoupled state. Our conclusion is also in agreement with the uncoupling characteristics of tetraphenylborate reported previously (2). Similar to picrate, this anion affects energy-dependent reactions in submitochondrial particles, but not in mitochondria, and increases proton permeability only marginally. Since a neutral tetraphenylboric acid is

[1] Based on an analytical value of 0.3 mg phospholipid/mg protein, a partial specific volume of 0.98 ml/g lecithin (16), and a membrane density of 1.21 g/ml (15).

apparently nonexistent even in extremely acidic media (17), it is obvious that the model of McLaughlin et al. (5,6) is neither applicable to picrate nor to tetraphenylborate as an uncoupler.

On the other hand, the results of Fig. 2 offer a possible explanation for the observed uncoupling by picrate (1). Whatever the mechanism, it is clear that picrate diminishes the membrane potential, and, as stated above, the extent of this effect agrees with the extent of uncoupling brought about by picrate in an energy-dependent reaction catalyzed by submitochondrial particles.

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