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Mitochondrial Membrane Potential: Evidence from Studies with a Fluorescent Probe

(3,3′-dihexyl-2,2′-oxacarbocyanine/*Drosophila virilis*/oxidative phosphorylation)

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ABSTRACT **The fluorescence of the probe 3,3′-dihexyl-2,2′-oxacarbocyanine (CC_6) has been found to indicate potentials across cell membranes. Results obtained in the present study using CC_6 and *Drosophila* mitochondria are in agreement with membrane potentials previously measured by Tupper and Tedeschi using microelectrodes. The results of both studies with *Drosophila* suggest that the potential across the mitochondrial membrane does not play a significant role in oxidative phosphorylation.**

Tupper and Tedeschi (1–4) using microelectrodes driven by a piezo-electric device observed in giant *Drosophila* mitochondria, membrane potentials in the range of 10–20 mV (inside positive). The potentials do not vary significantly with metabolic conditions (2). They depend on the integrity of the mitochondria (4), are quantitatively predictable from the ratio of internal to external concentrations of organic anions (3, 4) using the Nernst equation, and they vary predictably with osmotically active volume (1). The results are consistent with the interpretation that the potentials recorded are across the mitochondrial semi-permeable membrane and they represent a diffusion potential of anions distributed between the internal and external phases in accordances with a Donnan effect. The evidence is not consistent with the notion that the membrane potential plays a significant metabolic role.

This proposal is also supported by distribution of organic anions (5) and the cation methylamine (6) in rat liver mitochondria. These ions appear to permeate the mitochondria readily and to distribute according to a Donnan equilibrium.

The results obtained with microelectrodes have been questioned. Lassen *et al.* (7) found in Ehrlich ascites tumor cells using a similar microelectrode system, a membrane potential which depended on the K^+ concentration of the medium and which decayed in a few milliseconds. After the initial decay, the steady-state potential was no longer affected by the K^+ in the medium. They proposed that a similar perhaps faster decay took place in our experiments. However, in at least some other studies [e.g., Sekiya's (8) and Johnstone's (9)] the K^+-sensitive membrane potential decayed considerably more slowly (with a half-time of 10 sec in Sekiya's work). Consequently, the results of Lassen *et al.* may well not be typical even for Ehrlich ascites tumor cells and the results should not be extrapolated to mitochondria.

Liberman and Skulachev (10) question our findings on the ground that in our work the membrane resistance is too low (1–4 Ωcm^2, refs. 1 and 2) compared to that postulated by them for mitochondria. Since no measurements of specific resistance of the mitochondrial membrane have been carried out by others, this assumption is gratuitous and we consider this objection of doubtful significance. The magnitude of the resistance recorded by Tupper and Tedeschi (1, 2) is not unusual in biological systems. For example, erythrocytes have membrane resistances of 3–10 Ωcm^2 measured either with microelectrodes (11) or by an alternative method (12). Similarly, glial cells in tissue culture have resistances of 3–10 Ωcm^2 (13). Other cells have even lower resistances. In the electric plates of the electric eel, for example, resistances of 1–5 Ωcm^2 (14) and 4–13 Ωcm^2 (15) have been recorded from the excitable face and 0.1–0.4 Ωcm^2 (15) for the nonexcitable face.

A number of studies have attempted to estimate the mitochondrial membrane potential by indirect means. Harris and Pressman (5) have calculated membrane potentials in rat liver mitochondria from anion distributions. These estimates correspond in sign and order of magnitude to those measured in *Drosophila* by means of microelectrodes (1–4). Several other workers estimate much larger potentials in actively metabolizing mitochondria. In these cases, the inside of the mitochondria has been considered negative in relation to the outside. The estimates of the magnitude and direction of the membrane potential have been based on the movement of artificial, presumably highly permeable cations and anions, upon activation of metabolism in mitochrondria or submitochondrial particles (10, 16, 17). Similar high potentials have been estimated by Mitchell and Moyle for rat mitochondria (18) from the distribution of K^+ presumed to be in equilibrium after the addition of valinomycin.

The redistribution of the low concentration of highly permeable anions and cations is to be expected from the exchanges of H^+ accompanying metabolism (e.g., ref. 18) without the need for postulating a membrane potential. The calculations of Mitchell and Moyle assume an equilibrium or near equilibrium in relation to the concentration of K^+ (analogous to a Donnan equilibrium) and a diffusion potential of K^+ exactly matching the membrane potential produced by the electrogenic process. Since the loss of H^+ in response to valinomycin, is equivalent or in excess of the simultaneous K^+ gain (see 18), it is questionable whether the distribution of K^+ is in response to the postulated mechanism.

The present work takes advantages of the fluorescent probe 3,3′-dihexyl-2,2′-oxacarbocyanine (CC_6) which has been shown to respond to changes in membrane potentials in squid axons (19) and erythrocytes (20).

Abbreviations: 3,3′-dihexyl-2,2′-oxacarbocyanine, CC_6; *F*, fluorescence.

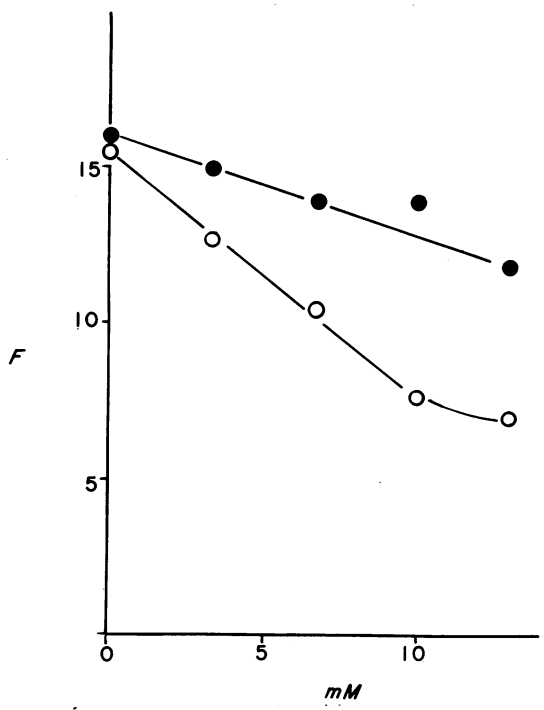

FIG. 1. The effect of sodium acetate (O) or sodium chloride (●) on the fluorescence F. The fluorescence was measured 2–4 min after the serial additions of sodium chloride or acetate shown in the figure.

METHOD

Giant mitochondria were isolated from *Drosophila virilis* as previously described (1). CC_6 was introduced in a concentration of approximately 0.1–0.2 ng/ml. In typical experiments, 0.05–0.1 mg of mitochondrial protein were present per ml. A Perkin–Elmer–Hitachi spectrofluorimeter was used for the measurements (model 204). Excitation was at 470 nm and the fluorescence (F) was measured at 505 nm.

TABLE 1. *Osmotic effect on the fluorescence*

	F	ΔF
Osmolality		
0.47	14.1	
0.12	9.0	
		4.2
Effect of acetate		
Control	14.6	
33 mM Na acetate	9.9	
		4.7

The fluorescence reported corresponds to 4 min of incubation.

TABLE 2. *Effect of salts on the fluorescence in the absence of mitochondria*

Treatment	F before	F 4 min after
Addition of 33 mM Na acetate	22.3	22.8
Addition of 33 mM NaCl	24.5	24.7

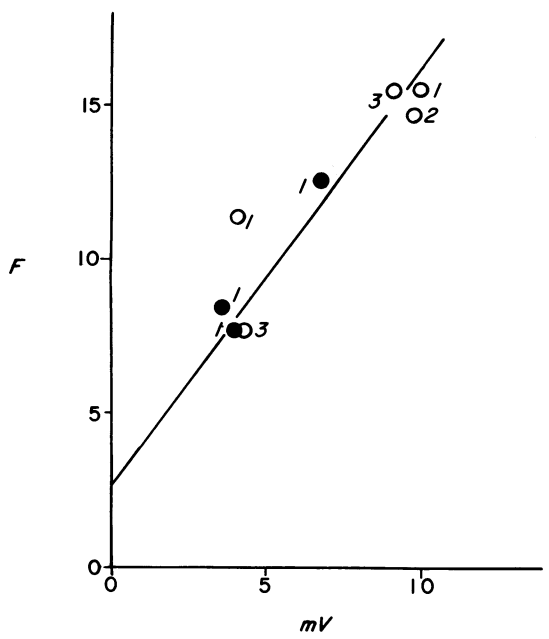

FIG. 2. A comparison between the fluorescence (taken from Fig. 1) and the membrane potential. *1* denotes that the potential was taken from the figure of ref. 3, *2* denotes results presented in the table of ref. 3, and *3* denotes the results presented in ref. 4. *Open circles* denote that the membrane potential shown corresponds to an experimental value. The *closed circles* are membrane potentials taken from the curve of the figure of ref. 3.

RESULTS AND DISCUSSION

The dependence of the membrane potential on the concentration of organic anions permits examining the relationship between membrane potential [as measured with microelectrodes (3, 4)] and fluorescence. As previously shown (3, 4) the membrane potential decreases when the concentration of acetate in the medium increases in approximate agreement with our theoretical expectations. Similarly, the fluorescence also varies with sodium acetate concentration as shown in Fig. 1 (lower curve). The NaCl concentration (upper curve) has a much smaller effect. Except where otherwise specified, the experimental conditions are those previously reported

TABLE 3. *Uptake of dye by mitochondria*

Preparation	F	Mitochondrial uptake (%F)
1. Mitochondria + dye	24.0	
2. Mitochondria + dye + 33 mM Na acetate	16.0	
3. Mitochondria + dye + 33 mM NaCl	21.7	
4. Supernatant of mitochondria + dye*	7.0 ± 1.5	71
5. Supernatant of mitochondria + dye + 33 mM Na acetate*	7.1 ± 0.3	71

* The mitochondria were removed using Millipore filters, 0.8 μm in pore size. Three to four determinations were carried out.

TABLE 4. *Metabolic dependence of the fluorescence**

Sequential additions (4–5 min apart)	F
1. Control	10.5
None	10.2
2. Control	10.6
1 mM pyruvate	7.8
3. Control	9.4
2 mM KCN + 1 mM pyruvate	6.7

* In this experiment the addition of 33 mM Na acetate lowered the fluorescence by a ΔF of 4.6.

(1–3). A plot of the values obtained from Fig. 1 are compared with the membrane potentials determined with microelectrodes (3, 4) in Fig. 2. The two correlate well. As expected from previous work (1), the fluorescence also responds to the osmotic pressure of the medium (Table 1). Sodium acetate has no significant effect on the fluorescence of the dye in the absence of mitochondria (Table 2). The mitochondria take up most of the dye (Table 3, items *4* and *5*). Although the fluorescence of the suspension is sensitive to the concentration of sodium acetate (Table 3; compare items *1*, *2*, and *3*), the acetate does not affect the uptake of the dye (Table 3; compare items *4* and *5*). The dependence of the fluorescence on metabolic conditions (Table 4) is minor, since the same ΔF was noted with the addition of pyruvate, either in the presence or the absence of KCN. The results correspond rather closely to those reported by Tupper and Tedeschi (ref. 2, Table 2) from studies with microelectrodes. The fluorescence of the dye is not significantly affected by its binding to mitochondria. For example, in one experiment acetate decreased the fluorescence of the mitochondria-dye system by 48%. The fluorescence of the dye in the medium alone was 25.4 ± 2.2 (SD). In the presence of mitochondria it was 24.2 ± 2.8 (four measurements each).

The results are entirely consistent with the notion that the mitochondrial membrane potential measured with microelectrodes is a reasonable approximation of the potential present in intact mitochrondria. The study further supports the notion that the membrane potential is the result of the diffusion of organic anions (3, 4) which in mitochondria distribute between the internal and external phase following a Donnan effect.

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1. Tupper, J. T. & Tedeschi, H. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 370–377.
2. Tupper, J. T. & Tedeschi, H. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 713–717.
3. Tupper, J. T. & Tedeschi, H. (1969) *Science* **166**, 1539–1540.
4. Tedeschi, H. (1971) in *Energy Transduction in Respiration and Photosynthesis*, eds. Quagliariello, E., Papa, S. & Rossi, C. S. (Adriatica Editrice, Bari), pp. 767–779.
5. Harris, E. J. & Pressman, B. (1969) *Biochim. Biophys. Acta* **172**, 66–70.
6. Harris, E. J. & Bassett, D. J. (1972) *FEBS Lett.* **19**, 214–216.
7. Lassen, U. V., Nielsen, A. M. T., Pape, L. & Simonsen, L. O. (1971) *J. Membrane Biol.* **6**, 269–288.
8. Sekiya, T. (1962) *Gann* **53**, 41–57.
9. Johnstone, B. M. (1959) *Nature* **183**, 411.
10. Liberman, E. A. & Skulachev, V. F. (1970) *Biochim. Biophys. Acta* **216**, 30–42.
11. Lassen, U. V. & Sten-Knudsen, O. (1968) *J. Physiol. (London)* **195**, 681–696.
12. Johnson, S. L. & Woodbury, J. W. (1964) *J. Gen. Physiol.* **47**, 827–837.
13. Hild, W. & Tasaki, J. (1962) *J. Neurophysiol.* **25**, 277–304.
14. Nakamura, Y., Nakajima, S. & Grundfest, H. (1965) *J. Gen. Physiol.* **49**, 321–349.
15. Keynes, R. D. & Martins-Ferreira, H. (1953) *J. Physiol.* **119**, 315–351.
16. Grinius, L. L., Jasaitis, A. A., Kadziauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M. & Vladimirova, M. A. (1970) *Biochim. Biophys. Acta* **216**, 1–12.
17. Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kuliene, V. V., Levitsky, D. O., Liberman, E. A., Severina, I. I. & Skulachev, V. P. (1970) *Biochim. Biophys. Acta* **216**, 13–21.
18. Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484.
19. Davila, H. V., Salzberg, B. M., Cohen, L. B. & Waggoner, A. (1973) *Nature New Biol.* **241**, 159–160.
20. Laris, P. C. & Hoffman, J. F. (1973) *Fed. Proc.* **32**, 271 Abstr.