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A CYANINE DYE TRI-S-C_7(5)

PHOSPHATE-DEPENDENT CATIONIC UNCOUPLER OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

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The trinuclear cyanine dye, tri-S-C_7(5), at about 10 μM stimulated State 4 respiration of rat liver mitochondria more than 6-fold and released oligomycin-inhibited respiration completely. Thus, the dye is concluded to be a very effective cationic uncoupler of oxidative phosphorylation in mitochondria. However, for exhibition of its uncoupling action, the presence of P_i (or arsenate) was necessary, and a phosphate-transport inhibitor, *N*-ethylmaleimide or mersalyl, inhibited its action. The stimulation of phosphate transport via the P_i carrier by the dye is suggested to be directly related to the uncoupling action.

Introduction

Many compounds are known to be uncouplers of oxidative phosphorylation in mitochondria. Almost all of them are weakly acidic and increase proton permeability across the mitochondrial membrane and model membrane systems [1,2]. Thus, they are called protonophoric uncouplers, and the dissipation of the electrochemical gradient across the membranes by their protonophoric action is generally regarded as decisive for exhibition of uncoupling.

However, little is known about the action of cationic uncouplers in mitochondria Hydrophobic cations, such as DDA^+ and TBA^+, are reported to act as uncouplers in mitochondria at about 0.1–1 mM [3,4], being far less effective than potent, weakly acidic uncouplers, which are effective at less than 1 μM

In preliminary studies [5], the trinuclear cyanine dye tri-S-C_7(5) was found to release State 4 respiration of mitochondria in the presence of P_i, but to cause acidification of the incubation medium either in the presence or absence of P_i. This dye has three thiazole rings, each of which has a hydrophobic *n*-heptyl chain. Two of the three nitrogen atoms in the three thiazole rings take the quaternary ammonium structure. Thus, the dye is a hydrophobic cation with two stable cationic moieties (for chemical structure, see Refs. 5 and 6). Some hydrophobic cations, such as the cyanine dye di-S-C_3(5), ethidium bromide and rhodamine 6G, are reported to act as inhibitors of oxidative phosphorylation in mitochondria [7–9], but their actions seem to be complex. Thus, the action of tri-S-C_7(5) on mitochondria is interesting in understanding the

Abbreviations 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, 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), DDA^+, *N*,*N*-dibenzyl-*N*,*N*-dimethylammonium, TBA^+, tetrabutylammonium, TPP^+, tetraphenylphosphonium, TPB^-, tetraphenylborate, SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile, FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

mechanism of action common to hydrophobic cations on mitochondria. This paper reports the effect of the cyanine dye in mitochondria as a P_i-dependent cationic uncoupler of oxidative phosphorylation. An important role of the P_i-transport system in the uncoupling by the cyanine dye is demonstrated.

Materials and Methods

Tri-S-C_7(5) and SF6847 were gifts from the Nippon Kankoshikiso Research Laboratory, Okayama, and Sumitomo Chemical Industry, Osaka (Japan), respectively. Other reagents were commercial products A concentrated solution of tri-S-C_7(5) in dimethyl sulfoxide was used as stock solution, since the presence of up to 60 μl dimethyl sulfoxide in 2.53 ml incubation medium had no effect on State 3 and 4 respirations of mitochondria with succinate as substrate.

Mitochondria were isolated from adult male Wistar rats by the method of Hogeboom [10] as described by Myers and Slater [11].

Consumption of oxygen in the reaction medium due to respiration of mitochondria was measured polarographically with a Clark-type oxygen electrode (Yellow Springs, YSI 5331 oxygen probe) We used two incubation media: $+P_i$ medium consisted of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM Na_2EDTA and 10 mM potassium phosphate buffer, pH 7.4; $-P_i$ medium had the same composition as $+P_i$ medium but with Tris-HCl buffer (pH 7.4) instead of phosphate buffer. In most cases, succinate (10 mM, sodium salt) with rotenone (1 μg/mg protein) was used as respiratory substrate All reactions were carried out at 25°C.

ATPase activity was determined by measuring the change in pH of the medium by the method of Bertina and Slater [12]. The mitochondria were incubated for 2 min with 2 mM ATP before addition of the dye to the reaction medium.

Change in volume of mitochondria was monitored spectrophotometrically as the change in absorbance at 700 nm in a Shimadzu recording spectrophotometer, model UV-300

The amount of dye bound to mitochondria was determined as follows. The mitochondrial suspension was incubated with the dye for a known period at 25°C in a polyethylene centrifuge tube, then the mitochondria were spun down quickly through a layer of silicon oil (Toray Silicon SH 5500) in a Kubota centrifuge, model KM 15000, and the concentration of the dye in the supernatant was determined spectrophotometrically. The difference between the amounts of dye before and after incubation was taken as the total amount bound to mitochondria

The movement of H^+ from mitochondria to the incubation medium on addition of the dye was measured with a combined pH electrode (Horiba, type 2535A) The amount of H^+ ejected was calibrated by addition of 10 mM oxalic acid solution The incubation medium for monitoring H^+ movement consisted of 150 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 1 mM Na_2EDTA and 1 mM potassium phosphate buffer (pH 7 4) as $+P_i$ medium, and the same medium with 1 mM Tris-HCl buffer (pH 7 4) instead of 1 mM phosphate buffer as $-P_i$ medium

The potential across the mitochondrial membrane was determined with a TPP^+ electrode prepared by the method reported previously [13,14] in reaction medium containing 10 μM TPP^+ It was confirmed that TPP^+ at 10 μM had no effect on mitochondrial State 3 and 4 respirations, and that tri-S-C_7(5) at up to 20 μM had little effect on the electrical response depending on the concentration of TPP^+ in the incubation medium in the absence of mitochondria Thus, the electrical response during the assay reflected the movement of TPP^+ across the mitochondrial membrane

In this study all experiments were repeated at least three times, and typical results from one experiment are shown

Results

Effect on mitochondrial function

When the cyanine dye tri-S-C_7(5) was added to State 4 mitochondria with succinate (plus rotenone) as substrate, it accelerated respiration in the presence of P_i, but had little effect in the absence of P_i, as observed previously [5]. The degree of stimulation of respiration increased with increase in concentration of the dye, and a maximum of more than 6-fold release of State 4 respiration was attained at a concentration of 10 μM, as shown in

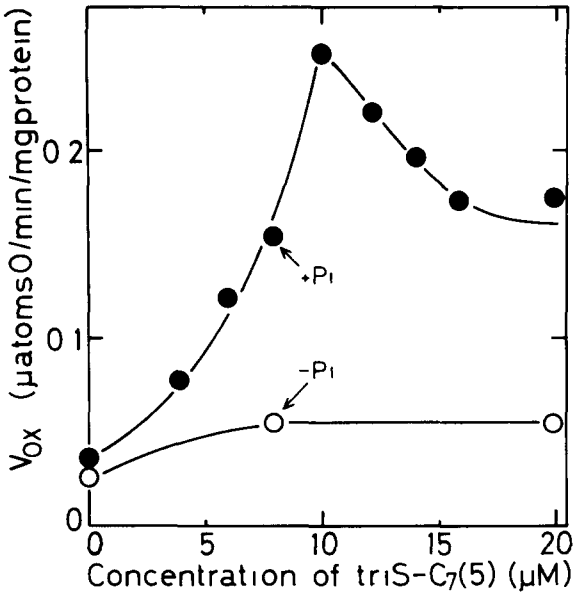

Fig 1 Effect of tris-S-C_7(5) on the respiration (V_{ox}) of mitochondria as a function of the dye concentration in media with (●——●) and without (○——○) 10 mM P_i The dye was added to State 4 respiring rat liver mitochondria with succinate (10 mM) plus rotenone (1 μg/mg protein) as substrate Mitochondria 0 7 mg protein/ml in a total volume of 2 53 ml

Fig. 1 Further increase in the dye concentration caused a progressive decrease in stimulation. Furthermore, at dye concentrations of more than 10 μM, the dye had a transient effect, causing first stimulation and then slight inhibition (cf. Fig. 3B) The inhibition was not completely reversed by addition of a weakly acidic uncoupler, such as SF6847 or FCCP (data not shown) This inhibition was not significant when the concentration of the dye was lower than 10 μM (cf. Fig 4). The V_{ox} shown in Fig. 1 is that induced just after addition of the dye. The figure shows that the change in V_{ox} vs dye concentration is not linear, but sigmoidal in the presence of P_i, although the effect of a protonophoric uncoupler is always linear, not sigmoidal. In the absence of P_i, V_{ox} did not exceed 3-fold the original level (V_{ox} in State 4) at dye concentrations of up to 20 μM A similar stimulatory effect of the dye on respiration in the presence of P_i was observed with glutamate plus malate as substrate (data not shown *).

* This result was reported in preliminary form in Ref 15

The cyanine dye released oligomycin-inhibited State 3 respiration completely, as shown in Fig. 2. It also stimulated ATPase activity at more than 20 μM, which is higher than the concentration required for stimulation of State 4 respiration (data not shown). The activation of ATPase was dependent on the P_i concentration. The mechanism of the ATPase activation by the dye appeared complex and will be the subject of further study From these results, we concluded that the effect of the dye was due to the uncoupling of oxidative phosphorylation, and that this uncoupling was P_i dependent.

We next examined the effects of various anions on induction of uncoupling to see whether the uncoupling is really specific to P_i. Fig 3 shows the effects of anions such as picrate, Cl^-, SCN^-, I^-, ClO_4^- and acetate on the uncoupling by tri-S-C_7(5) in $-P_i$ medium. These anions permeate the mitochondrial membrane by different mechanisms [16]. The effect of P_i is also shown for comparison. All the anions (at 2 mM) were found to be far less effective than P_i for induction of uncoupling by the dye when the dye concentration was less than 10 μM (Fig. 3A). At dye concentrations greater than 10 μM, these anions induced some uncoupling, but always much less in extent than that caused by P_i (Fig. 3B) In the case of P_i, respiratory inhibition was followed after the first acceleration of State 4 respiration Thus, the effect of the

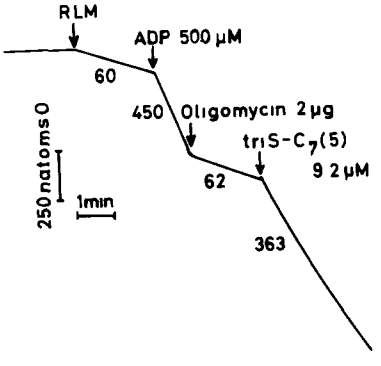

Fig 2 Release of oligomycin-inhibited respiration of mitochondria by tri-S-C_7(5) RLM, rat liver mitochonria (0 7 mg protein/ml in a total volume of 4 35 ml) Substrate 10 mM succinate (plus 1 μg rotenone/mg protein) Incubation medium + P_i medium Numbers beside the curve indicate velocities of respiration in ngatom O/min

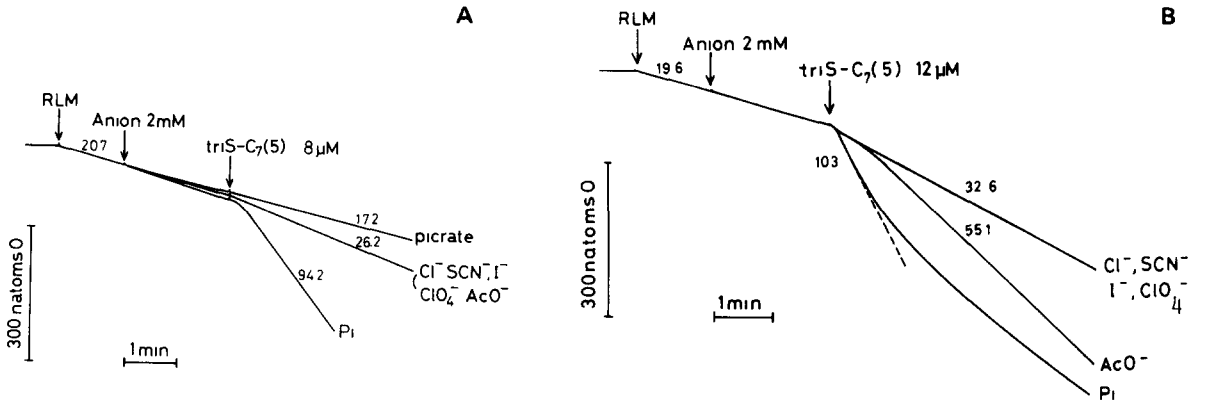

Fig 3 Effects of various anions on the release of State 4 respiration of mitochondria induced by tri-S-C_7(5) RLM, rat liver mitochondria (0 7 mg protein/ml) The anion was added to the mitochondrial suspension in $-P_i$ medium with succinate (plus rotenone) as substrate at the time indicated in the figure Experimental conditions were as for Fig 1 Numbers beside the curve indicate velocities of respiration in ngatom O/min per mg protein (A) With 8 μM tri-S-C_7(5), (B) with 12 μM tri-S-C_7(5) AcO^-, acetate

cyanine dye on mitochondrial functions is characterized by P_i-associated uncoupling Only arsenate, which is known to be transported into mitochondria via the P_i carrier [17,18], had an effect similar to that of P_i (data not shown).

To determine the role of P_i in the uncoupling action of tri-S-C_7(5), we next examined the effects of the P_i-transport inhibitors *N*-ethylmaleimide and mersalyl As shown in Fig. 4, when 100 μM *N*-ethylmaleimide, which did not have any inhibitory effect on electron flow through the respiratory chain monitored as acceleration of respiration by the protonophoric uncoupler, was added 1.5 min before addition of the dye, it abolished the uncoupling action of the dye in $+P_i$ medium (curve C) In $-P_i$ medium, the respiration of mitochondria in the presence of the dye was not affected at all by *N*-ethylmaleimide (curve D) Results in the absence of *N*-ethylmaleimide with P_i (curve A) and without P_i (curve B) are shown for comparison. Thus, the transport process of P_i via a P_i carrier (P_i/H symporter) into the matrix space of mitochondria is suggested to be very important for exhibition of uncoupling by tri-S-C_7(5)

Swelling of mitochondria

The swelling and shrinkage of mitochondria are usually monitored as a decrease and increase, respectively, in the optical absorbance at about 520 nm [19,20]. However, tri*S*-C_7(5) has its maximum absorption at 588 nm, and its absorption spectrum changes depending on the energy state of the mitochondria Thus, to exclude the effect of

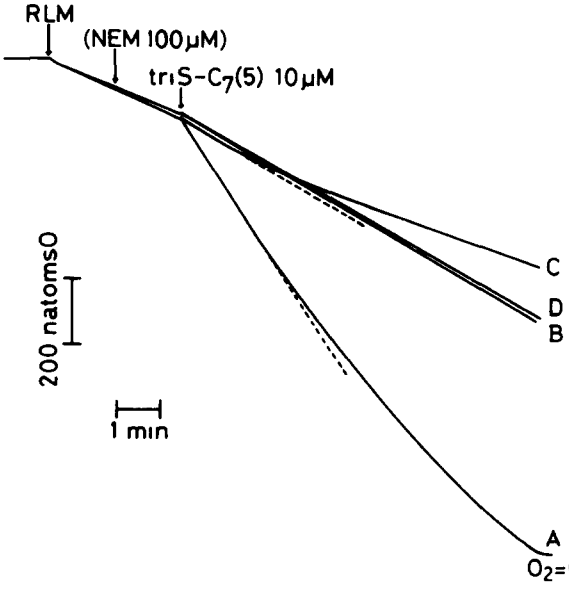

Fig 4 Effect of tri-S-C_7(5) on State 4 respiration of mitochondria under various conditions (A) In $+P_i$ medium, (B) in $-P_i$ medium, (C) in $+P_i$ medium in the presence of 100 μM *N*-ethylmaleimide (NEM), (D) in $-P_i$ medium in the presence of 100 μM *N*-ethylmaleimide RLM, rat liver mitochondria Succinate (10 mM) plus rotenone (1 μg/mg protein) was used as substrate Mitochondria 0 7 mg protein/ml in a total volume of 2 53 ml

metachromasy on the absorbance change associated with the change in volume of mitochondria, we measured the absorbance at 700 nm. As seen in Fig. 5, the dye caused a great decrease in absorbance in the presence of P_i, indicating that under these conditions marked swelling occurs. The swelling is generally interpreted as being a result of ion transport into mitochondria [16,19–21] In the absence of P_i, the dye induced slight shrinkage rather than swelling The P_i-transport inhibitor *N*-ethylmaleimide completely abolished the effect of P_i on swelling. In the presence of anions which pass through the mitochondrial membrane by different mechanisms [16], such as Cl^-, I^-, SCN^-, ClO_4^- and acetate at 2 mM, only slight swelling of mitochondria was observed in the presence of less than 10 μM tri-S-$C_7(5)$ in $-P_i$ medium (data not shown). Only arsenate was as effective as P_i, as in respiration. Thus, the transport of such anions as P_i and arsenate via P_i carrier [17,18] is responsible for the swelling induced by the cyanine dye.

For the P_i-supported swelling, the supply of energy from a respiratory substrate or ATP (when electron transfer was inhibited) was necessary. As shown in Fig. 6, when succinate was used as substrate, treatment of mitochondria with antimycin A inhibited the induction of swelling by the dye. Addition of antimycin A during the process of swelling arrested the swelling, with maintenance of the volume of mitochondria at the level at the time of addition of inhibitor, and no reversal to the original level was observed However, in the case of the permeant cation TBA^+ [4], addition of antimycin A to mitochondria undergoing TBA^+-supported swelling stopped the swelling and reversed the mitochondrial volume to the original level (Fig. 6)

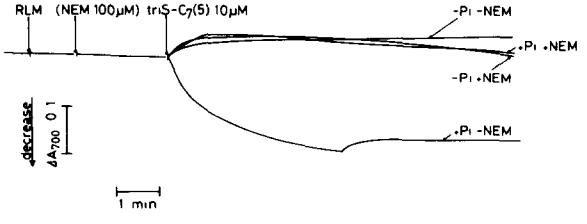

Fig 5 Induction of mitochondrial swelling by tri-S-$C_7(5)$ in the presence of P_i The dye was added to suspensions of rat liver mitochondria (RLM) under various conditions with succinate (plus rotenone) as substrate, and the absorbance at 700 nm was monitored The total volume of the reaction mixture was 3 0 ml NEM, *N*-ethylmaleimide

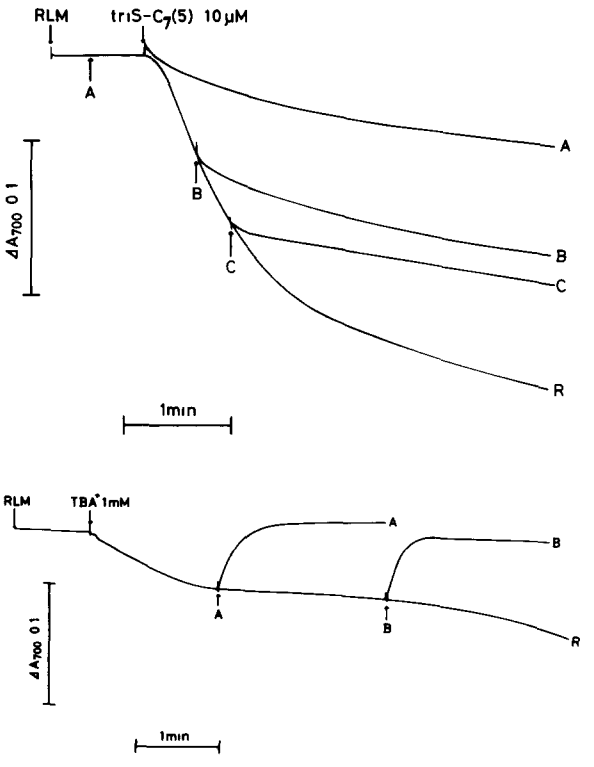

Fig 6 Effect of antimycin A on the swelling induced by tri-S-$C_7(5)$ and the penetrating cation TBA^+ in the presence of P_i Succinate (plus rotenone) was used as substrate, and the incubation medium was $+P_i$ medium Experimental conditions were as for Fig 5 At the times indicated by arrows A–C, antimycin A (3 μg) was added to give curves A–C Curve R was obtained without addition of antimycin A RLM, rat liver mitochondria

The rate and magnitude of swelling in the presence of P_i increased with increase in concentration of the dye, reaching a maximum at about 10 μM, and further increase in the concentration of the dye caused decrease in swelling. A linear relationship was observed between the rate of swelling (V_{swell}) and the rate of respiration (V_{ox}) in the presence of P_i, as shown in Fig. 7, suggesting a close relation between ion transport and uncoupling induced by the dye.

Binding to mitochondria

The binding of tri-S-$C_7(5)$ to mitochondria was

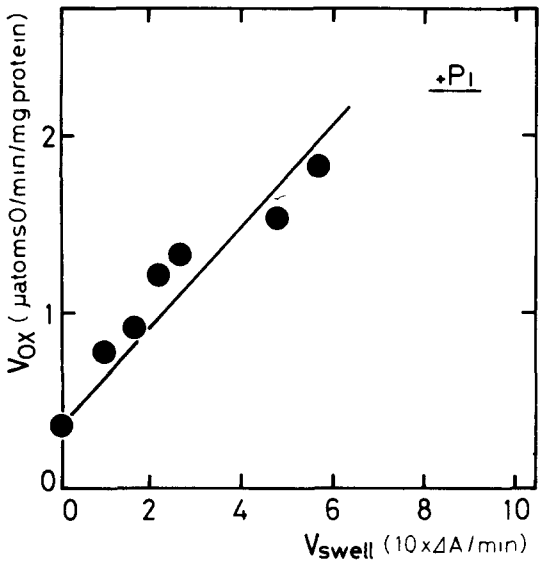

Fig 7 Linear relation between the rate of swelling (V_{swell}) and the rate of uncoupled respiration (V_{ox}) induced by tri-S-C_7(5) in $+P_i$ medium Experimental conditions were as for Figs 1 and 5

TABLE I

BINDING OF TRI-S-C_7(5) TO MITOCHONDRIA

Mitochondria (0 7 mg protein/ml) were incubated with 10 μM tri-S-C_7(5) in 1 0 ml of $+P_i$ medium or $-P_i$ medium at 25°C Total amount of dye, 14 5 nmol/mg protein Energized mitochondria were incubated with 10 mM succinate in the presence of rotenone (1 μg/mg protein) Deenergized mitochondria were incubated by addition of 1 μg/mg protein of antimycin A and oligomycin to energized mitochondria

Incubation period (min)	Bound tri-S-C_7(5) (nmol/mg protein)			
	Energized mitochondria		Deenergized mitochondria	
	$+P_i$	$-P_i$	$+P_i$	$-P_i$
0 5	10 4	12 2	11 5	12 3
1 0	10 7	12 5	12 7	13 2
3 0	11 0	12 6	–	–
5 0	11 0	12 8	–	–
6 0	11 2	–	13 5	13 7
10 0	11 6	12 7	–	–
13 5	–	–	13 6	13 8
20 0	12 7	13 0	13 7	14 0

studied under various conditions at 25°C. The dye at 10 μM was added to mitochondria energized with succinate and to those deenergized by the inhibitors rotenone, antimycin A and oligomycin in media with and without P_i. The amount of binding was determined from the concentration difference of the dye in the medium before and after incubation. Table I summarizes the time course of binding. Just after its addition to the mitochondrial suspension, most of the dye added was bound to mitochondria, irrespective of the energy state of the mitochondria or the incubation medium: about 72% of the dye was bound to energized mitochondria in the presence of P_i, and about 85% to energized and deenergized mitochondria in the absence of P_i Then the binding gradually increased with time of incubation in all cases. The fact that the binding to uncoupled mitochondria (energized mitochondria in $+P_i$ medium) was always the lowest suggests that the affinity of the dye for mitochondria is not related to the exhibition of uncoupling, or that the manner of binding is different in the presence and absence of P_i

Proton ejection and membrane potential

Preliminary studies [5] showed that tri-S-C_7(5) induced acidification of the incubation medium when added to the mitochondrial suspension, as observed with other hydrophobic cations, such as DDA^+ and TBA^+ [4]. This H^+ ejection required a supply of energy from respiratory substrates, and the ejected H^+ was reabsorbed to mitochondria when the respiratory inhibitor antimycin A was added to mitochondria energized with succinate (data not shown) As shown in Fig. 8, the amount of H^+ ejected from mitochondria became greater as the concentration of the dye was increased in medium with or without P_i The value in $-P_i$ medium was always much greater than that in $+P_i$ medium, contrary to results with DDA^+, where H^+ ejection becomes greater on addition of penetrating anions [4]

When *N*-ethylmaleimide was added before addition of the dye in $+P_i$ medium, the amount of H^+ ejected became very close to, but slightly less than, that in $-P_i$ medium, as shown in Fig. 8 The enhancement of H^+ ejection by *N*-ethylmaleimide is interpreted as showing that during uncoupling,

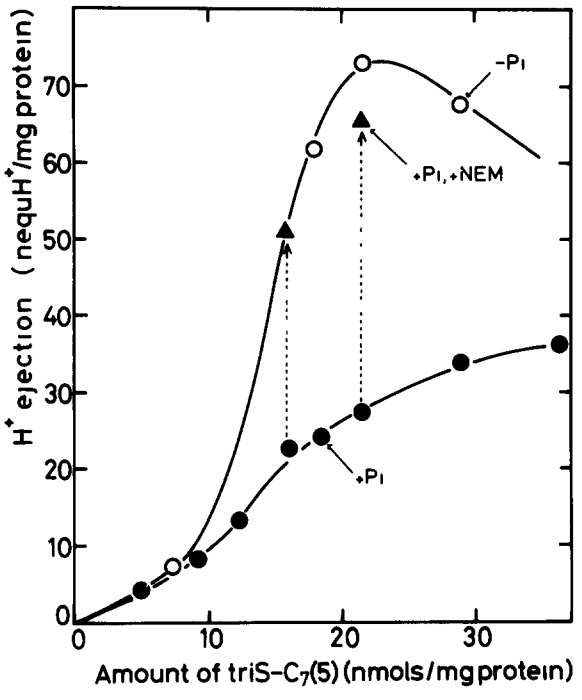

Fig 8 Ejection of H^+ from mitochondria induced by tri-S-$C_7(5)$ Rat liver mitochondria (1 4 mg protein/ml) were incubated with succinate (5 mM) plus rotenone (1 μg/mg protein) at 25°C in medium with or without P_i in a total volume of 5 81 ml In the figure the total amount of H^+ ejected is plotted against the amount of added tri-S-$C_7(5)$ (●———●) $+P_i$ medium, (○———○) $-P_i$ medium, (▲) mitochondrial suspension in $+P_i$ medium to which 70 μM *N*-ethylmaleimide (NEM) was added before addition of dye

transport of P_i into mitochondria via the P_i/H symporter is accelerated, and the increase of H^+ caused by *N*-ethylmaleimide corresponds to the amount of H^+ transported into mitochondria via the P_i/H symporter The amount of P_i transported during uncoupling is expected to be the same as that of H^+. It is noteworthy that the amount of H^+ ejected is always much greater than the amount of dye added to the medium The highest molar ratio of H^+ ejected to dye added was about 3 with about 20 nmol dye/mg protein, as shown in Fig. 8. However, in the case of DDA^+, this ratio is reported to be less than unity [4].

Next we measured the change in the membrane potential during the action of the dye. As shown in Fig. 9A, addition of succinate to a mitochondrial suspension caused uptake of TPP^+ from the reaction medium into the mitochondria In the presence of P_i, addition of tri-S-$C_7(5)$ was accompanied by the release of incorporated TPP^+ into the incubation medium, with concomitant acceleration of respiration. In the absence of P_i (Fig. 9B), the dye had little effect on the release of incorporated TPP^+, and the protonophoric uncoupler SF6847 induced extrusion of the TPP^+ taken into the mitochondria These results show a close relation between uncoupling and dissipation of the membrane potential monitored by uptake of TPP^+. It is suggested that in dissipation of the membrane potential, cations in the incubation medium are

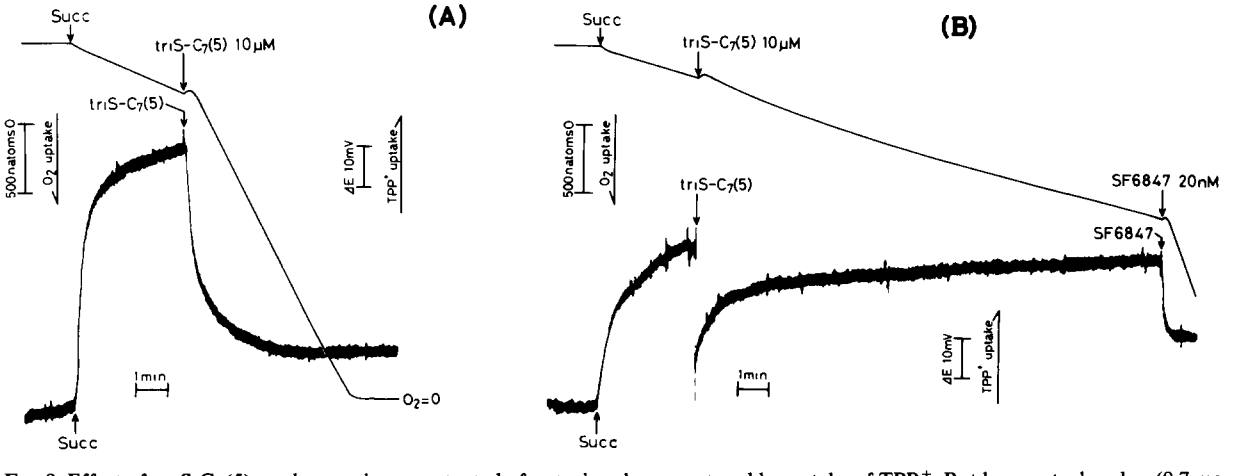

Fig 9 Effect of tri-S-$C_7(5)$ on the membrane potential of mitochondria monitored by uptake of TPP^+ Rat liver mitochondria (0 7 mg protein/ml) were suspended in $+P_i$ medium (A) or $-P_i$ medium (B) in a total volume of 5 0 ml Oxygen consumption was monitored concomitantly In the figure, Succ indicates 10 mM succinate plus 1 μg rotenone/mg protein

pulled across the membrane according to the electrical gradient to the negatively charged inner side

Discussion

The present study indicates that the trinuclear cyanine dye tri-S-C_7(5) is a potent cationic uncoupler of oxidative phosphorylation in mitochondria However, exhibition of the uncoupling action requires P_i or arsenate. The action of the cationic uncoupler DDA^+ in mitochondria has been extensively studied [3,4]. DDA^+ induces stimulation of State 4 respiration and swelling of mitochondria in the presence of P_i or other penetrating anions, such as TPB^-, though the molecular role of these anions has not been clarified DDA^+ also causes ejection of H^+ from mitochondria, and this ejection is enhanced in the presence of anions, and is reversed to the original level by addition of the respiratory inhibitor antimycin A [4] Skulachev et al. [3] proposed that the uncoupling by hydrophobic cations such as DDA^+ is the result of dissipation of the membrane potential established across the energized mitochondrial membrane by the electrophoretic transfer of the cationic uncoupler from the positively charged outside to the negatively charged inside; during the uncoupling they suggested that P_i or TPB^- is transported into the matrix space of the mitochondria as an ion-pair complex with DDA^+ On the inner side of the mitochondrial membrane they considered that this ion-pair complex dissociates, and that the resulting anion moves back electrophoretically toward the positively charged outer surface of the membrane Thus, the cycling of the anion is suggested to be responsible for the penetration of DDA^+ through the membrane [4]

The action of tri-S-C_7(5) is very similar to that of the permeant cation DDA^+ However, it appears to differ from that of the latter in some respects, especially in H^+ movement, affinity of the dye to uncoupled mitochondria and the effect of antimycin A on the swelling of mitochondria. The results in Figs. 4 and 5 suggest that P_i is transported into mitochondria via the P_i carrier, and thus that transport of the dye into mitochondria in the form of an ion-pair complex with P_i is unlikely As shown in Fig 6, mitochondria retained a consistent ion composition, reflected by their constant extent of swelling, when the progress of swelling was arrested by antimycin A, and no reversal of the swelling to the original level was observed. It is logical to speculate that if the dye is permeant, the swelling should be reversed by outflow of the dye accumulated in the matrix space when the inside-negative electrical potential is dissipated by inhibition of energy supply, as observed with the permeant cation TBA^+ (cf. Fig 6) and valinomycin-mediated K^+ transport [22]. Thus, it is concluded that the dye itself is not permeant, but that it binds to the mitochondrial membrane, inducing the intrusion of cations from the incubation medium into the inner space of the mitochondria in the presence of P_i. The findings that the extents of binding of the dye and the binding-induced H^+ ejection are slightly smaller with uncoupled mitochondria in the presence of P_i than with mitochondria in the absence of P_i, but that uncoupling takes place only in the presence of P_i suggest that the manner of the binding is different in the presence and absence of P_i. It is interesting to note that the similar trinuclear cyanine dye tri-S-C_4(5) causes time-dependent fluctuation of the electrical current across a phospholipid bilayer membrane and marked increase in the electrical conductance of the membrane in the presence, but not absence, of P_i [6].

Thus, we propose as a mechanism of action of the cyanine dye tri-S-C_7(5) that in the presence of P_i the membrane-bound dye causes perturbation of the integrity of the membrane structure in cooperation with P_i, and that this perturbation stimulates P_i translocation via the P_i/H symporter, associated with penetration of cations from the incubation medium into the mitochondria The transported P_i then returns to the positively charged outer side of the mitochondria, probably via the dicarboxylate-P_i carrier The cation intrusion accompanied by P_i translocation causes swelling and dissipation of the membrane potential which leads to uncoupling of oxidative phosphorylation in mitochondria.

At present, the role of H^+ extrusion in the uncoupling is not clear However, the findings that H^+, corresponding to about 3-fold the amount of added dye, was actually ejected from mitochondria both in the presence and absence of P_i (cf. Fig. 8), and that the uncoupling is P_i dependent suggest

that the movement of H^+ is not directly related to the uncoupling. Possibly H^+ is extruded from the mitochondrial membrane, not from the matrix space

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