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SPECIFIC REQUIREMENT FOR INORGANIC PHOSPHATE FOR INDUCTION OF BILAYER MEMBRANE CONDUCTANCE BY THE CATIONIC UNCOUPLER CARBOCYANINE DYE

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The trinucleous divalent cationic cyanine dye triS-C_4(5) was shown to be an uncoupler of oxidative phosphorylation in mitochondria only in reaction medium containing inorganic phosphate (P_i). This dye also induced marked increase in the electrical conductance of a phospholipid bilayer membrane in bathing solution containing P_i, but not in solution containing Tris-HCl buffer without P_i. Time-dependent fluctuation of the electrical current across the bilayer membrane was observed in the presence of triS-C_4(5) only in bathing solution containing P_i. This fluctuation could be due to perturbation of the bilayer membrane structure induced by the cooperative action of the cyanine dye and P_i, and this perturbation should be directly related to their effects in increasing membrane conductance and also causing uncoupling in mitochondria.

With the widespread use of cyanine dyes as fluorescent probes for monitoring membrane potential in various biomembrane systems [1–5], the direct effects of the dyes as hydrophobic cations on various biomembrane functions have been receiving considerable attention. The most commonly used dye is the dinucleous mono-valent cation diS-C_3(5) (3,3′-dipropylthiocarbocyanine), which interferes with bacterial motility [6], blocks the Ca^{2+}-activated K^+-channel in human red blood cells [7] and inhibits oxidative phosphorylation in mitochondria by acting as an uncoupler and an inhibitor of electron transport [9–11].

Recently, we found that the divalent cationic trinucleous carbocyanine dye triS-C_7(5) (for chemical structure, see Fig. 1), is a unique cationic uncoupler of oxidative phosphorylation in mitochondria, showing activity only in the presence of inorganic phosphate (P_i) [12–14]. Owing to the similarity in the chemical structures of diS-C_3(5) and triS-C_7(5), it is reasonable to consider that these two dyes have the same mechanism of action, at least on energy-transducing membranes, including the requirement for P_i.

This paper deals with the effects of the trinucleous carbocyanine dye triS-C_4(5) on mitochondrial function and on the conductance of bilayer phospholipid membranes. This dye is more soluble in water than triS-C_7(5), because it has shorter alkyl chains (*n*-butyl chains), and so its effect on bilayer membranes can be studied easily.

TriS-C_4(5) was a gift from the Nippon Kankoshikiso Research Laboratory, Okayama (Japan), and was used as a solution in dimethyl sulfoxide. Rat liver mitochondria were isolated as described previously [15]. Oxygen uptake by mitochondria was monitored

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Abbreviations: triS-C_7(5), 2,2′-[3-[2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]bis[3-heptyl-4-methyl thiazolinium iodide]; triS-C_4(5), 2,2′-3-[2-(3-butyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]bis[3-butyl-4-methyl thiazolinium iodide]. TriS-C_7(5) has been referred to as NK-19 or Platonin, and TriS-C_4(5), as NK-24 or butyl-Platonin.

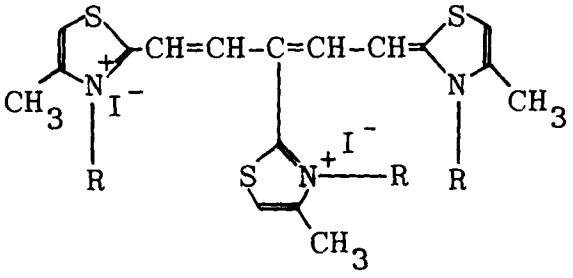

Fig. 1. Chemical structure of the carbocyanine dyes: triS-$C_4(5)$, R = $-CH_2(CH_2)_2CH_3$; and triS-$C_7(5)$, R = $-CH_2-(CH_2)_5CH_3$.

with a Clark oxygen electrode (Yellow Spring Instruments) at 25°C in medium consisting of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA (Na or K salt according to the incubation medum) and buffer of pH 7.4 with 10 mM succinate (Na or K salt according to the incubation medium) plus 1 μg/mg protein rotenone as substrate. The effect of the cyanine dye on mitochondrial function in the presence of various ions was examined by using 10 mM potassium phosphate buffer, 10 mM sodium phosphate buffer, 10 mM Tris-HCl buffer plus 10 mM KCl (Tris-KCl medium), or 10 mM Tris-HCl buffer plus 10 mM NaCl (Tris-NaCl medium). The bilayer membrane was formed by the brush method [16] with a solution of 10 mg soybean lecithin (Nakarai Chemical Co. Ltd., Kyoto) and 5 mg cholesterol (Sigma Chemical Co., St. Louis) in one ml of *n*-decane (distelled and passed through a column of activated aluminum), across a hole of about 1 mm diameter in the wall of a Teflon cell. Electrical measurements were carried out as described by Hanai et al. [16] at 25°C. The following buffer solutions at pH 7.0 were used as bathing solutions: 100 mM potassium phosphate buffer, and 25 mM Tris-HCl buffer plus 100 mM KCl (Tris-KCl medium). The cyanine dye was always added to the outer chamber with gentle stirring.

As shown in Table I, the cyanine dye triS-$C_4(5)$ at 50 μM accelerated State 4 respiration only in the presence of P_i. This stimulation is due to the uncoupling of oxidative phosphorylation as observed with triS-$C_7(5)$. The cations K^+ and Na^+ had essentially no effect on the uncoupling. Thus, for inducing uncoupling, it is concluded that only P_i is a requisite, at least under the present experimental conditions.

It had been proposed that some lipophilic cations,

TABLE I

EFFECT OF 50 μM TRIS-$C_4(5)$ ON STATE 4 RESPIRATION OF RAT LIVER MITOCHONDRIA IN INCUBATION MEDIA OF VARIOUS ION COMPOSITION

Mitochondria, 0.7 mg/ml in a total volume of 2.53 ml. K-P_i, potassium phosphate buffer; Na-P_i, 10 mM sodium phosphate buffer; Tris-KCl, 10 mM Tris-HCl buffer plus 10 mM KCl; Tris-NaCl, 10 mM Tris-HCl buffer plus 10 mM NaCl.

triS-$C_4(5)$ (μM)	Respiratory rate (natom O/min per mg protein) in incubation medium of			
	K-P_i	Na-P_i	Tris-KCl	Tris-NaCl
Nil	20	21	14	16
50	117	110	27	25

such as DDA^+ (dimethyldibenzylammonia), uncouple oxidative phosphorylation by causing collapse of the membrane potential as a result of their electrophoretic transfer from the positively charged outside to the negatively charged inside of the energized mitochondrial membrane [17]. The transfer of the lipophilic cation is known to cause an increase in the electrical conductance of the lipid bilayer membrane. Thus, we next examined the effect of triS-$C_4(5)$ on the conductance of phospholipid membranes in bathing solutions of various ionic compositions.

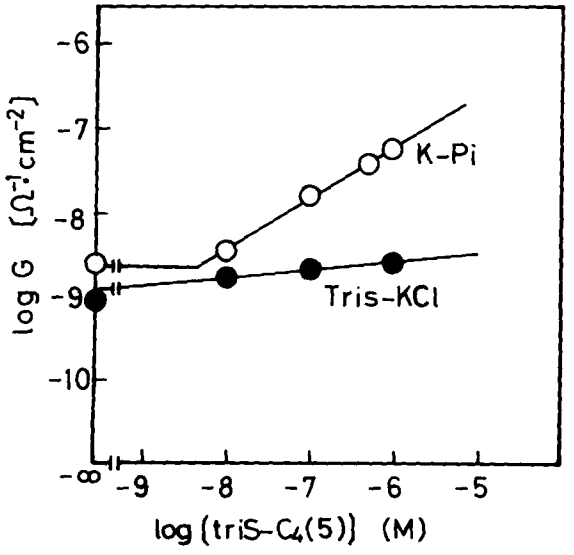

Fig. 2. Effect of triS-$C_4(5)$ on the electrical conductance (G) of phospholipid bilayer membrane. Bathing solutions are Tris-KCl medium and potassium phosphate buffer (K-P_i medium).

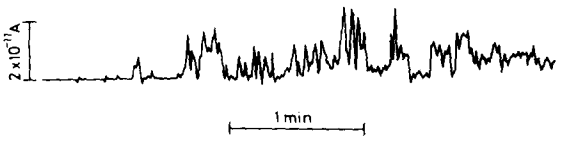

Fig. 3. Fluctuation of the electrical current across the bilayer membrane in the presence of 1 μM triS-C_4(5) in 100 mM potassium phosphate buffer. The electrical current was monitored under application of a constant electrical potential of 50 mV.

As shown in Fig. 2, the cyanine dye at more than about 10 nM increased the conductance concentration-dependently, when the bathing solution contained K^+ and P_i (potassium phosphate buffer), but had little effect on conductance in medium containing Tris-HCl buffer and KCl (Tris-KCl medium); in the former medium, the slope of the plot for log (conductance) (mho . cm^{-2}) vs. log (dye concentration) (M) was about 0.6, whereas in Tris-KCl medium, it was only about 0.1. These results again demonstrated the specific role of P_i in the effect of the cyanine dye on the membrane system.

The electrical current monitored under application of a constant electrical potential of 50 mV to the bilayer membrane was constant in potassium phosphate buffer or Tris-KCl medium in the absence of the dye and in Tris-KCl medium in the presence of 1 μM triS-C_4(5). However, fluctuation of the electrical current was observed in the presence of 1 μM dye in potassium phosphate buffer (Fig. 3). This current fluctuation with time could be due to the formation of channels, as observed with gramicidin A [18] and alamethicin [19], or to perturbation of the phospholipid bilayer structure of the membrane. Since the size of a single molecule of triS-C_4(5) is small compared with the thickness of the bilayer membrane, and since dye molecules aggregate in aqueous solution at a concentration more than 100 μM, the latter possibility seems more probable. The results in Fig. 3 indicate that for perturbation of the bilayer structure, both the cyanine dye and P_i are necessary, and that neither of them alone has any influence on the integrity of the membrane structure. This perturbation should render the membrane permeable to various ions, and hence increase the conductance of the bilayer membrane as shown in Fig. 2. In this case, the dye cation could also be permeant.

Since the effect of triS-C_4(5) on the mitochondrial membrane is similar to that on the phospholipid bilayer membrane with respect to the specific requirement for P_i, it is possible that uncoupling by the cyanine dye in mitochondria is based on the same mechanism as that observed in the model bilayer membrane. However, it is not certain at present whether the perturbation of the membrane structure is directly related to the dissipation of the membrane potential due to electrogenic ion transport, or whether the perturbation causes direct modification of the function of proteins concerned with oxidative phosphorylation. The effects of cyanine dyes on a wide variety of biological functions may be based essentially on a common mechanism to that on the model bilayer or mitochondrial membranes.

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