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Uncoupling of oxidative phosphorylation by divalent cationic cyanine dye. Participation of phosphate transporter

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The trinuclear cationic cyanine dye tri-S-C_4(5) was found to be an uncoupler of oxidative phosphorylation. Its uncoupling required inorganic phosphate (P_i) or arsenate, which is transported into mitochondria via the P_i transport system, and was abolished by the P_i-transport inhibitor *N*-ethylmaleimide or mersalyl. The dye stimulated P_i uptake into mitochondria, and its uncoupling action was accompanied by swelling of the mitochondria. The adenine nucleotides ADP and ATP protected mitochondria from uncoupling by the dye. The dye taken up by mitochondria was released into the incubation medium on induction of uncoupling. In the absence of P_i, the dye did not cause uncoupling, but its uptake was much greater than in the presence of P_i. The cyanine dye is suggested to induce uncoupling by acting on the membrane, rather than after its electrophoretic transfer into the mitochondria.

Introduction

Hydrophobic cations, such as alkylbiguanides [1], rhodamine 6G [2], DDA^+ and TBA^+ [3,4], the alkaloid chelerythrine [5] and cyanine dyes [6–8] have been reported to act as uncouplers of oxidative phosphorylation in mitochondria. Though the action of weakly acidic uncouplers has been studies extensively, little is known about the action of cationic uncouplers [9,10]. This is mainly because the activities of the latter depend greatly on the experimental conditions, especially on the anion species of the incubation medium. For instance, DDA^+ alone does not have any uncoupling activity even at 1 mM, but in the presence of a small amount of the permeant anion tetraphenylborate (about 20 μM) it induces uncoupling [4]. Furthermore the hydrophobic divalent cation tri-S-C_7(5) acts as an uncoupler only in the presence of P_i or arsenate [7,8]. The uncoupling by hydrophobic cations, such as DDA^+ and TBA^+, has been proposed to be due to their electrophoretic transfer across the mitochondrial membrane with consequent dissipation of the inside-negative membrane potential [3,4]. However, the precise mechanism of action of cationic uncouplers, including the role of anions in their action, has not been clarified.

This paper deals with the effect of the trinuclear cyanine dye tri-S-C_4(5) on mitochondrial function. This dye is similar in chemical structure to tri-S-C_7(5), but it is more hydrophilic with three butyl chains instead of the three heptyl chains in tri-S-C_7(5) (for chemical structures, see Ref. 11). In a preliminary study, we found that tri-S-C_4(5) accelerates state 4 respiration in the presence of P_i with

Abbreviations: P_i, inorganic phosphate; tri-S-C_4(5), 2,2′-[3-[2-(3-butyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]-bis[3-butyl-4-methylthiazolinium iodide]; tri-S-C_7(5), 2,2′-[3-[2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]-propenylene]bis[3-heptyl-4-methylthiazolinium iodide], also named NK-19 or Platonin, DDA^+, *N*,*N*-dibenzyl-*N*,*N*-dimethyl-ammonium, TBA^+, tetrabutylammonium; TPP^+, tetraphenylphosphonium, SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene-malononitrile.

succinate as substrate [11]. Use of this more polar dye allows study of the effect of the dye in terms of its affinity to mitochondria; tri-S-C_7(5) is so hydrophobic that it binds almost completely to mitochondria, irrespective of their energy state [8].

Materials and Methods

Tri-S-C_4(5) was a gift from Nippon Kankoshikiso Research Laboratory, Okayama (Japan), and SF 6847 was obtained from Wako Pure Chemical Industries Ltd., Osaka (Japan). Concentrated solutions of tri-S-C_4(5) in dimethyl sulfoxide, and SF 6847 in ethanol were used as stock solutions. At the concentrations used, these organic solvents were confirmed not to have any effect on mitochondrial function under the present experimental conditions. [^{32}P]Orthophosphoric acid was purchased from Amersham International, U.K.

Mitochondria were isolated from adult male Wistar rats as reported by Myers and Slater [12]. The amount of protein in mitochondria was determined from the absorbance difference between reduced and oxidized cytochrome aa_3 [13].

Respiration of mitochondria was monitored polarographically with a Clark oxygen electrode (Yellow Springs, YSI 5331) at 25°C. The composition of the incubation medium was as follows: 200 mM sucrose/2 mM $MgCl_2$/1 mM Na_2EDTA/10 mM Tris-HCl (pH 7.4). This medium is referred to as $-P_i$ medium. In studies on the effect of P_i, we used 10 mM potassium phosphate buffer (pH 7.4) instead of Tris-HCl buffer. This medium is referred to as $+P_i$ medium.

The volume change of mitochondria was monitored as the absorbance change at 700 nm in a Shimadzu recording spectrometer, model UV-300 or model UV 3000. At this wavelength the dye had little absorption [8].

The uptake of P_i by mitochondria was determined by use of [^{32}P]P_i. Mitochondria were added to 1 ml of $-P_i$ medium containing 10 mM succinate (plus rotenone at 1 μg/mg mitochondrial protein) in a small plastic centrifuge tube (volume: 1.5 ml) at 25°C and after 1 min [^{32}P]P_i (0.3 μCi) was added to a concentration of 10 mM in the presence or absence of 100 μM tri-S-C_4(5) to initiate P_i transport. After a definite period, P_i transport was arrested by addition of 150 μM mersalyl. Since swollen mitochondria were sometimes found not to sediment well through a silicone layer, the mitochondria were centrifuged without addition of silicone oil at 12000 rpm for 1 min in a Kubota centrifuge, model KM 15000. The supernatant was then removed, 0.5 ml of silicone oil (Toray Silicone SH 550) was added and the tube was centrifuged at 12000 rpm for 1 min. The water in the pellet of mitochondria rose to the top of the silicone layer, and was removed by four washed with distilled water and then blotting with tissue paper. Then 1 ml of 15% $HClO_4$ was added and the tube was centrifuged for 1 min. The radioactivity of [^{32}P]P_i in the $HClO_4$ layer was assayed in an Aloka liquid scintillation spectrometer LSC-602. Results on P_i incorporated into mitochondria without dye determined by this method were confirmed to agree well with those determined by the conventional method with centrifugation of mitochondria through a layer of silicone oil into an $HClO_4$ layer [14,15].

The membrane potential of mitochondria was determined with a TPP^+-electrode prepared as reported previously [16]. In this case 10 μM TPP^+ was added to the incubation medium. TPP^+ at this concentration was confirmed not to have any effect on State 4 respiration of mitochondria [8].

The amount of dye retained by mitochondria was determined from the difference between the absorbance of the dye at 600 nm in the medium before and after incubation with mitochondria. In this case mitochondria in the incubation medium were promptly precipitated by centrifugation at 12000 rpm for 1 min in a Kubota centrifuge, model KM 15000, after the desired incubation period at 25°C and the absorbance of the dye in the supernatant was measured.

Results

Requirement of P_i for uncoupling

First, the effect of the cyanine dye tri-S-C_4(5) on State 4 respiration of mitochondria with succinate (plus rotenone) as substrate was examined. In the presence of 10 mM P_i, the dye at 50 μM stimulated the respiration greatly as shown in Fig. 1, trace '−NEM', while in the absence of P_i it had little effect. These effects are essentially the same as those observed with the more hydrophobic dye

tri-S-C_7(5) [8]. However, unlike in the case of tri-S-C_7(5), after addition of tri-S-C_4(5) to the incubation medium, there was a lag-phase before acceleration of respiration and then the respiratory rate increased with time to a maximal level. After attaining this maximum level, the respiratory rate remained constant until all the oxygen had been consumed by the mitochondria, as shown in Fig. 1.

The dye released oligomycin-inhibited respiration completely and activated ATPase (data not shown). Thus tri-S-C_4(5) is concluded to be an uncoupler of oxidative phosphorylation in mitochondria. The activation of ATPase was also P_i-dependent, but the degree of activation was not as great as that observed with the protonophoric uncouplers 2,4-dinitrophenol and SF 6847 (data not shown).

Fig. 2 depicts the acceleration of the rate of State 4 respiration (V_{ox}) by tri-S-C_4(5) as a function of dye concentration in the presence of various amounts of P_i. Values for V_{ox} are shown as maximum rates attained after the lag-phase. In the absence of P_i, the dye caused only about 2-fold release of State 4 respiration even at very high concentrations, such as 100 μM. However, in the presence of P_i, it caused considerable release of respiration. Its effect increased with increase in the P_i concentration to a maximum with 10 mM: in the presence of 10 mM P_i and about 50 μM dye, State 4 respiration was increased more than 6-fold. The lag phase became shorter with increase in the concentrations of both dye and P_i. The increase in

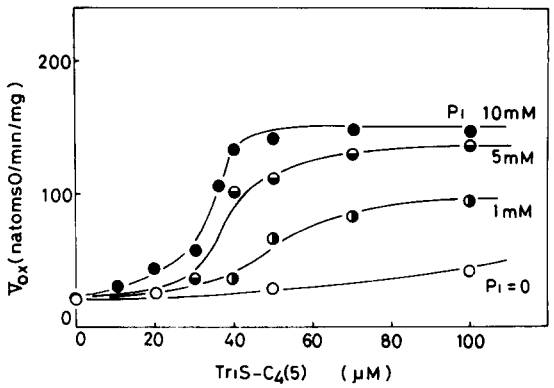

Fig. 2. Effect of tri-S-C_4(5) on the State 4 respiration rate (V_{ox}) of rat liver mitochondria with various concentrations of P_i. The maximal rate of respiration induced by the dye is plotted against the dye concentration. Experimental conditions were as for Fig. 1.

V_{ox} with dye concentration was always sigmoidal, unlike that with protonophoric uncouplers [13].

The results in Fig. 2 suggest that the uncoupling by tri-S-C_4(5) is P_i-dependent. To confirm this, we next examined the effects of various anions on the uncoupling by the cyanine dye. Fig. 3 depicts the release of state 4 respiration with succinate (plus rotenone) as substrate by 50 μM tri-S-C_4(5) in the presence of various amounts of the anions Cl^-, SCN^-, HCO_3^-, AcO^- (acetate ion) and P_i. It is apparent from the figure that P_i was the most effective. AcO^- was less than half as effective as P_i, and induced less than 3-fold stimulation of

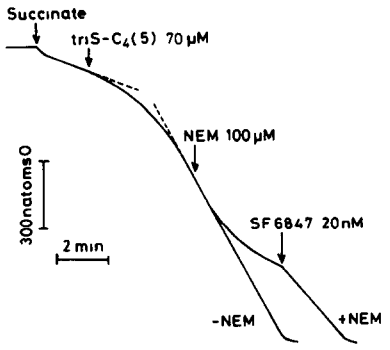

Fig. 1. Effect of the P_i-transport inhibitor *N*-ethylmaleimide (NEM) on the uncoupling by tri-S-C_4(5) Mitochondria were suspended in $+P_i$ medium. Rat liver mitochondria (0.7 mg protein/ml in a total volume of 2.53 ml) were energized with 10 mM succinate (plus rotenone at 1 μg/mg mitochondrial protein).

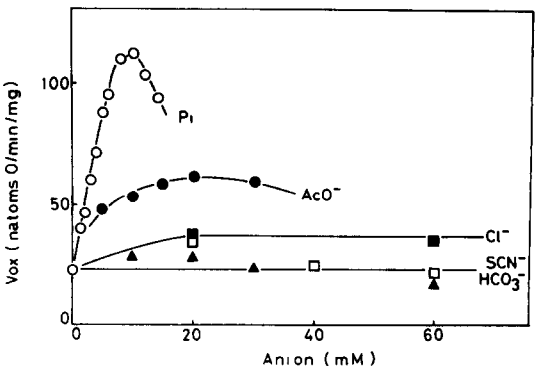

Fig. 3. Effects of various anions on the release of State 4 respiration of mitochondria induced by 50 μM tri-S-C_4(5) Anions were added to $-P_i$ medium. Experimental conditions were as for Fig. 1.

State 4 respiration by tri-S-C_4(5). Other anions, such as Cl^-, SCN^- and HCO_3^-, induced little, if any, uncoupling by the cyanine dye. Arsenate, which is known to be transported into mitochondria via the P_i-transport system [17,18], induced uncoupling as effective as P_i (data not shown). It is noteworthy that not all the proton-donating anions tested (AcO^-, HCO_3^-, P_i and arsenate) were effective for inducing uncoupling by the cyanine dye, although these anions all induce respiratory release by Ca^{2+} [18].

Next, the effect of tri-S-C_4(5) on P_i transport across mitochondrial inner membranes was examined. Fig. 4 shows the time courses of P_i uptake into respiring mitochondria with succinate (plus rotenone) as substrate in medium with 10 mM P_i in the presence and absence of 100 μM dye. It is apparent that the dye stimulated the uptake of P_i, which increased to a steady-state after 30 s of about 3-fold that without dye. The incorporated P_i should be transported back again into the incubation medium via the P_i-dicarboxylate antiporter [20]. Note that under these experimental conditions (with a high cyanine dye concentration and large amount of mitochondria, cf. legend for Fig. 4), the dye first induced mitochondrial shrinkage and then after about 2 min it induced swelling of mitochondria (cf. Fig. 7) and uncoupling. Thus the steady-state of the P_i-uptake was attained in the period of shrinkage of mitochondria before initiation of uncoupling.

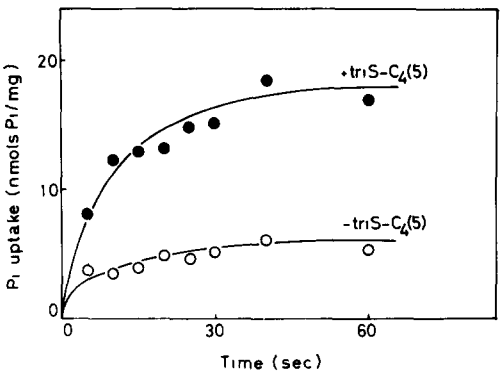

Fig 4. Effect of tri-S-C_4(5) on the time-course of P_i-transport into mitochondria. The time-course of P_i incorporation into mitochondria was measured in the presence or absence of 100 μM tri-S-C_4(5). Mitochondria (1.7 mg/ml in a total volume of 1 ml) were incubated with 10 mM succinate (plus rotenone at 1 μg/mg protein) in $-P_i$ medium for 1 min Then 10 mM [^{32}P]P_i (0.3 μCi) with or without dye was added. The total amount of P_i nonspecifically adsorbed to mitochondrial membranes and present in the sucrose space was 20 nmol/mg protein

When the P_i-transport inhibitor N-ethylmaleimide or mersalyl was added to the incubation medium, the P_i-associated uncoupling by the dye was abolished, as shown for the case of N-ethylmaleimide in Fig. 1. The repiration was released again almost completely on addition of the weakly acidic uncoupler SF 6847. Thus the P_i-transport system is concluded to play an essential role in the uncoupling. However, in view of the results in Fig. 4, P_i-transport itself does not seem to induce uncoupling and swelling of mitochondria directly, but rather to act as a trigger for inducing the uncoupling and uncoupling-associated responses.

Effect of ADP and ATP

Addition of ADP to state 4 mitochondria in $+P_i$ medium with succinate (plus rotenone) as substrate in the presence of oligomycin inhibited the release of respiration induced by 50 μM tri-S-C_4(5). ADP was effective at over 100 μM and inhibited the uncoupling almost completely at 4 mM (Fig. 5A). ADP was similarly inhibitory in the presence of oligomycin and the adenine nucleotide transport inhibitor atractyloside (Fig. 6A). Since the effect of ADP took place immediately after its addition, it can be concluded that it is not ATP converted from ADP by the adenylate kinase present in the mitochondrial intermembrane space, but ADP itself that exerts an inhibitory effect on

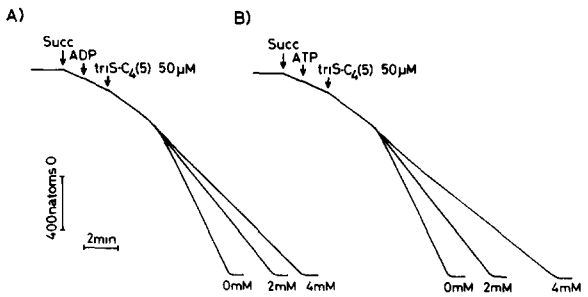

Fig. 5. Effects of ADP(A) and ATP(B) on uncoupling by 50 μM tri-S-C_4(5). Succinate (plus rotenone) was used as substrate in $+P_i$ medium and oligomycin at 2 μg/mg protein was added. Concentrations shown under traces are those of ADP or ATP. Experimental conditions were as for Fig. 1.

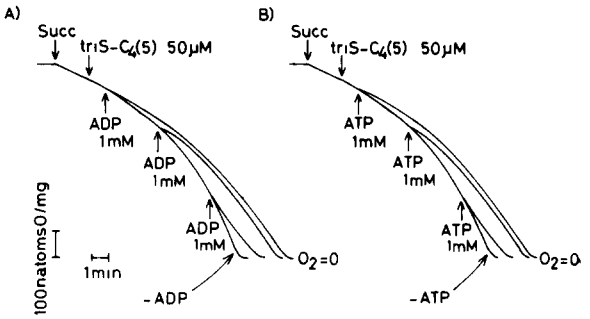

Fig. 6. Effects of ADP(A) and ATP(B) during uncoupling by 50 μM tri-S-C_4(5). To state 4 mitochondria with succinate (plus rotenone) as substrate in $+P_i$ medium and in the presence of oligomycin (2 μg/mg protein) and 20 μM atractyloside, 50 μM tri-S-C_4(5) was added to induced uncoupling ADP or ATP at 1 mM was added at the times indicated in the figure. Experimental conditions were as for Fig. 1.

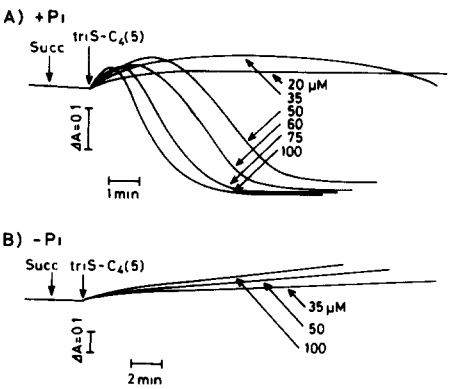

Fig. 7 Induction of volume change of mitochondria by 50 μM tri-S-C_4(5) in the presence (A) and absence (B) of 10 mM P_i. The dye was added to suspensions of rat liver mitochondria (0 7 mg protein/ml) energized with 10 mM succinate (plus rotenone at 1 μg/mg protein), and the absorbance change at 700 nm was monitored. The total volume of the reaction mixture was 3.0 ml. $+P_i$ medium (A) and $-P_i$ medium (B) were used

the uncoupling by the cyanine dye.

Like ADP, ATP protected mitochondria from uncoupling by the cyanine dye both in the presence and absence of oligomycin and atractyloside (Figs. 5B and 6B). Since ADP and ATP do not penetrate the mitochondrial membrane in the presence of atractyloside, the adenine nucleotide seem to exert their effects on the outer surface of the membrane. This idea is consistent with the fact that ADP and ATP exhibited their effects immediately after their addition during uncoupling by the cyanine dye (Fig. 6). Moreover, since the absorption spectrum of tri-S-C_4(5) was not changed by addition of ATP or ADP, these nucleotides did not form complexes with the dye. Therefore, the dye and the nucleotides affect mitochondrial function independently.

Volume change of mitochondria

Fig. 7 shows the effect of tri-S-C_4(5) on the volume of mitochondria energized with succinate (plus rotenone), monitored as the change in the optical absorption at 700 nm. In the presence of 10 mM P_i, the dye at less than 35 μM induced gradual increase in the absorbance (Fig. 7A). This increase can be interpreted as due to shrinkage of mitochondria [19]. At concentrations of more than 35 μM, the dye induced shrinkage followed by swelling of the mitochondria, as indicated by increase and then decrease in absorbance. With increase in the concentration of the dye, the period of shrinkage became shorter and the rate of swelling became greater. The time at which shrinkage changed to swelling corresponded to that of initiation of acceleration of State 4 respiration by tri-S-C_4(5). The swelling stopped when the incubation medium became anaerobic, and in the anaerobic state the mitochondria maintained a constant volume. In contrast, as shown in Fig. 7B, in the absence of P_i, the dye did not cause swelling but concentration-dependent shrinkage.

In the presence of the P_i-transport inhibitor *N*-ethylmaleimide or mersalyl, the dye did not induce swelling of mitochondria. Moreover, on addition of these inhibitors during swelling, the swelling stopped and the mitochondrial volume then remained constant, as shown for the case of

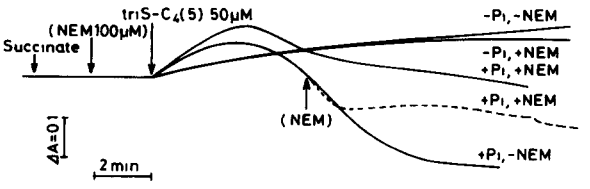

Fig. 8. Effect of *N*-ethylmaleimide (NEM) on the volume change of mitochondria induced by 50 μM tri-S-C_4(5). Experimental conditions were as for Fig. 7.

N-ethylmaleimide in Fig. 8. Anions, such as Cl^-, ClO_4^-, SCN^- and I^-, at 10 mM were ineffective in inducing shrinkage and then swelling by the dye at 50 μM, while acetate at 10 mM was slightly effective, and arsenate was as effective as P_i. Thus the relative effects of these anions on swelling were parallel with their relative effects on stimulation of State 4 respiration by the dye, suggesting that the swelling is directly related to the uncoupling.

Membrane potential-sensitive transmembrane movement of TPP^+

The permeant cation TPP^+ is taken up into the inner side of mitochondria when they are energized with succinate (plus rotenone). In the presence of 10 mM P_i (Fig. 9A), addition of tri-S-C_4(5) caused immediate loss of some of the incorporated TPP^+, and then after a certain lag phase, dependent on the concentration of the dye, the rest of the incorporated TPP^+ was completely extruded from the mitochondria. The second extrusion of incorporated TPP^+ took place at the time when the release of State 4 respiration attained a maximum level. At a concentration of dye that had no effect on State 4 respiration, such as 30 μM dye (trace a in Fig. 9A), all the TPP^+ that had been ejected from mitochondria just after addition of the dye was gradually taken up again. The weakly acidic uncoupler SF 6847 caused prompt ejection of almost all this incorporated TPP^+.

When the P_i-transport inhibitor N-ethylmaleimide was added to uncoupled mitochondria from which TPP^+ had been released, it caused the reincorporation of TPP^+. The incorporated TPP^+ was then released again on addition of SF 6847 (Fig. 9B). In the absence of P_i, the manner of TPP^+ movement in mitochondria in the presence of the cyanine dye was essentially the same as that in the presence of P_i with an ineffective amount of the dye (cf. Fig. 9A, trace a).

It is generally thought that TPP^+ is incorporated electrophoretically into the matrix space of mitochondria down the inside-negative membrane potential, and that dissipation of the membrane potential results in its extrusion [16]. Thus the uncoupling by tri-S-C_4(5) is concluded to be directly related to dissipation of the inside-negative membrane potential of energized mitochondria.

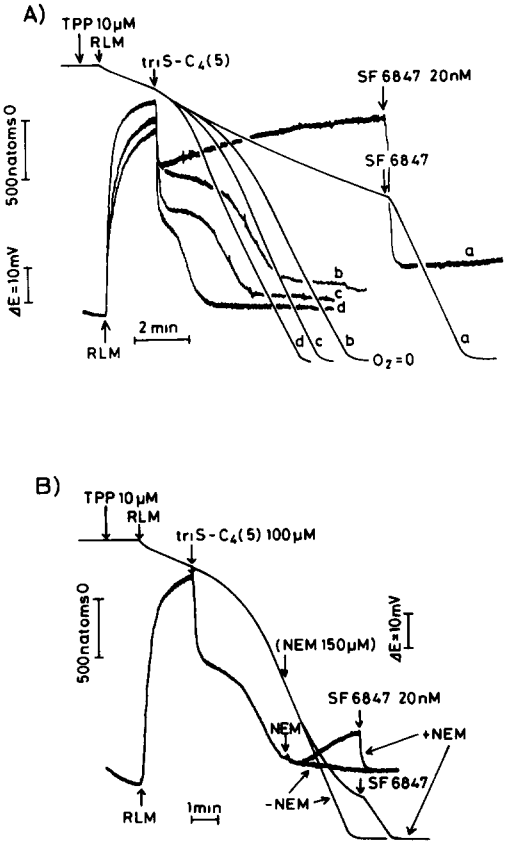

Fig. 9. Effect of tri-S-C_4(5) on the movement of TPP^+ across the mitochondrial membrane. (A) Effects of various concentrations of tri-S-C_4(5). a, 30 μM; b, 75 μM; c, 100 μM; d, 200 μM. (B) Effect of 150 μM *N*-ethylmaleimide (NEM) in uncoupled mitochondria induced by 100 μM tri-S-C_4(5). Rat liver mitochondria (RLM, 0.7 mg protein/ml) were suspended in $+P_i$ medium containing 10 mM succinate plus rotenone (1 μg/ml mitochondrial protein) in a total volume of 5.0 ml. Oxygen consumption was monitored concomitantly.

Amount of dye retained by mitochondria

Finally, we determined the amount of tri-S-C_4(5) retained by mitochondria under various conditions by measuring the decrease of the dye concentration in the incubation medium. This amount does not necessarily represent the amount penetrating the mitochondria, but is the sum of this and the amount bound to mitochondrial membranes. Results with 50μM cyanine dye are shown in Fig. 10. Only about 14% of the dye was taken up by mitochondria de-energized by rotenone, antimycin A and oligomycin either in the presence or absence of P_i, and the uptake was not time-depen-

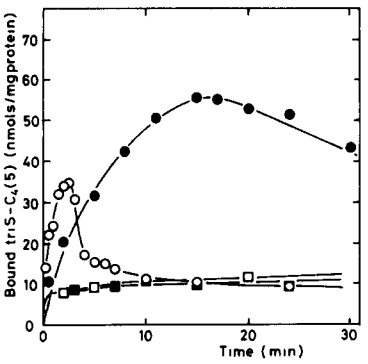

Fig. 10. Time-course of uptake of tri-S-$C_4(5)$ by mitochondria under various conditions. ○, energized mitochondria in $+P_i$ medium; ●, energized mitochondria in $-P_i$ medium; □, de-energized mitochondria in $+P_i$ medium, ■, de-energized mitochondria in $-P_i$ medium. Rat liver mitochondria (0.7 mg protein/ml in a total volume of 1.5 ml) were energized with 10 mM succinate (plus rotenone), and were de-energized with rotenone, antimycin A and oligomycin (each at 1 μg/mg mitochondrial protein). Concentration of tri-S-$C_4(5)$: 50 μM.

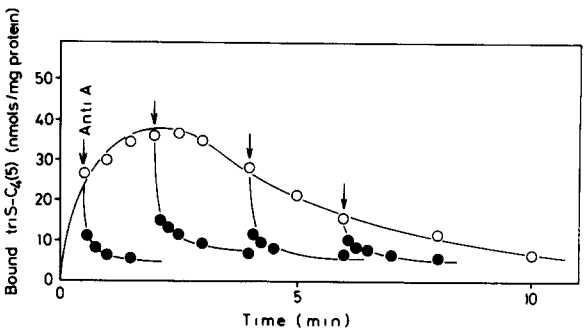

Fig. 11. Effect of antimycin A on the affinity of tri-S-$C_4(5)$ to uncoupled mitochondria. At the times indicated in the figure, 2 μg of antimycin A was added to samples of mitochondrial suspension in the presence of 50 μM tri-S-$C_4(5)$ in $+P_i$ medium The amount of dye was determined without (open circles) and with (closed circles) antimycin A. Experimental conditions were as for Fig 10.

dent. In contrast, the uptake by energized mitochondria was dependent on the incubation time. In the absence of P_i, where the dye has no effect in inducing uncoupling, its uptake by mitochondria energized with succinate (plus rotenone) increased gradually to a maximal of about 90% of the total dye after 15 min, and then decreased gradually. This decrease was because the incubation medium became anaerobic.

In the presence of P_i, the uptake was more rapid than that without P_i. However, about 2 min after addition of dye, the time-dependent uptake was reversed, and the dye began to be released from mitochondria into the incubation medium, the amount of dye taken up by mitochondria finally decreasing to the level in de-energized mitochondria. The time when the reversal of the interaction took place corresponded to that of initiation of uncoupling.

Addition of antimycin A to mitochondria with succinate as substrate in $+P_i$ medium at a various times after addition of 50 μM tri-S-$C_4(5)$ consistently resulted in immediate release of the retained dye to the level in de-energized mitochondria, as shown in Fig. 11. This results suggests that the uncoupling-dependent release of dye was not due to damage of the mitochondria.

Discussion

We showed that the uncoupling action of tri-S-$C_4(5)$ was dependent on P_i (or arsenate) and was associated with swelling of the mitochondria, and that the P_i-transport inhibitors N-ethylmaleimide and mersalyl abolished both the uncoupling and the swelling. These findings suggest that the P_i-transport system participated directly in the P_i-dependent uncoupling, and that the swelling was closely related to the uncoupling. However, the uncoupling was apparently not due to incorporated P_i itself. The action of tri-S-$C_4(5)$ was very similar to that of the more hydrophobic dye tri-S-$C_7(5)$ (Ref. 8), but tri-S-$C_4(5)$ was about 5-times less effective than tri-S-$C_7(5)$ in inducing maximal release of State 4 respiration (minimum effective concentration: tri-S-$C_4(5)$, 50 μM; tri-S-$C_7(5)$, 10 μM) and it required about 10-times more P_i (about 10 mM) than tri-S-$C_7(5)$ (1 mM) for its maximal effect.

The differences in the effective concentrations of P_i and of these two cyanine dyes for their uncoupling actions could be due to the difference in their hydrophobicities: higher concentrations of the less hydrophobic tri-S-$C_4(5)$ and of P_i are necessary for access of this dye to the mitochondrial membranes. The observed lag-phase before uncoupling and the shrinkage before swelling with tri-S-$C_4(5)$, but not with tri-S-$C_7(5)$, would

be reflections of the more polar property of the former dye. Possibly cyanine dyes perturb the membrane organization in the presence of P_i, since tri-S-C_4(5) caused marked increase in the electrical conductance of phospholipid bilayer membranes and fluctuation of the electrical current of these membranes in incubation medium containing P_i, but not in medium without P_i [11]. Furthermore, P_i was found to decrease the interfacial tension between water and phospholipid (Terada, H. et al., unpublished data).

Although the affinity of tri-S-C_7(5) to mitochondria was independent of their energy state [8], that of tri-S-C_4(5) was dependent on their energy state owing to its lower hydrophobicity. The dye interacted more rapidly with mitochondria in the presence than absence of P_i, probably because P_i increased its accessibility to the mitochondria. However, the dye began to be released from mitochondria when uncoupling and swelling started to occur and it lost affinity to mitochondria almost completely under full uncoupling. This loss of its affinity on uncoupling could not be due to mitochondrial damage by the dye, as described at the last part of this section. In contrast, in the absence of P_i, the amount of dye taken up by mitochondria continued to increase until all the oxygen was consumed, and the maximum uptake of dye was about 90% of the total amount added to the incubation medium. It is noteworthy that without P_i the affinity of dye to mitochondria was greater than that with P_i, but the dye had no appreciable effect on mitochondrial function.

The hydrophobic cations DDA^+ and TPP^+ are taken up by mitochondria on energization of the mitochondria with a respiratory substrate or ATP. However, these cations are released into the incubation medium when the mitochondria are de-energized either by respiratory inhibitors or uncouplers [8,16,21]. Furthermore, DDA^+ in the presence of P_i causes uncoupling and swelling of mitochondria [4]. Thus, the uncoupling actions of DDA^+ and other hydrophobic cations have been interpreted as due to dissipation of the membrane potential by their electrophoretic transfer into the mitochondria [3,4].

However, the retention of dye by mitochondria always caused shrinkage of mitochondria, either with or without P_i, while induction of uncoupling was accompanied by release of the dye and swelling. Furthermore, some of the incorporated TPP^+ (about one-third to a half, depending on the concentration of added dye, cf. Fig. 9) was instantly released on addition of dye and the rest of the TPP^+ remained incorporated into the mitochondria during uptake of dye and then was all released on induction of uncoupling. These results suggest that the dye acts on the mitochondrial membranes rather than after it has penetrated into the mitochondria. The swelling could result from penetration of ions, such as K^+, from the incubation medium. Since the uptake of P_i took place while the mitochondria were shrinking, the action of the dye was not due simply to phosphate-induced swelling and uncoupling [22].

Thus, the following alternative mechanism for the uncoupling action of the cyanine dye is possible. The dye first binds to mitochondrial membranes. Its binding site could be the P_i-H symporter itself or proteins adjacent to it as well as the phospholipid region of the membrane. In the presence of P_i, the dye bound to the mitochondria perturbs the membranes, whereas in the absence of P_i, it does not. This perturbation stimulates the P_i-H symporter, and plays as a trigger to induce the penetration of cations across membranes from the incubation medium, causing dissipation of the membrane potential. The transported P_i then returns to the outside of the mitochondria in exchange with succinate used as respiratory substrate via the P_i-dicarboxylate antiporter. The importance of the P_i-transporter for this uncoupling mechanism is stressed.

Finally, it is interesting to note that the action of tri-S-C_4(5) on mitochondria is very similar to 'calcium uncoupling' in its requirement for P_i [23], induction of swelling of mitochondria [24], and protection by adenine nucleotides [23,25,26]. The effect of Ca^{2+} is regarded as the result of damage of the mitochondrial membrane by Ca^{2+} in the matrix space. At present it is not clear whether cyanine dye damages mitochondria. However, the findings that *N*-ethylmaleimide abolished the swelling (Fig. 8) and uncoupling of mitochondria that were able to respond the weakly acidic uncoupler SF 6847 (Fig. 1), and that it also caused reincorporation of TPP^+ that had been released from uncoupled mitochondria (Fig. 9B) suggest

that most of the mitochondria are still intact, and that the dye caused little, if any, damage of mitochondria. It is noteworthy that in the case of 'calcium uncoupling', irreversible depolarization of the membrane potential by Ca^{2+} monitored by the transmembrane movement of TPP^{+} was taken as an indication of mitochondrial damage [27]. The immediate loss of bound dye from mitochondria on addition of antimycin A (Fig. 11) also indicated that the respiratory chain was not damaged. The mechanism of 'calcium uncoupling' is not fully clarified [28], and studies on uncoupling by the divalent cation cyanine dyes should be helpful in understanding the effect of Ca^{2+} on oxidative phosphorylation. We are now comparing actions of cyanine dye and Ca^{2+} under identical experimental conditions.

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