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Effects of the Cyanine Dye 3,3′-Dipropylthiocarbocyanine on Mitochondrial Energy Conservation

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Mitochondrial respiration and oxidative phosphorylation were inhibited by the membrane potential probe 3,3′-dipropylthiocarbocyanine [diS-C_3-(5)]. Evidence is presented that suggests that the dye acts as both an inhibitor of electron transport and an uncoupler of oxidative phosphorylation.

Cyanine dyes have been employed as fluorescent probes of membrane potential in a variety of biological applications (Waggoner, 1976). The fluorescence of the dyes has been shown to correlate linearly with known membrane potentials (Sims *et al.*, 1974); this property has been used to estimate the magnitude of membrane potentials and to monitor potential changes coupled with biological functions. For example, the dyes have been used in suspensions of mitochondria (Tedeschi, 1974; Laris *et al.*, 1975), yeast (Eddy *et al.*, 1977), tumour cells (Eddy *et al.*, 1977; Laris *et al.*, 1976), *Rhodospirillum rubrum* chromatophores (Pick & Avron, 1976) and various bacteria (Kashket & Wilson, 1974; Brewer, 1976; Manson *et al.*, 1977). The cyanines have also been employed to monitor membrane potentials in excitable cells (Cohen *et al.*, 1974). An outstanding review of this entire field is provided by Waggoner (1976).

With the increasing and widespread use of these dyes, it is important to recognize and isolate any biological side effects. Waggoner (1976) has noted that the 3,3′-dipropylthiocarbocyanine [diS-C_3-(5); see Sims *et al.* (1974) for nomenclature] inhibits oxidation at Complex I in rat liver mitochondria. Pick & Avron (1976) have observed uncoupling of *R. rubrum* chromatophore respiration together with inhibition of photophosphorylation at high concentrations of 3,3′-dipropyloxocarbocyanine [diO-C_3-(5)]. Manson *et al.* (1977) and Miller & Koshland (1978) have more recently reported that several cyanine dyes interfere with bacterial motility through undetermined effects, both photodynamic and non-photodynamic.

We now report on the effects of 3,3′-dipropylthiocarbocyanine on the respiration of isolated mitochondria from turnips (*Brassica napus* L.) and rat liver. Results were obtained for the NAD-linked substrate malate (malate+glutamate for rat liver mitochondria) and the flavoprotein-linked substrate succinate.

Experimental

Turnip mitochondria were prepared by the method of Wilson & Moore (1973). Rat liver mitochondria (Wistar strain) were prepared by the method of Chance & Hagihara (1961). Mitochondria were used only if they met the usually accepted criteria for intact mitochondria, i.e. for turnip mitochondria succinate oxidation rates of 100–120nmol of O_2/min per mg of protein and ADP/O ratios of 1.5–1.7 with respiratory control ratios of 2.0–2.6. The corresponding values for rat liver mitochondrial succinate oxidation are 50–100nmol of O_2/min per mg of protein with ADP/O ratios of 1.8–2.0 and respiratory control ratios of 2.0–3.0, depending on the preparation used. Malate or malate+glutamate oxidation rates are similar in both cases. O_2 consumption was measured polarographically as described by Estabrook (1967), and ADP/O ratios and respiratory control ratios were calculated as described by Chance & Williams (1956). 3,3′-Dipropylthiocarbocyanine was titrated into the reaction cuvette from stock solutions of 0.5mg/ml and 0.05mg/ml in ethanol. Adenosine triphosphatase activity was measured by the method of Takeuchi (1975), by monitoring the release of P_i from the hydrolysis of ATP. H^+ permeability of the inner membrane was measured with rat liver mitochondria (2mg of protein) suspended in 3ml of 250mM-sucrose/50mM-mannitol / 10mM-KCl / 5mM-$MgCl_2$ / 0.2mM-KCN/1mM-potassium phosphate buffer, pH7.4. pH was measured with a glass combination electrode for 5min after the addition of a pulse of 250nmol of NaOH or HCl, and the $t_{\frac{1}{2}}$ for proton decay was calculated from semi-logarithmic plots (Mitchell & Moyle, 1967). $t_{\frac{1}{2}}$ varied between 30 and 40s for different

batches of mitochondria. Mitochondrial protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

All reagents were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. 3,3'-Dipropylthiocarbocyanine was a gift from Dr. Alan Waggoner, of the Department of Chemistry, Amherst College, MA 01002, U.S.A.

Results and Discussion

3,3'-Dipropylthiocarbocyanine has a marked inhibitory effect on malate oxidation by turnip mitochondria in State 3 and in State 4 plus uncoupler (Fig. 1*a*), and on the ADP/O ratio and respiratory control ratio (not shown). Malate-driven State 4 and succinate oxidation (Fig. 1*b*) are unaffected except for a slight stimulation of State 4 and a lowering of ADP/O ratio at high dye concentrations. The addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone gave a partial release of the inhibition of State 3, the final rate being higher than that obtained for the inhibited State 4 plus uncoupler. Neither valinomycin nor nigericin released the inhibition.

Similar results were obtained for rat liver mitochondria (Figs. 2*a* and 2*b*) except that malate+glutamate-driven State 4 was also inhibited by 3,3'-dipropylthiocarbocyanine and the inhibition of malate+glutamate-driven State 3 and ADP/O ratio and respiratory control ratio was more pronounced.

These results suggest that 3,3'-dipropylthiocarbocyanine is acting on the site I (Complex I) region of the respiratory chain, as suggested by Waggoner (1976). Another group of inhibitors acting at this level of the respiratory chain, the barbiturates, can also be partially released by uncouplers of oxidative phosphorylation (Chance & Hollunger, 1963). Release by uncouplers has been taken to indicate that the inhibitor might be being accumulated at the site of action in response to the pH gradient or the membrane potential. However, such an explanation fails to account for the action of 3,3'-dipropylthiocarbocyanine, since the inhibition is not released by valinomycin or by nigericin alone and the inhibition also occurs in the uncoupled State 4. It is not clear why uncoupler added after inhibition has occurred results in a partial release to give a final rate greater than that obtained when inhibitor is added to State 4 mitochondria in the presence of uncoupler.

Since ADP/O ratios are not affected by simple inhibition of electron transport, additional explanations are required for the action of 3,3'-dipropylthiocarbocyanine. Release of the inhibition of State 3 by uncoupler indicates an oligomycin-like action;

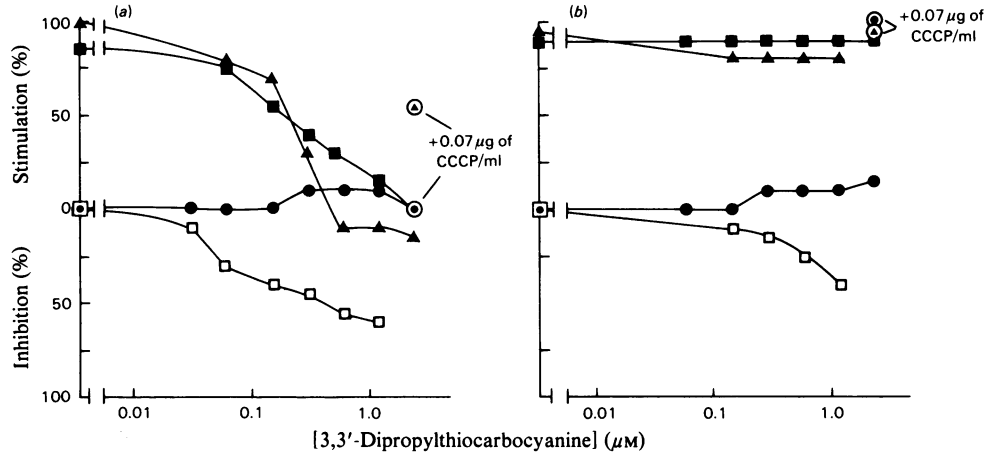

Fig. 1. *Effect of* 3,3'-*dipropylthiocarbocyanine on the rate of* O_2 *consumption and the ADP/O ratio of isolated turnip mitochondria respiring on* (*a*) 16.7 mM-*malate and* (*b*) 8.3 mM-*succinate*

Inhibition and stimulation are expressed relative to the State 4 rate and ADP/O ratio measured in the absence of 3,3'-dipropylthiocarbocyanine. Respiratory medium contained 300mM-mannitol, 10mM-KCl, 5mM-$MgCl_2$ and 10mM-potassium phosphate buffer, pH 7.2. Mitochondrial protein was 0.56mg/ml. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (8 μM) was added after the final titration of 3,3'-dipropylthiocarbocyanine, at a concentration shown to completely uncouple respiration. The turnip mitochondria used for these experiments oxidized succinate at a rate of 100–120nmol of O_2/min per mg of protein with an ADP/O ratio of 1.5–1.7 and a respiratory control ratio of 2.0–2.6. The rate of malate oxidation was similar, with correspondingly higher values of the ADP/O ratio and the respiratory control ratio, depending on the preparation used. ●, State 4; ■, State 4 uncoupled; ▲, State 3; □, ADP/O ratio.

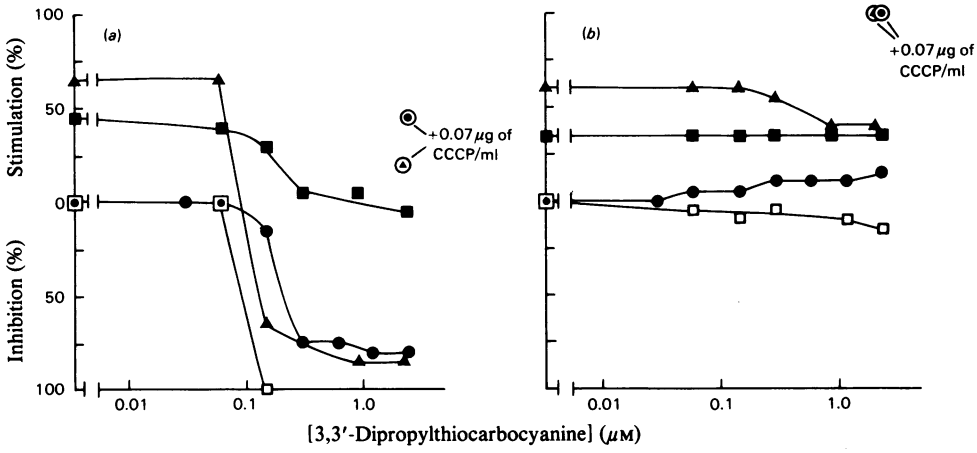

Fig. 2. *Effect of* 3,3'-*dipropylthiocarbocyanine on the rate of* O_2 *consumption and the ADP/O ratio of isolated rat liver mitochondria respiring on* (*a*) 16.7 mM-*malate+glutamate and* (*b*) 8.3 mM-*succinate*

Inhibition and stimulation are expressed relative to the State 4 rate and the ADP/O ratio measured in the absence of 3,3'-dipropylthiocarbocyanine. Respiratory medium contained 250mM-sucrose, 50mM-mannitol, 10mM-KCl, 5mM-$MgCl_2$ and 10mM-potassium phosphate buffer, pH7.4. Mitochondrial protein was 0.9mg/ml for the respiration experiments and 1.4mg/ml for the ADP/O-ratio experiments. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added after the final titration of 3,3'-dipropylthiocarbocyanine, at a concentration (8μM) shown to completely uncouple respiration. The rat liver mitochondria used for these experiments oxidized succinate at a rate of 50–100nmol of O_2/min per mg of protein with ADP/O ratios of 1.8–2.0 and respiratory control ratios of 2.0–3.0. The malate+glutamate oxidation rates are similar with correspondingly higher values of the ADP/O ratio and respiratory control ratio, depending on the preparation used. ●, State 4; ■, State 4 uncoupled; ▲, State 3; □, ADP/O ratio.

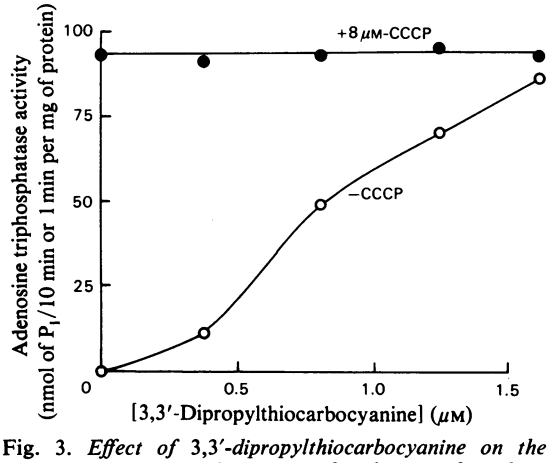

Fig. 3. *Effect of* 3,3'-*dipropylthiocarbocyanine on the oligomycin-sensitive adenosine triphosphatase of rat liver mitochondria in the presence and absence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone*

Mitochondria (1.5mg of protein) were incubated at 25°C in 300mM-KCl/10mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl] amino} ethanesulphonic acid) buffer, pH7.4, containing 2mM-ATP. The reaction was terminated after 10min by the addition of trichloroacetic acid (10%, w/v), and the P_i released was assayed by the method of Lowry & Lopez (1946). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (8μM) and oligomycin (4μg) were added when required. Results are expressed as P_i released per mg of protein for 10min or 1min in the absence or in the presence of uncoupler respectively.

however, with rat liver mitochondria the malate+glutamate-driven State 4 is also inhibited by the dye, showing that the inhibition is not a direct inhibition of phosphorylation. This was confirmed by measuring the effect of dye on the uncoupler-stimulated oligomycin-sensitive adenosine triphosphatase of rat liver mitochondria: no inhibition of the adenosine triphosphatase occurred over the range of concentrations used in these experiments (Fig. 3).

In the light of the slight stimulation of State 4, particularly with succinate as the substrate for turnip mitochondria, we suggest that uncoupling might be occurring. This was confirmed by measuring the rate of proton permeation out of the mitochondria after an alkali pulse (Mitchell & Moyle, 1967). 3,3'-Dipropylthiocarbocyanine decreased the half-time for the decay of pH after an alkali pulse (at 125μM-dye $t_{\frac{1}{2}}$ decreased from 36 to 24s in a typical preparation). Similarly, measurement of the ability of the dye to induce an oligomycin-sensitive adenosine triphosphatase in rat liver mitochondria shows (Fig. 3) that 3,3'-dipropylthiocarbocyanine increases the adenosine triphosphatase activity.

The results given in the present paper clearly show

that 3,3'-dipropylthiocarbocyanine used at probe concentrations inhibits mitochondrial oxidations at the site I (Complex I) level of the respiratory chain. Although its primary action is on the electron-transport system, the dye also acts as an uncoupler, increasing both the proton permeability and the adenosine triphosphatase activity of the mitochondria. Both of these parameters are classical measures of uncoupler activity. 3,3'-Dipropylthiocarbocyanine is thus like several other inhibitors, e.g. the barbiturates, in its ability to both inhibit and uncouple. However, the extent of uncoupling as measured by the effect of probe concentrations of 3,3'-dipropylthiocarbocyanine on the adenosine triphosphatase or the proton permeability was always less than that obtained with carbonyl cyanide *m*-chlorophenylhydrazone. Thus in the presence of carbonyl cyanide *m*-chlorophenylhydrazone the adenosine triphosphatase activity and the $t_{\frac{1}{2}}$ for proton permeation are approximately 10 times and less than one-tenth respectively the values obtained with 1.5 μM-3,3'-dipropylthiocarbocyanine.

Since the experiments described in the present paper were completed Kinnally & Tedeschi (1978) have reported similar results to those in this paper, concluding that 3,3'-dipropylthiocarbocyanine 'dissociates' phosphorylation from respiration while also acting as an inhibitor of Complex I. 3,3'-Dihexyl-2,2'-oxacarbocyanine was identified as an uncoupler and an inhibitor. Differentiation between uncoupling and dissociation was based on the ability or non-ability of the dyes to stimulate the succinate-driven State 3. However, stimulation of State 3 succinate oxidation appears to depend on the ability of the dyes to activate the succinate dehydrogenase. In the experiments performed by Kinnally & Tedeschi (1978) succinate-driven State 3 rates were lower than those obtained with β-hydroxybutyrate, indicating that the oxidation was rate-limited by the dehydrogenase. Activation therefore would measure the action of the dye on the dehydrogenase and not any effect on the phosphorylation system. In the experiments reported in the present paper the effect of the dye on the phosphorylation system was measured directly by studying the adenosine triphosphatase activity and the proton permeability. Kinnally & Tedeschi (1978), in addition to the inhibition of β-hydroxybutyrate oxidation by the cyanine dyes, also observed decreased phosphorylation with succinate at very high (non-probe) dye concentrations. In the experiments reported in the present paper, particularly with turnip mitochondria, diminished phosphorylation occurred at very much lower (probe) concentrations of the dye.

Although it is not possible to compare these results for mitochondria directly with those for intact cells or other subcellular preparations, our experiments were conducted over a range of dye concentrations including those commonly employed in biological systems.

Since the cyanine dyes are membrane-permeable (Waggoner, 1976; Sims *et al.*, 1974) and can thus interact with mitochondria *in situ*, the above observations suggest that caution be exercised in using 3,3'-dipropylthiocarbocyanine and perhaps other cyanine dyes with mitochondria and intact cell preparations. Experimental design and interpretation should seek to minimize the potential for inhibitory and uncoupling effects. NAD-linked substrates should be avoided, and low dye concentrations should be used when possible, perhaps even at the expense of some sacrifice of signal size (Waggoner, 1976).

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