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METABOLIC EFFECTS OF SOME ELECTROFLUORIMETRIC DYES

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Summary

The effect of five electrofluorimetric dyes on mitochondrial metabolism was examined to determine their suitability for mitochondrial studies and other biological uses. The dyes merocyanine 540, 8-anilino-1-naphthalene sulfonic acid and bis(1,3-dibutyl barbituric acid-(5))-pentamethane oxonol were found to be inhibitors of the respiratory chain. However, the first two exerted their effect only at high concentrations. 3.3′-Dihexyl-2,2′-oxacarbocyanine was found to act as an uncoupler. 3,3′-Dipropyl-thiocarbocyanine inhibited β-hydroxybutyrate respiration while dissociating succinate supported respiration from the phosphorylation of ADP. Merocyanine 540 and 8-anilino-1-naphthalene sulfonic acid may be the best suited for studies of membrane potentials in mitochondria since their effect on metabolism is negligible.

Introduction

Fluorescent probes have been used in many biological and model systems in a variety of studies involving, e.g., protein conformational changes [1] and membrane fluidity [2]. A diverse selection of probes are available, and dyes with specific properties, e.g. degree of hydrophobicity, can be designed to probe different environments. For example, Waggoner et al. [3] synthesized a number of fluorescent analogs of membrane lipids which permitted an investigation of different transverse regions of the lipid bilayer. For the most part, the fluorescence of extrinsic probes is a function of the number of dye molecules and their microenvironment and therefore can provide information on the multifaceted nature of biological membranes.

Abbreviations: diBa-C_4-(5), bis(1,3-dibutyl barbituric acid-(5))-pentamethane oxonol; diS-C_3-(5), 3,3′-dipropylthiocarbocyanine; diO-C_6-(3), 3,3′-dihexyl-2,2′-oxacarbocyanine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; S-13, *S*-chloro-3-*t*-butyl-2′chloro-4′-nitrosalicylanilide; Tris, tris-(hydroxymethyl)-aminomethane.

Electrofluorimetric dyes have been used extensively to study electrical potentials across membranes in such systems as nerve [4], artificial bilayers [5], red blood cells [6—8], liposomes [6,9], bacteria [10—12] and mitochondria [13—17]. The application of these probes to the study of biological function, e.g. energization processes in mitochondria, requires an investigation of their effect on metabolism. The present study is an attempt to evaluate the effect of 8-anilino-1-naphthalene sulfonate, merocyanine 540, diS-C_3-(5), diBa-C_4-(5), and DiO-C_6-(3) on mitochondrial oxidative phosphorylation. Some of these dyes have been used previously with mitochondria [13—17].

Methods

Mitochondria were isolated from rat liver by the method previously described by Tedeschi [18]. The final suspension medium was generally 0.3 osmolal sucrose, 10 mM Tris, pH 7.4. *Drosophila virilis* mitochondria were isolated by the method of Tupper and Tedeschi [19].

The effect of the dyes, 8-anilino-1-naphthalene sulfonate, diS-C_3-(5), diBa-C_4-(5), diO-C_6-(3) and merocyanine 540 on phosphorylation and respiration was examined. When assaying for phosphorylative ability, mitochondria were suspended in 3 ml of 0.3 osmolal sucrose, 10 mM Tris, 0.5 mM sodium phosphate, 5 mM KCl, pH 7.4. Rotenone, 0.85 μM, was present in all incubations in which succinate was used. When used, antimycin A, FCCP and S-13 were in final concentrations of 0.61, 0.06 and 0.004 μM, respectively. Succinate and β-hydroxybutyrate in 10 mM Tris, pH 7.4, were added to final concentrations of 3.3 mM and ADP in a Tris (pH 7.4) solution was in a final concentration of 1.3 mM. When a hexokinase trap was used, 1 mM glucose and 75 units of hexokinase were added to the reaction mixture. Dyes were added in small aliquots of ethanol. Controls (zero dye concentration) correspond to samples containing aliquots of ethanol equivalent to those used to deliver the dye. All incubations were at 25° C.

The phosphorylative ability was estimated by monitoring the disappearance of orthophosphate (P_i) from the whole suspension by the method of Hurst [20] as described previously [16]. Metabolic studies of *Drosophila* mitochondria were carried out using the method of Estabrook [21]. Oxygen concentration was monitored polarigraphically using a Clarke oxygen electrode (Yellow Springs Instruments Model 53). ATPase activity was estimated by following the appearance of orthophosphate from the whole suspension. Mitochondria were suspended in 0.3 osmolal sucrose, 10 mM Tris, 5 mM KCl, pH 7.4, with 0.85 μM rotenone and 0.67 or 2.3 mM ATP. The appearance of orthophosphate was found to be entirely oligomycin-sensitive.

At some concentrations, binding of the dyes to the test tubes was noted. However, no attempts were made to correct for this binding. The concentrations reported are calculated on the basis of the amount of dye added. Generally, oxygen uptake and phosphate disappearance were measured in parallel, in identical chambers in the presence of the oxygen electrode probes. The Biuret reaction [22], with bovine serum albumin standards, was used for protein analysis.

Results and Discussion

The metabolic effect of five dyes (8-anilino-1-naphthalene sulfonate, merocyanine 540, diO-C_6-(3) diBa-C_4-(5) and diS-C_3-(5)) was studied since the fluorescence of these probes appears to respond to changes in diffusion potentials of mitochondria. To facilitate comparisons of different studies, the conditions used previously in the study of mitochondrial membrane potentials are displayed in Table I.

The effect of the dyes on electron transport and phosphorylation was examined using two substrates, β-hydroxybutyrate and succinate. Extensive studies with β-hydroxybutyrate were difficult because, in our hands, the mitochondria were found uncoupled when tested with this substrate after storage for about 4 h. Therefore, most experiments were carried out with succinate as the substrate.

In the present study, the dyes displayed a variety of effects on mitochondrial suspensions. Therefore, the effect of each dye on electron flow and phosphorylation was examined and both respiratory rates (right hand ordinate and curve 1) and P : O ratios (left hand ordinate and curve 2) are depicted in Figs. 1—6.

Three dyes, 8-anilino-1-naphthalene sulfonate, merocyanine 540 and diBa-C_4-(5), were found to act as electron transport inhibitors. However, neither 8-anilino-1-naphthalene sulfonate nor merocyanine 540 significantly affected succinate or β-hydroxybutyrate oxidation under conditions in which these dyes' fluorescence has been used to estimate diffusion potential changes, i.e. in the μM range (compare Table I to Figs. 1 and 2 and Table II, Expts. 1 and 2). The oxonol dye, diBa-C_4-(5) does not inhibit at the concentrations used to measure diffusion potentials (see Table I). However, if the dye effect is examined as a function of mg protein, it is clear that oxidation is partially blocked and phosphorylation partially inhibited at concentrations approximating those used in the measurements of diffusion potentials (20—24 μM dye · mg^{-1} protein).

Generally, for merocyanine 540 (Fig. 1 and Table II, Expt. 1), 8-anilino-1-naphthalene sulfonate (Fig. 2, Table II, Expt. 2) and diBa-C_4-(5) (Fig. 3), the

TABLE I

CONDITIONS USED TO ESTIMATE MITOCHONDRIAL MEMBRANE POTENTIAL

Samples from literature of conditions used to study membrane potentials in mitochondria. diO-C_6-(3) has been used with *Drosophila virilis* mitochondria. All other dyes have been used with rat liver mitochondria. diS-C_3-(5) has been used with both rat [16] and hamster [14] mitochondria. Ref. refers to the references. The protein concentration is approximate.

Dye	[Dye] (μM)	Protein (mg/ml)	Ref.
diO-C_6-(3)	0.003	0.15	15
diS-C_3-(5)	3	0.3	14
	3	0.15	16
8-Anilino-1-naphthalene	42	0.15	26
sulphonate	12	2.5	13
Merocyanine 540	1.5	0.8	26
diBa-c_4-(5)	3	0.15	26

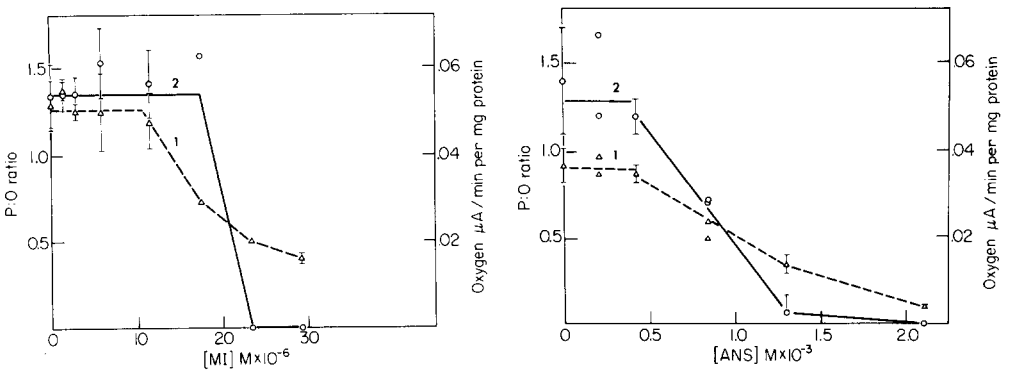

Fig. 1. Concentration-dependent effect of merocyanin 540 (MI) on succinate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphotylative ability. Mitochondria (0.84 mg protein/ml) were suspended in 0.3 osmolal sucrose, 10 mM Tris, 5 mM KCl, 0.5 mM sodium phosphate pH 7.4, with succinate, rotenone, 1.3 mM ADP and dye.

Fig. 2. Concentration-dependent effect of 8-anilino-1-naphthalene sulfonate (ANS) on succinate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphorylative ability. Mitochondria (0.95 mg protein/ml) were suspended as described in Fig. 1.

phosphorylation of ADP appeared to be more sensitive to the action of the dyes than respiration; some oxygen uptake remained when phosphorylation was completely blocked. This is also the case with diO-C_6-(3) and diS-C_3-(5) using succinate as a substrate (Figs. 4 and 6, respectively). A similar dissociation seems to take place with both dyes when β-hydroxybutyrate is the sub-

TABLE II

EFFECT OF DYES ON THE METABOLISM OF β-HYDROXYBUTYRATE

Concentration-dependent effect of merocyanine 540, 8-anilino-1-naphthalene sulfonate, and diO-C_6-(3) on β-hydroxybutyrate metabolism. Mitochondria were suspended in the medium of Fig. 5 with 1.3 mM ADP.

Dye	[Dye] (μM)	Oxygen (μA · min^{-1} · mg^{-1} · protein)	P : O
1. Merocyanine 540	0	0.030 ± 0.003	2.1 ± 0.2
	0.15	0.028	2.0
	1.46	0.026 ± 0.004	2.3 ± 0.4
	2.93	0.026	1.9
	5.85	0.027	1.52
2. 8-anilino-1-naphthalene sulfonate	0	0.027 ± 0.002	2.9 ± 0.3
	1.1	0.022	3.4
	42	0.025 ± 0.002	2.6 ± 0.4
	84	0.024	2.1
	210	0.023 ± 0.003	2.0 ± 0.2
	420	0.021	1.6
3. diO-C_6-(3)	0	0.023 ± 0.002	2.6 ± 0.2
	0.01	0.023 ± 0.001	2.6 ± 0.2
	0.11	0.024 ± 0.001	2.3 ± 0.2
	1.12	0.025 ± 0.001	1.6 ± 0.2

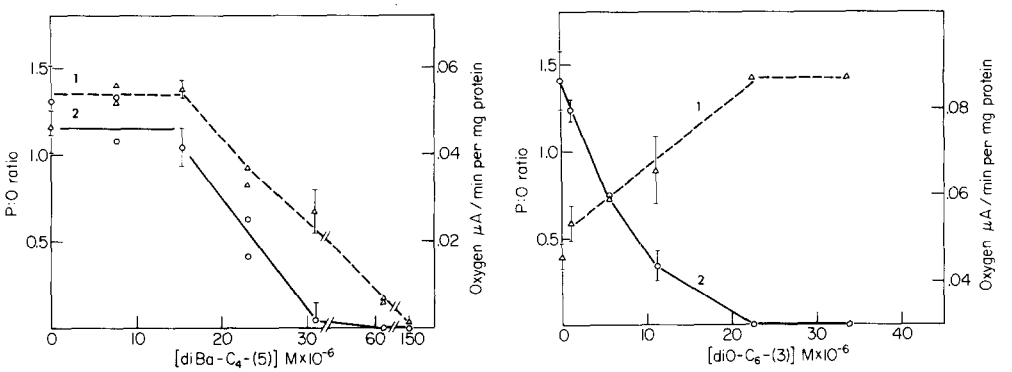

Fig. 3. Concentration-dependent effect of diBa-C_4-(5) on succinate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphorylative ability. Mitochondria (0.83 mg protein/ml) were suspended as in Fig. 1.

Fig. 4. Concentration-dependent effect of diO-C_6-(3) on succinate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphorylative ability. Mitochondria (1.0 mg protein/ml) were suspended as in Fig. 1.

strate (Fig. 5, Table II, Expt. 3). It is conceivable that the phosphorylation corresponding to the low respiratory rates could be masked by the ATPase activity of the preparation. However, for all five dyes tested, the ATPase activity was found to account only for a portion of the missing phosphorylation. The ATPase activity was found to generate less than 0.004 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein (when in the presence of 0.67 mM ATP, a concentration much higher than that obtained under optimal phosphorylating conditions). Although the presence of high concentrations of merocyanine 540 doubled the ATPase activity, the other dyes did not increase the activity significantly

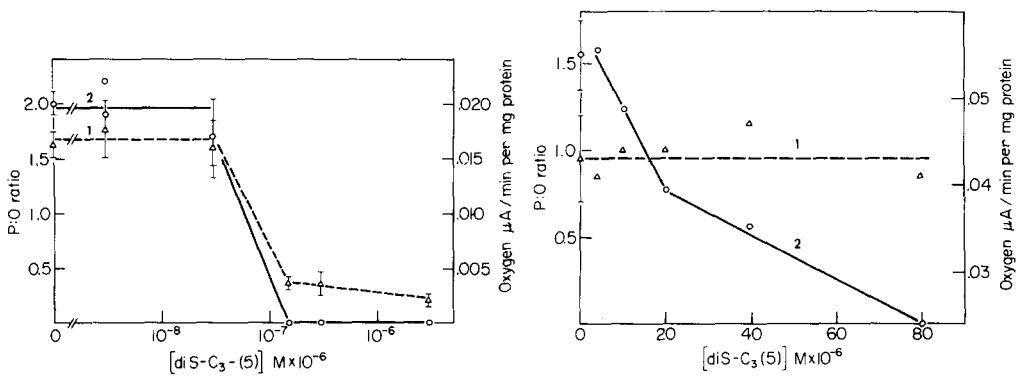

Fig. 5. Concentration-dependent effect of diS-C_3-(5) on β-hydroxybutyrate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphorylative ability. Mitochondria (0.99 mg protein/ml) were suspended in 0.3 osmolal sucrose, 10 mM Tris, 5 mM KCl, 0.5 mM sodium phosphate, pH 7.4, with β-hydroxybutyrate, 1.3 mM ADP and dye.

Fig. 6. Concentration-dependent effect of diS-C_3-(5) on succinate metabolism. Curve 1: respiratory rate in the presence of ADP. Curve 2: P : O ratios calculated from the phosphorylative ability. Mitochondria (0.87 mg protein/ml) were suspended as in Fig. 1.

(below 25%) and diBa-C_4-(5) inhibited the ATPase activity. Furthermore, at high concentrations of 8-anilino-1-naphthalene sulfonate (and diS-C_3-(5), see below), some respiration was observed in the absence of phosphorylation regardless of whether or not a glucose-hexokinase trap was used. These results agree with the notion that a dissociation between oxidation and phosphorylation distinct from classical uncoupling has taken place. This effect was most clearly seen with diS-C_3-(5) and is discussed in more detail below.

The dye diO-C_6-(3) has been used as a membrane potential indicator in *Drosophila virilis* mitochondria [15]. Under the conditions of Tedeschi, i.e., at low concentrations, diO-C_6-(3) appeared to have no effect on *Drosophila* (not shown) and rat liver mitochondria when succinate is used as the substrate. However, since increased respiration and concomitant decrease in P : O ratios were observed with high diO-C_6-(3) concentrations (see Fig. 4), this dye apparently can act as an uncoupler of succinate supported metabolism in rat liver mitochondria.

diS-C_3-(5) has been used in the study of mitochondrial membrane potentials [14,16]. Additionally, a more thorough study of its metabolic effects seemed indicated by its inhibition of β-hydroxybutyrate oxidation (Pressman, B., personal communication). As shown in Fig. 5, this probe effectively blocked the oxidation of β-hydroxybutyrate (but not succinate, see Fig. 6) at concentrations at which it responds best as an indicator of diffusion potential changes.

A dramatic decrease in both phosphorylation and respiration was evident in the presence of β-hydroxybutyrate and concentrations of diS-C_3-(5) less than 10^{-6} M (Fig. 5). Similarly, diS-C_3-(5) reduced succinate supported phosphorylation (Table III) but at higher concentrations halving this ability at about $15 \cdot 10^{-6}$ M. In contrast, in the presence of ADP, generally no effect of the dye on succinate supported respiration was evident at concentrations as high as 10^{-4} M diS-C_3-(5) and at dye concentrations which eliminated any measurable ADP phosphorylation (see Table III and Fig. 6 and discussion below; note, however, one expection in Expt. 2 of Table III). When measured at dye concentrations that completely inhibited phosphorylation, the low ATPase activity of the preparation (which was entirely oligomycin sensitive) did not appear to be affected by diS-C_3-(5), and in fact, the same results were obtained in the presence of a glucose-hexokinase trap. These data suggest that diS-C_3-(5) may act as a dissociator of phosphorylation from respiration.

The effect of diS-C_3-(5) on succinate metabolism was examined in further detail. The experimental procedure used can be best illustrated by an actual oxygen electrode tracing (see Fig. 7).

Several features of interest, in addition to the independence of state 3 respiration and phosphorylation (Fig. 6), are shown in Fig. 7 and Table III. Generally, high concentrations of the dye which inhibited phosphorylation completely (Table III, column 3, Expt. 1b, 2b, 2c, 3b) increased the state 4 respiratory rate (Table III, column 4, Expt. 1b and 3b) but not over the level observed in the presence of ADP (column 5). Occasionally, similar results were observed at lower concentrations (e.g., Expt. 3a but not 1a and 2a). Interestingly, oligomycin did not decrease the respiratory rate in the presence of ADP and the uncoupler FCCP did not significantly increase the respiratory rate (Fig. 7 and Table III, column 7, Expts. 1b and 2b). The ineffectiveness of

TABLE III

EFFECT OF diS-C_3-(5) ON RESPIRATION UNDER VARIOUS CONDITIONS

Conditions and methods are as described in Fig. 7. Phosphorylative ability was estimated in the presence of 1.3 mM ADP. State 4 to state 3 transitions were induced by the addition of 0.4 μmol ADP. In expts. 2a and b, one ADP pulse was used. In Expts. 4 and 5, respiratory rates were measured in the presence of 1.3 mM ADP. Mitochondrial protein ranged from 0.47 to 0.75 mg/ml. Antimycin A results were omitted since the respiratory rate was consistently zero in its presence. Results obtained with the second ADP pulse were omitted since they were comparable to those observed with the first pulse.

Expt.	Concn. (μM)	Phosphorylation (nM · min^{-1} · mg^{-1} · protein)	Respiration (μA oxygen · min^{-1} · mg^{-1} · protein)			
			State 4	State 3	+ Oligomycin	+ FCCP
1a	0	114.9	0.040 ± 0.001	0.072 ± 0.016	0.046 ± 0.001	0.160 ± 0.025
	15.0	61.6	0.043 ± 0.004	0.060 ± 0.011	0.072 ± 0.014	0.158 ± 0.006
b	0	89.4	0.050 ± 0.006	0.064 ± 0.010	0.053 ± 0.006	0.157 ± 0.012
	90.0	0	0.088 ± 0.018	0.082 ± 0.016	0.088 ± 0.011	0.094 ± 0.014
2a	0	92.9	0.037 ± 0.003	0.057 ± 0.007	0.037 ± 0.004	0.138 ± 0.009
	15.0	53.9	0.037 ± 0.002	0.055 ± 0.002	0.048 ± 0.006	0.142 ± 0.010
b	0	78.0	0.051 ± 0.002	0.069 ± 0.008	0.050 ± 0.006	0.145 ± 0.002
	75.0	0	0.085 ± 0.011	0.081 ± 0.014	0.095 ± 0.016	0.093 ± 0.005
c	0	78.0	0.040 ± 0.005	0.079 ± 0.006		
	75.0	0	0.049 ± 0.010	0.051 ± 0.007		
3a	0	61.4	0.034 ± 0.008	0.066 ± 0.011		
	15.0	31.4	0.061 ± 0.016	0.058 ± 0.014		
b	0	77.1	0.041, 0.041	0.061, 0.065		
	45.0	0	0.061 ± 0.010	0.062 ± 0.007		
4	0	78.8 ± 9.7		0.055 ± 0.002		
	15.0	31.4 ± 4.9		0.054 ± 0.012		
5	0	61.5 ± 13.7		0.079 ± 0.005		
	15.0	24.5 ± 8.4		0.079 ± 0.004		

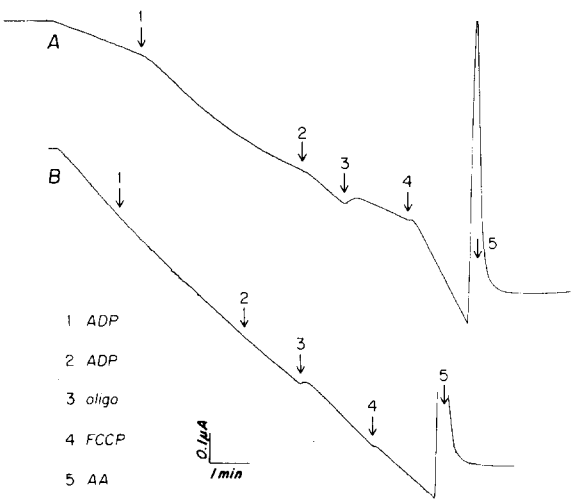

Fig. 7. Effect of ADP and metabolic reagents on oxygen uptake in the presence of diS-C_3-(5). Curve A: alcohol control. Curve B: 90 μM diS-C_3-(5). Mitochondria (0.47 mg protein/ml) were suspended in 0.3 osmolal sucrose, 10 mM Tris, 5 mM KCl, 0.5 mM sodium phosphate, pH 7.4, with succinate and rotenone. 0.4 μmol ADP was added to initiate state 3. The following serial additions were made in small aliquots of alcohol to their final concentrations after a second pulse of ADP: 16.6 μM oligomycin, 0.06 μM FCCP and 0.61 μM antimycin A. Tracings were taken from Expt. 1, Table III.

uncouplers could be explained by a binding of the uncoupler to the dye. However, increasing concentrations of FCCP (up to 7 μM) or S-13 (up to 5.2 μM, not shown) do not seem to increase respiration, suggesting that this might not be the case. In contrast to the absence of an effect of uncouplers at high concentrations of dye, FCCP had the usual uncoupling effect of increasing respiration at lower dye concentrations (Table III Expts. 1a and 2a). At all diS-C_3-(5) concentrations tested, the inhibitor antimycin A was effective in blocking respiration. The effectiveness of this inhibitor indicates that the dye does not bypass the respiratory chain, at least before the antimycin A binding site.

These results are difficult to explain without a complex model unless the assumption is made that diS-C_3-(5) acts on the coupling mechanisms before the oligomycin block and simultaneously locks the respiratory chain at the state 3 level. The most straight forward interpretation of these data is that the dye acts at more than one site. In the case of succinate oxidation, the dye could be acting both as an inhibitor of electron flow (which may be similar to the effect on β-hydroxybutyrate oxidation) and as an uncoupler (similar to the effect on succinate supported state 4 respiration). A mechanism of dual action could account for the lack of effect by FCCP at high dye concentrations. since FCCP should not increase oxidation when the respiratory chain is blocked. However, a simple mechanism such as this is not supported by the experiments at lower dye concentrations (see Table III, column 3, Expts. 1a and 2a). In these cases, the dye decreases phosphorylation dramatically. Nevertheless, respiration in the presence of ADP usually remained the same as the control and FCCP was effective in increasing respiration in the presence of the dye.

The activity of diS-C_3-(5) as a dissociator seems reminiscent of the action of some of the other dyes already discussed, i.e. those dyes that block phosphorylation but permit a reduced yet significant level of respiration. However, the two dyes diS-C_3-(5) and diO-C_6-(3) which are similar in structure exhibit very different metabolic effects. As already discussed, diO-C_6-(3) acts as an uncoupler while diS-C_3-(5) acts as a dissociator. Further study of the effects of these dyes (and derivatives) on metabolism may provide new insights into the mechanism of energy transduction.

Experiments from other studies [16,25] show that all these five dyes can be used effectively to measure diffusion potentials. However, since they have metabolic effects, they should be used with caution. When the dye/protein ratios are considered, diS-C_3-(5) and diBa-C_4-(5) express some of their metabolic effects under conditions (20 μM/mg protein) approaching or below those used to study membrane potentials. However, the inhibition of succinate oxidation is not complete with either dye. In contrast, 8-anilino-1-naphthalene sulfonate and merocyanine 540 do not seem to have a significant metabolic effect, in the concentrations shown in Table I, suggesting that they are more suitable for metabolic studies.

Acknowledgements

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