INHIBITION OF ENERGY-LINKED UPTAKE OF ACRIDINE DYES BY PERMEANT ANIONS

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1. Introduction

Energization of submitochondrial particles results in uptake of H^+, K^+, NH_4^+, inorganic and organic anions [1–9], and a large number of protonated ligands [10–13]. The most widely accepted mechanism for this effect involves an electrophoretic diffusion of anions down the electrical gradient (membrane potential positive inside) and an electroneutral exchange between internal H^+ and external cation [7–9]. As to the uptake of $NH_4^+$and acridines, this should be due to diffusion of the neutral base followed by protonation in the inner space. A number of observations are compatible with this model: a) stimulation of uptake of H^+, K^+ and NH_4^+ by permeant anions; b) uptake of inorganic anions parallel to that of H^+ and c) uncoupling of particles by simultaneous addition of one agent permitting an electrophoretic transport (such as valinomycin or inorganic anions) and of another favouring an electroneutral exchange (such as nigericin or NH_3).

However the anion requirement for the uptake of protonated dyes presents a number of features which are not compatible with the electrogenic proton pump [11–13]. In this paper we report on some aspects of this incompatibility concerning the effect of lipohilic anions on the uptake of either protonated dyes or organic anions.

Abbreviations: AO, acridine orange; NR, neutral red; TPB^-, tetraphynylboron; BTB, bromthymolblue; ANS, 8-aminonaphthalene-1-sulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PSSA, polystyrene sulfonic acid.

2. Experimental

Beef heart mitochondria were prepared as described previously [11]. Submitochondrial particles were obtained by sonication in pyrophosphate [12]. The sonicated particles were kept frozen for several months without loss of activity. Each experiment was carried out with a particle preparation thawed only once and used immediately after thawing, since either refreezing or storing at 0°C implied a loss of activity. The kinetics of the change of absorbance and of fluorescence were measured in a dual wavelength spectrophotometer or in a Perkin Elmer MP F2A spectrofluorimeter. The binding of the dyes to the particles was measured by centrifugation as described previously. All chemicals were of the highest purity available.

3. Results

The pyrophosphate particles used in the present study possess the following general properties: a) although they have a high rate of resting respiration, oligomycin-inhibition and FCCP stimulation of the respiration are still present (ratio FCCP-rate/oligomycin-rate = 3); b) inorganic cation movements, such as those of H^+ and K^+, are practically absent; c) high rate of P_i–ATP exchange and extensive response with various types of anionic and protonated ligands are present.

The interpretation of the nature of the energy-linked response of the acridine dyes and of bromthymol blue has been discussed in previous papers from our laboratory.

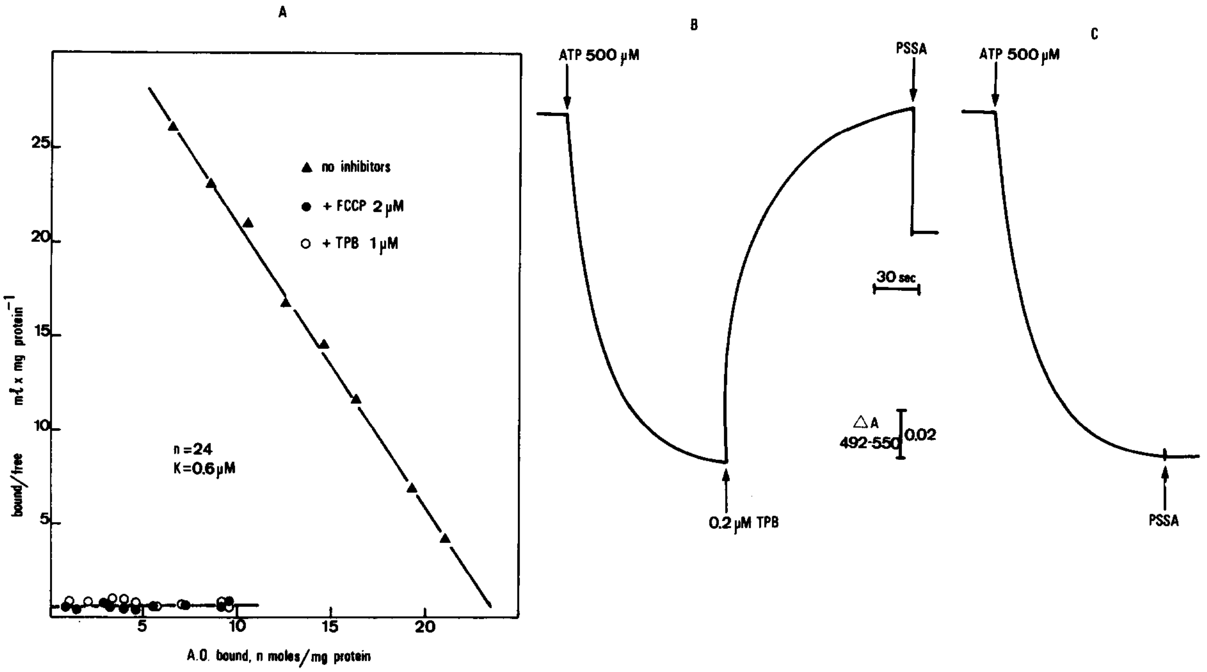

Fig. 1. Inhibitory effect of TPB$^-$ on active binding of acridine orange. The medium contained in A 0.1 M KCl, 5 mM $MgSO_4$, 10 mM HEPES pH 7.4, 1 mM ATP and submitochondrial particles, 0.9 mg/ml. When indicated 2 μM FCCP or 1 μM TPB$^-$. In B and C, the medium contained 30 mM KCl, 5 mM $MgSO_4$, 10 mM HEPES pH 7.4, and particles, 0.25 mg/ml, 5 μM acridine organge When indicated 2 μl 0.1% PSSA; *n* is the number of sites in μmoles X g protein^{-1} and *K* the dissociation constant.

In fig. 1 A it is seen that addition of either FCCP or TPB$^-$ induced a release of A0 from the membrane, as measured by centrifugation experiments. This is further seen in fig. 1B which shows that when A0 was bound to the membrane, the further addition of PSSA caused no spectral change since the dye was already in a stacked state. On the other hand when A0 was released from the membrane after addition of TPB$^-$, addition of PSSA induced a change of absorbance of the free dye [11]. The PSSA technique allows measurement of the amount of bound dye without centrifugation and to correlate binding and kinetic experiments.

In contrast to a number of previous observations where the energy-linked uptake of H^+, K^+ and of acridine dyes have been shown to be markedly enhanced by, or completely dependent on, the addition of permeant anions, figs. 2 and 3 further indicate that permeant anions may cause also an inhibition. Fig. 2 shows the sensitivity to TPB$^-$ of the energy linked responses of NR and atebrine. The concentrations of TPB$^-$ required to completely abolish the responses were: 0.2 μM in the case of A0 (fig. 1B), 2 μM in the case of NR (A) and 40 μM in the case of atebrine (B). This means a difference in sensitivity greater than two orders of magnitude between the various ligands. Fig. 3 shows that also other anions such as SCN$^-$ and NO_3^- can act as inhibitors and that also in this case the inhibition depends on the nature of the probe. For example 5 mM SCN$^-$ is already inhibitory for AO while 15 mM is required for NR. Note that atebrine is much more resistant to inhibition by SCN$^-$. On the other hand 20 mM $NO_3{}^-$ does not affect the response of NR, while it inhibits partially that of AO.

In contrast ot a number of previous observations where the simultaneous addition of nigericin (supposed to abolish the Δ pH) and of permeant anions (supposed to collapse the membrane potential) induces uncoupling, fig. 4 shows that an increased energy-linked response of anionic probes can be obtained in presence of the two agents. This effect is FCCP sensitive. In fig. 4 A there was no effect of 0.2 μM

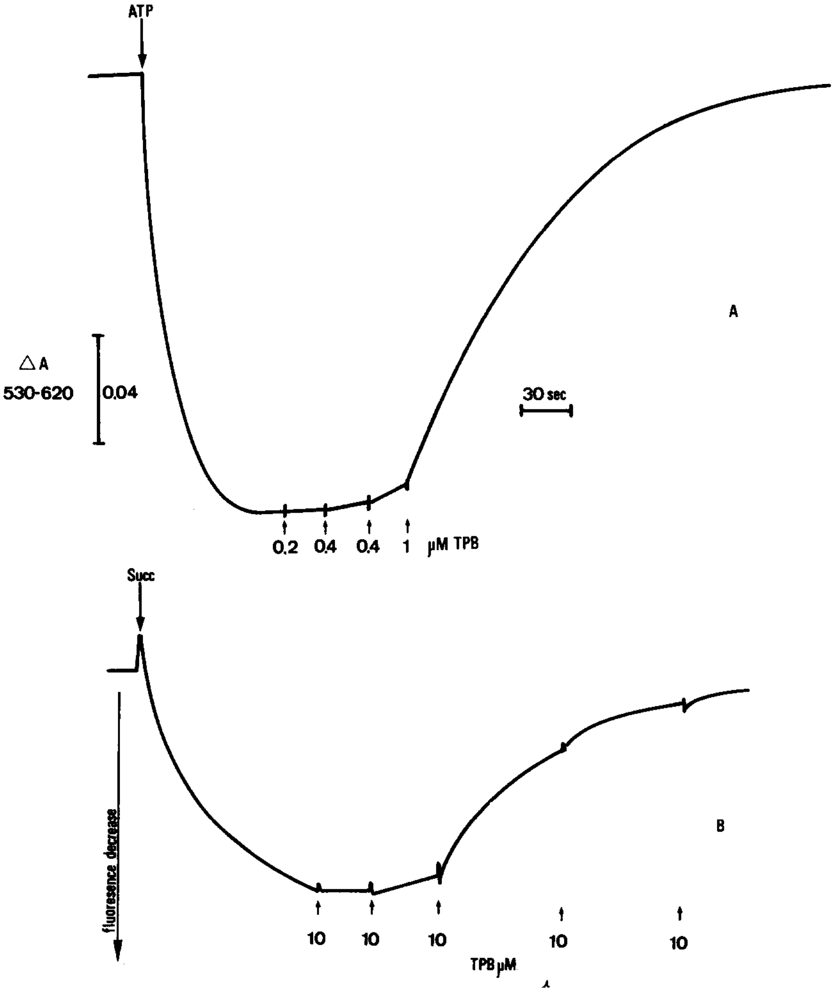

Fig. 2. Comparison of the effects of TPB^- on neutral red and atebrine. The medium contained 0.1 M KCl, 5 mM $MgSO_4$, and 0.65 mg/ml submitochondrial particles. In A, 10 μM neutral red and 10 mM HEPES pH 6.5. In B 3 μM atebrine, 10 mM HEPES pH 7.4. The reaction was initiated by 500 μM ATP in A, 1 mM succinate in B. Additions of TPB^- as indicated.

TPB^- (which abolished the response of AO, fig. 1B) and an enhancement by nigericin. In B, there was partial inhibition by 8 μM TPB^- and enhancement by nigericin. In C there was no effect by 10 mM NO_3 and again enhancement by nigericin. Responses similar to those of BTB were obtained with ANS.

4. Discussion

Horton and Packer [14] have reported that addition of TPB^- to illuminated chloroplasts induces a cyclic release of H^+ ion, the dimension of the cycle being proportional to the amount of TPB^- added. This is exactly what one would have predicted by assuming that the uptake of H^+ ion is the expression of the energy-linked binding of H^+ ion to the membrane and not of the formation of a Δ pH. In the former case the release of H^+ ions should be proportional to the amount of energy required for the uptake of TPB^-. In the latter case the uptake of a permeant anion should increase the Δ pH because of its collapsing effect on the membrane potential.

In the case of the submitochondrial particles,

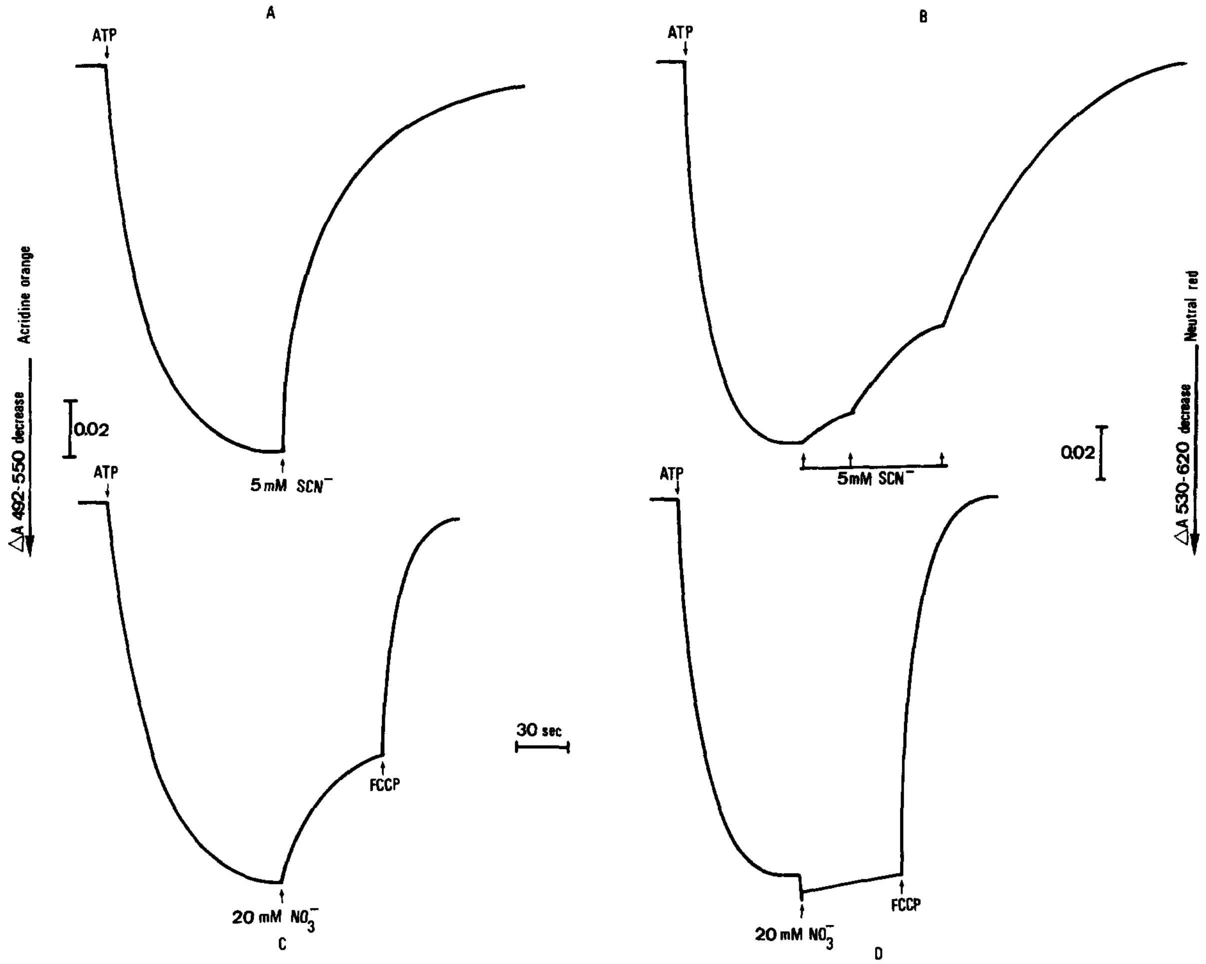

Fig. 3. Comparison of anion effects on neutral red and acridine orange. Experimental conditions as in fig. 2 A. In B and D 10 μM neutral red; in A and C 5 μM acridine orange. Other additions as indicated.

application of the electrogenic pump model leads to the following predictions [5,7–9]: a) addition of a limited amount of permeant anions should result in a state 4—state 3—state 4 transition (analogous to that observed with permeant cations in mitochondria), while impermeant anion should have no effect; consequently there should be no uptake of the protonated dye in the presence of Cl^- (an impermeant anion) and an enhancement of the uptake by TPB^-, SCN^- and NO_3^- in order of lipophilicity; b) large amounts of lipophilic anions when added together with a cation capable of electroneutral exchange should result in an irreversible state 4—state 3 transition (uncoupling) analogous to that observed in mitochondria in the presence of an excess K^+ (+ valinomycin) and P_i; consequently the BTB and ANS responses should be abolished when nigericin is added in the presence of TPB^- or NO_3^-. The present paper shows that both predictions are not satisfied. First there is no enhancement but inhibition of the uptake of the acridines by permeant anions and the effect increases with the lipophilicity of the anion. Second, there is no abolition of the response of anionic dyes in the presence of nigericin and permeant anions, but rather enhancement. Another prediction of the electrogenic model has already been reported not to hold

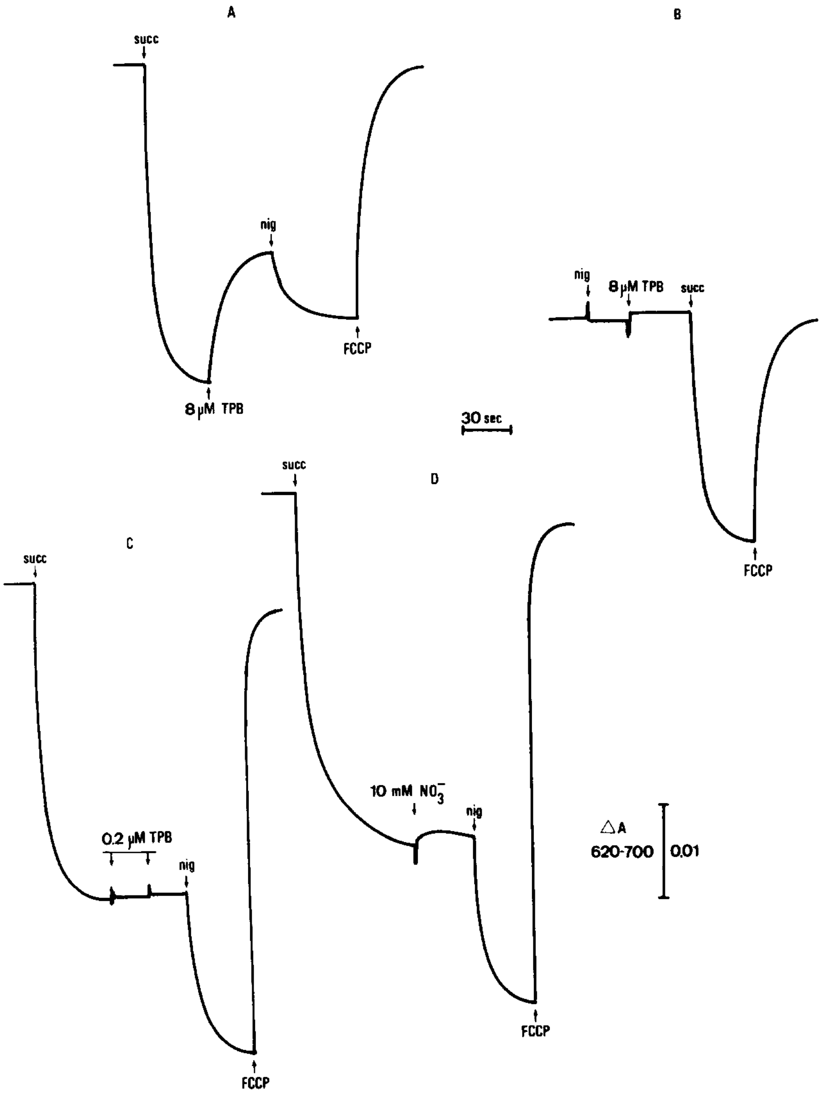

Fig. 4. Effect of permeant anions and nigericin on the interaction of bromthymol blue. The medium contained: 0.25 M sucrose, 30 mM KCl, 10 mM HEPES pH 8.0, 10 μM BTB, submitochondrial particles 1.2 mg/ml and 0.5 μg/ml oligomycin. Other additions as indicated. 0.08 μg/ml Nigericin. 1 mM succinate, 2 μM FCCP.

namely the lack of stoichiometric uptake of inorganic anions during the energy linked accumulation of NR. [13].

It may be argued that a fundamental difference exists between transmembrane and intramembrane phenomena, the first studied mainly with the inorganic ions, used in previous studies, and the second with the organic ions used in the present studies [10–13, 15]. We do not deny that the interaction of the organic ions *with* the membrane is different from the transport of inorganic ions *across* the membrane. This leads however to an alternative: i) The movements of organic ions are not related to transport and therefore cannot be explained by the mechanism for mitochondrialion transport; ii) The movement of organic ions do reflect intermediate steps in the transport of ion across the membrane and therefore the electrogenic pump does not account for the molecular mechanism of transport.

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