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MEMBRANE POTENTIALS IN MITOCHONDRIAL PREPARATIONS AS MEASURED BY MEANS OF A CYANINE DYE

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SUMMARY

Changes in the fluorescent intensity of the dye 3,3'-dipropylthiodicarbocyanine iodide were measured in suspensions of hamster liver mitochondria upon the development of a K^+ diffusion potential by the addition of valinomycin and upon the development of the energized state by the addition of succinate or ATP. The changes (large decreases) seen with the addition of succinate or ATP (inhibitable by NaCN and oligomycin respectively) were comparable to those recorded upon the addition of valinomycin to mitochondria suspended in media containing low concentrations of K^+. The change observed with succinate was partially reversed by the addition of either 2,4-dinitrophenol or ADP. Oligomycin prevented the reversal seen with ADP. Decreases in fluorescent intensity were also recorded when succinate was added to suspensions of inner membranes (prepared from rat liver mitochondria) containing the dye. With submitochondrial particles (also from rat liver mitochondria), however, increases in fluorescent intensity were seen upon the addition of succinate or ATP. These observations are consistent with the idea that a large negative (internal) potential develops across the inner membrane of the mitochondrion during energization and with other aspects of the chemiosmotic hypothesis.

INTRODUCTION

Estimations of membrane potentials of mitochondria have ranged from internal positive to internal negative values. These estimates were made in State 4 utilizing a variety of techniques. Tupper and Tedeschi [1, 2] measured the electrical potential by impaling giant mitochondria from Drosophila with microelectrodes and recorded a small potential (+10 to +20 mV, internal positive). Harris and Pressman [3] arrived at similar values (+30 to +40 mV, internal positive) by monitoring the distribution of permeant anions. Mitochondria do not normally transport K^+ but can be induced to do so by the addition of the antibiotic valinomycin. Mitchell and Moyle [4] assumed that in the presence of valinomycin K^+ would attain its equilibrium distribution so that the membrane potential can be equated to the Donnan potential determining the distribution of K^+. Applying this principle they calculated

values for the potential ranging from −150 to −200 mV (internal negative). Skulachev and his associates [5] studied the movement of synthetic lipid-soluble anions and cations and found that they were distributed across mitochondrial membranes to an extent consistent with a high potential, internally negative. Similar results were reported for mitochondria (viz. high potential, internally negative) by Jasaitis et al. [6] and independently by Azzi and coworkers [7], based on studies with the fluorescent probe anilinonaphthalene sulfonate (ANS).

Since criticisms of these methods have been raised by a number of investigators [8–12], we sought to measure potentials in these organelles employing the fluorescent cyanine dye 3,3′-dipropylthiodicarbocyanine iodide and fluorometric methods developed by Hoffman and Laris [13]. Evidence that the fluorescent intensity of the dye is sensitive to membrane potential has been described previously [13]. Since this study was initiated a report [11] has appeared in which the cyanine dye 3,3′-dihexyloxacarbocyanine iodide (CC_6) was used to estimate membrane potentials in *Drosophila* mitochondria. It was concluded from this investigation that the fluorometric measurements confirmed the values of +10 to +20 mV and that metabolism does not influence membrane potential. The experiments described below, however, indicate that mitochondrial membrane potential in State 1 is negative and that the potential becomes more negative with added substrate (succinate or ATP).

METHODS

Mitochondrial preparations

The livers were removed from adult male hamsters (*Mesocricetus auratus*) and rats of the Wistar strain, and mitochondria were isolated using the technique described by Kennedy and Lehninger [14]. Inner membranes of rat liver mitochondria were isolated and purified by the methods of Parson and Williams [15] and Caplan and Greenwalt [16]. Submitochondrial particles were prepared from rat liver mitochondria according to the method of Azzi et al. [7]. Mitochondrial respiratory control was determined as described by Estabrook [17]. All the mitochondrial preparations used in these experiments exhibited normal respiratory control with P/O ratios between 1.65 and 1.85 with succinate as substrate. Protein content of mitochondria preparations were determined by the method of Lowry et al. [18].

Measurements of fluorescence

The fluorescent dye 3,3′-dipropylthiodicarbocyanine iodide (designated diS-C_3-(5)), kindly supplied by Dr Alan Waggoner of Amherst College, was used in these experiments. The methods used to record fluorescence have been described previously [13]. Two different incubation media were used: one prepared as directed by Estabrook [17] (0.225 M sucrose, 0.01 M potassium phosphate (pH 7.4), 0.005 $MgCl_2$, 0.02 M KCl and 0.002 M triethanolamine buffered to pH 7.4 with KOH) containing K salts (referred to as K-medium) and another (referred to as Na-medium) also prepared according to Estabrook except that K salts were replaced by Na salts. The order in which materials were added to the cuvette was: (1) 3 ml Na- or K-medium, (2) an aliquot of the mitochondrial preparation, (3) 10 μl dye (0.5 mg/ml ethanol) and (4) other materials as described in the text. Stock solutions of valinomycin ($3 \cdot 10^{-5}$ M), rotenone ($1.2 \cdot 10^{-3}$ M), oligomycin (1 mg/ml) and 2,4-dinitrophenol ($6 \cdot 10^{-2}$ M) were added

as solutions in ethanol; succinate (0.5 M), ATP (0.02 M), ADP (0.018 M) and NaCN (0.12 M) were added as solutions in Na-medium. All experiments were carried out at room temperature (22–23 °C). Other details are given in the figure legends and text.

RESULTS

Intact mitochondria

Addition of K^+ and valinomycin. The intensity of fluorescence of the dye attained a constant level approx. 50–60 s after its addition to suspensions of intact hamster liver mitochondria. The constant level of intensity reached in Na-medium was either equal to or less than the intensity recorded with K-medium (Fig. 1). The difference between the intensities in K- and Na-media varied with different preparations of mitochondria and was frequently seen to increase with aging. In cases where a difference in fluorescent intensity was observed between the two media, the addition of KCl (added as aliquots of 2 M KCl solution) to mitochondria suspended in Na-medium led to an increase in intensity (Fig. 1). The addition of comparable amounts of NaCl to these suspensions resulted in a small (less than 5 %) decrease.

Upon the addition of valinomycin (final concentration $2 \cdot 10^{-8}$ M), an ionophore known to induce a marked increase in K^+ permeability in mitochondria [19], a rapid decrease in the fluorescent intensity of mitochondrial suspensions in Na medium or an increase in intensity with K-medium was observed (Fig. 1). New steady levels of fluorescent intensity were attained within 5 or 10 s after the addition of valinomycin. If KCl was then added to either of these suspensions, the level of fluorescent intensity increased (Fig. 1). The effect of valinomycin was maximal at $2 \cdot 10^{-8}$ M. The metabolic inhibitors rotenone (final concentration $2 \cdot 10^{-6}$ M), NaCN (final concen-

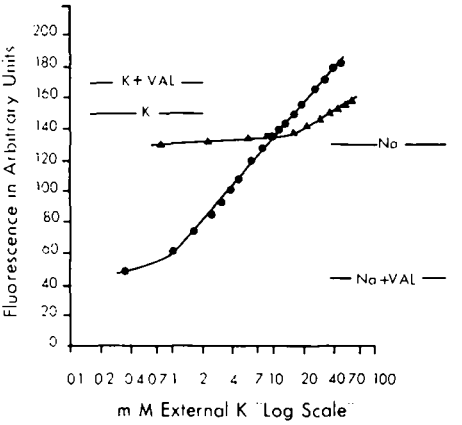
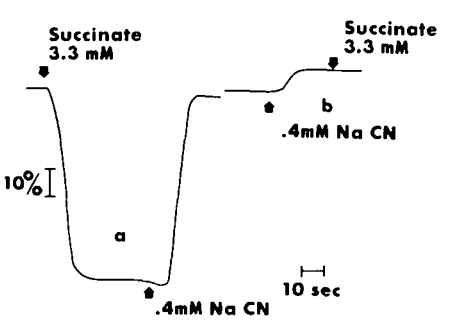


Fig. 1. The steady level of fluorescent intensity of the dye diS-C_3-(5) (final concentration $2.9 \cdot 10^{-6}$ M) in suspensions of intact hamster liver mitochondria (0.33 mg protein/ml suspension) as a function of $\log[K^+]_o$ in the absence and presence of valinomycin (final concentration $2 \cdot 10^{-8}$ M). Horizontal lines indicate levels of fluorescence in Na-medium and K-medium in the absence (–Na–, –K–) and presence (–Na + VAL–, –K + VAL–) of valinomycin, respectively. Aliquots of 1 M or 2 M KCl solutions were added to mitochondrial suspensions in Na-medium in the absence (▲ – ▲) and presence (● – ●) of valinomycin, to bring the concentrations in the cuvette to the values given on the abscissa.

tration $4 \cdot 10^{-4}$ M) or oligomycin (final concentration 2 μg/mg mitochondrial protein) did not prevent the changes seen with the addition of valinomycin.

Addition of succinate. When sodium succinate (final concentration 3.3 mM) was added to suspensions of dye and hamster liver mitochondria in either Na- or K-medium, there was a rapid decrease in fluorescent intensity (Fig. 2a). The percentage decrease observed with succinate varied with the amount of mitochondria present. The amount (0.33 mg/ml) which gave the largest decrease (see Table I) was employed in all experiments described below. This amount of mitochondria was also optimal for the change observed with valinomycin. The concentration of succinate in the range 0 to 30 μM also influenced the decrease in fluorescent intensity. In the range 30 μM to



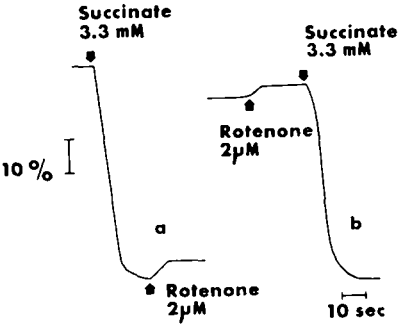


Fig. 2. Relative changes of fluorescent intensity of diS-C_3-(5) (final concentration $2.9 \cdot 10^{-6}$ M) of suspensions of intact hamster liver mitochondria (0.33 mg protein/ml suspension) in K-medium are shown following the addition of disodium succinate (final concentration 3.3 mM) and NaCN (final concentration 0.4 mM). All additions were made in the order shown after the fluorescent intensity had attained a steady level.

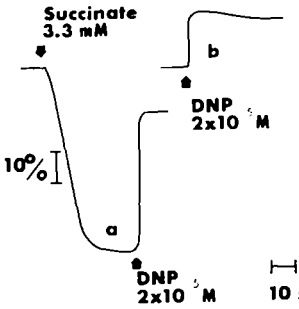
Fig. 3. Effect of rotenone (final concentration 2 μM) on fluorescence of diS-C_3-(5) with intact mitochondria. Same conditions as in Fig. 2. Additions as indicated.

TABLE I

INFLUENCE OF CONCENTRATION OF MITOCHONDRIAL PROTEIN ON CHANGE IN FLUORESCENCE WITH VALINOMYCIN

The percentage change in the fluorescent intensity of dis-C_3-(5) (final concentration $2.9 \cdot 10^{-6}$ M) upon the addition of valinomycin (final concentration $2 \cdot 10^{-8}$ M) to mitochondria suspended in Na-medium as a function of the amount of intact mitochondria present.

Mitochondrial protein (mg/ml suspension)	% change
1.7	−3
1.3	−3
0.83	−13
0.50	−70
0.33	−80
0.03	−54
0.008	−9
0.003	0



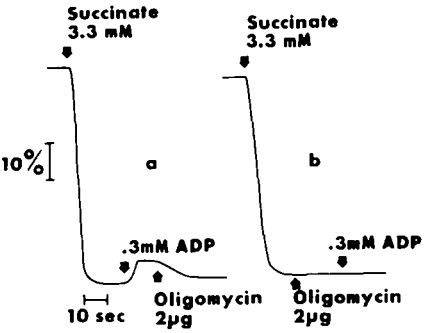


Fig. 4. Effect of 2,4-dinitrophenol (final concentration $2 \cdot 10^{-5}$ M) on fluorescence of diS-C_3-(5) with intact mitochondria. Same conditions as in Fig. 2. Additions as indicated.

Fig. 5. Effects of ADP (final concentration 0.3 mM) and oligomycin (2 μg added to cuvette) on fluorescence of diS-C_3-(5) with intact mitochondria. Same conditions as in Fig. 2. Additions as indicated.

16 mM succinate the decreases recorded were maximal.

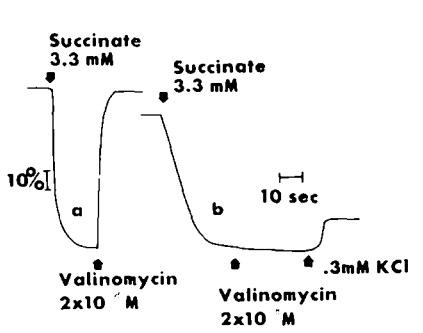
The influence of succinate on the fluorescent intensity could be prevented or fully reversed by the addition of NaCN (Fig. 2) but not by rotenone (Fig. 3) or oligomycin (not shown). In the absence of succinate the addition of either NaCN (Fig. 2b) or rotenone (Fig. 3b) resulted in a small increase in fluorescence while oligomycin had no effect.

The addition of 2,4-dinitrophenol (final concentration $2 \cdot 10^{-5}$ M) partially reversed the decrease in fluorescent intensity observed with succinate (Fig. 4a). This concentration of 2,4-dinitrophenol also increased fluorescent intensity in the absence of succinate (Fig. 4b). Changes seen with 2,4-dinitrophenol, NaCN, and rotenone in the absence of succinate can be attributed to the capacity of these agents to reverse an effect of endogenous metabolism on the level of fluorescent intensity. Higher concentrations of 2,4-dinitrophenol were found to change the fluorescent intensity of dye solutions and hence were not employed.

A partial reversal (Fig. 5a) of the decrease in response to succinate was also seen upon the addition of ADP (final concentration 0.3 mM). The reversal was maximal at this concentration. The effect of ADP was prevented or reversed by the addition of oligomycin (Fig. 5). In the absence of ADP, oligomycin had no influence on the change in fluorescent intensity resulting from the addition of succinate (Fig. 5b).

The decrease with succinate could also be reversed by the addition of valinomycin provided that the mitochondria were suspended in K-medium (Fig. 6a). In Na-medium, however, the addition of valinomycin resulted in either no change in intensity or a small decrease, so that if KCl was now added to the suspensions in Na-media, the fluorescent intensity increased (Fig. 6b). For example, the addition of KCl to a final concentration of 0.3 mM brought the fluorescent intensity to a level higher than that recorded prior to the addition of valinomycin (i.e. with succinate alone).

ATP addition. The addition of ATP to hamster liver mitochondria suspended in either Na- or K-media (Fig. 7) led to a rapid decrease in fluorescent intensity. This change was completely reversed by the addition of oligomycin (Fig. 7a) and partially



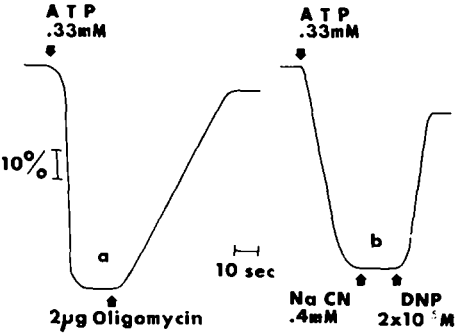
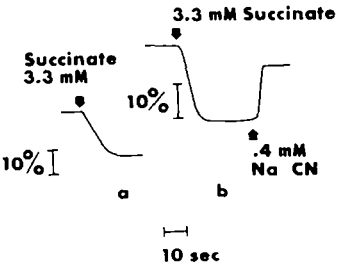


Fig. 6. Effects of valinomycin (final concentration $2 \cdot 10^{-8}$ M) on fluorescence of diS-C_3-(5) with intact mitochondria suspended: (a) in K-medium, and (b) in Na-medium. Other conditions same as in Fig. 2. Additions as indicated.

Fig. 7. Effects of: (a) oligomycin, and (b) NaCN and 2,4-dinitrophenol on fluorescence of diS-C_3-(5) with intact mitochondria. Final concentrations and order of additions are shown. Conditions same as in Fig. 2 except that ATP (final concentration 0.33 mM) was added initially instead of succinate.

reversed with $2 \cdot 10^{-5}$ M 2,4-dinitrophenol (Fig. 7b). The change in fluorescent intensity found with ATP was not prevented or reversed by the addition of NaCN (Fig. 7b). The addition of valinomycin led to a reversal of the change with ATP if the mitochondria were suspended in K-medium but not in Na-medium.

Sucrose addition. Many of the manipulations described above could have induced shrinkage or swelling of the mitochondria, and hence it was important to determine whether or not the changes in fluorescent intensity could be attributed to changes in volume. Since mitochondria are known to shrink in hypertonic sucrose [20], fluorescent intensity was measured while the medium was made hypertonic by the addition of sucrose. Successive additions of sucrose were made to increase its concentration in K-medium up to 300 mM. Only very small changes in fluorescence intensity were observed and these small changes were the same as changes seen upon dilution of the sample with an equal volume of K-medium.



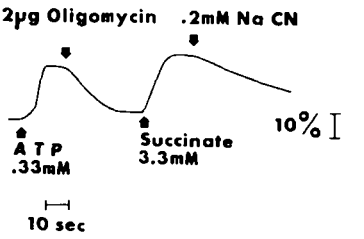


Fig. 8. Effects of succinate (final concentration 3.3 mM) on fluorescence of diS-C_3-(5) with inner mitochondrial membranes (8 mg protein/ml suspension) prepared from rat livers: (a) by the method of Parson and Williams [15], and (b) by the method of Caplan and Greenwalt [16]. Other conditions same as in Fig. 2.

Fig. 9. Effects of substrates (ATP, succinate) and inhibitors (oligomycin, NaCN) on fluorescence of diS-C_3-(5) with submitochondrial particles (0.11 mg protein/ml suspension) prepared from rat livers by the method of Azzi et al. [7]. Final concentrations and order of additions are shown. Other conditions same as in Fig. 2.

Inner membrane preparations

A decrease in fluorescent intensity was also seen when succinate was added to inner membranes prepared using two different methods [15, 16] (Fig. 8a). The percentage change was always smaller than that recorded with intact mitochondria and was also reversed by the addition of NaCN (Fig. 8b). With submitochondrial particles the addition of either ATP or succinate led to an increase in fluorescent intensity. The influence of ATP was blocked by oligomycin and that of succinate by NaCN (Fig. 9). The addition of sucrose to 100 mM final concentration had no effect on the fluorescent intensity of suspensions of inner membranes or submitochondrial particles.

Other observations

Intact mitochondria isolated from rat livers responded in an identical fashion to the same experimental procedures employed above where hamster liver mitochondria were used. Whenever the effects of a substance were examined in our system, suitable control experiments were performed which demonstrated that any effect observed on the fluorescent intensity of the dye was due to the substance itself and not to the solution in which it was dissolved. Furthermore, the substance was shown to have no effect on the fluorescent intensity of the dye in the absence of mitochondria.

DISCUSSION

The fluorescent intensity of several cyanine dyes has been correlated with membrane potential in a number of different preparations [13, 21–23]. In all of these studies a decrease in intensity was observed as the potential became more negative (internally) and an increase was seen as it became more positive. The experiments reported above with valinomycin were performed to see whether or not this analytical approach was also applicable to the mitochondrion. Application of this ionophore specifically increases K^+ permeability and hence should shift the potential towards the K^+ equilibrium potential.

$$E_K = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}$$

where E_K is the K^+ equilibrium potential; R, the gas constant; T, the absolute temperature; F. Faraday's constant; $[K^+]_o$, the K^+ concentration of the medium and $[K^+]_i$, the K concentration of the mitochondrion.

If the pattern seen with other preparations applies to the mitochondrion, then upon the addition of valinomycin the fluorescent intensity should vary with log $[K^+]_o$ and should be at its lowest level in K^+-free media. These predictions were confirmed by the data presented in Fig. 1. The relationship between fluorescent intensity and log $[K^+]_o$ in the presence of valinomycin was found to be linear over most of the range studied. A linear relationship would be expected provided that two conditions are fulfilled: (1) the potential induced by valinomycin is equivalent to the K^+ equilibrium potential, and (2) fluorescent intensity is directly proportional to membrane potential. At lower (below 1 mM) external $[K^+]$ at least one of these conditions is not met.

We would, of course, like to calibrate the fluorescent intensity in terms of potential. With previous preparations [13, 22] the membrane potential was calculated from the Nernst equation using values of $[K^+]_i$ and the values of $[K^+]_o$ in the presence of

valinomycin for which the fluorescent intensity was equivalent to that of the experimental system. Since we are not certain that the potential in the presence of valinomycin is precisely equal to the K^+ equilibrium potential, these estimates are approximate and probably represent limiting values. The choice of a reliable value for $[K]_i$ is complicated because the water in mitochondria occurs in more than one compartment and K^+ is not uniformly distributed through these compartments (for a critical review see ref. 24). K^+ in mitochondria is located within a barrier which excludes sucrose and chloride [25, 26]. Since a second barrier excludes carboxydextran but not sucrose, Harris [27] concluded that K^+ is located within the inner membrane of the mitochondrion. Hence the membrane potential seen upon the addition of valinomycin apparently occurs across the inner membrane. We have taken the value for $[K^+]_i$ the K concentration within the sucrose-impermeable space) to be 100 mM. This value was determined for rat liver mitochondria [27, 28] and our preliminary observations indicate that the comparable value for hamster liver mitochondria is at least this high. It should be noted that calculations of the potential using the K ratio are not very sensitive to errors in the estimations of $[K^+]_i$. For example, if the estimate (100 mM) for $[K^+]_i$ is wrong by a factor of 2, the error in the potential would be 20 mV. With these reservations in mind, the potential was estimated to be -64 mV in Na-medium and -48 mV in K-medium (Fig. 1).

Energization of the mitochondrion by the addition of succinate or ATP also influenced the fluorescent intensity of the dye and in both cases resulted in a large decrease in intensity, reflecting marked hyperpolarizaton. The change in intensity following the addition of succinate was inhibited by NaCN but not by rotenone or oligomycin. The change with ATP, on the other hand, was blocked by oligomycin but not by NaCN or rotenone. These observations are compatible with the chemiosmotic hypothesis developed by Mitchell [29], which states that electron transport or ATPase activity results in an electrogenic transfer of protons across the inner membrane.

Our results with the uncoupler 2,4-dinitrophenol and the phophate acceptor ADP are also in agreement with Mitchell's concept. According to the chemiosmotic hypothesis 2,4-dinitrophenol acts as a proton conductor allowing protons to move rapidly across the membrane collapsing the proton gradient and consequently the membrane potential. We observed that 2,4-dinitrophenol reversed the effects of both succinate and ATP. In terms of Mitchell's scheme, the addition of ADP should also lead to a more rapid return of protons through the ATP synthetase system which would depolarize the membrane. Addition of ADP partially reversed the effects of succinate and when the utilization of the "proton-motive force" to synthesize ATP was blocked by the addition of oligomycin, the depolarization obtained with ADP was prevented or reversed. According to the chemiosmotic theory the potential is generated across the inner membrane, and in fact when we tested inner membranes, the expected decrease in fluorescent intensity was observed. Furthermore, Mitchell's theory predicts a reversal of the potential in submitochondrial particles where the orientation of the membrane is reversed. Indeed, we found that the addition of succinate or ATP to these particles resulted in an increase in fluorescence.

Valinomycin is also known to uncouple oxidation and phosphorylation in the presence of higher concentrations of K^+ (see ref. 30). Rottenberg [28] attributed the uncoupling to a depolarization of the membrane which lowered the proton-motive force. Our studies with mitochondria in State 4 (Fig. 6) provide substantive evidence

for a depolarization under these conditions.

The fluorescent intensity and hence the potential generated by the addition of succinate is equal to the intensity (and potential) observed in 0.1–0.3 mM KCl with valinomycin. If we assume that the potential with valinomycin is equal to the K^+ equilibrium potential, then the potential developed with succinate is estimated to be of the order of -150 mV to -180 mV, again assuming a $[K^+]_i$ value of 100 mM. Since these values fall within the non-linear portion of the curve which relates fluorescence (potential) to $\log[K^+]_o$, they probably represent lower (most negative) limits for the mitochondrial membrane potential. By the same reasoning, the addition of ADP reduced the potential to the -130 mV range. The values obtained here are comparable to those obtained by Mitchell and Moyle [4].

Others have arrived at conclusions similar to ours by using anilinonaphthalene sulfonate [6, 7]. The addition of valinomycin to mitochondria and submitochondrial particles also influenced the fluorescent intensity of this fluorochrome, and these changes were attributed to changes in membrane potential. In studies with submitochondrial particles Azzi et al. [7] found the percent change in the fluorescence of anilinonaphthalene sulfonate in the presence of valinomycin to be a linear function of $\log[K^+]_o$ (1–100 mM). The authors stated that intact mitochondria also gave linear plots of $\log[K^+]_o$ versus changes in fluorescence induced by valinomycin. Unfortunately, the data were not given so we were unable to compare our measurements with theirs. Changes in the fluorescence of anilinonaphthalene sulfonate also occurred upon the initiation of electron transport or ATPase activity. Furthermore, their studies indicated a hyperpolarization (negative inside) with energy conservation in intact mitochondria. The changes in fluorescence with energy conservation were reversed in submitochondrial particles where the orientation of the inner membrane was known to be reversed. Azzi and coworkers [7] also attempted to relate the change in fluorescent intensity seen with succinate to an equivalent change with valinomycin. They suggested that the potential induced by succinate in submitochondrial particles would be of the order of -180 mV. They did not demonstrate, however, that anilinonaphthalene sulfonate fluorescence was a linear function of $\log[K^+]_o$ in a range that would include -180 mV. No estimate was given for intact mitochondria.

Tedeschi [11] has employed the cyanine dye CC_6 to study membrane potentials in *Drosophila* mitochondria. He reported that the results of his fluorometric study were in agreement with his electrophysiological data [1, 2]. From his observations he concluded that the membrane potential was approx. ± 10 to ± 20 mV and was not influenced by metabolism. Since his conclusions are diametrically opposed to ours, we shall review the possible reasons for the differences. First, the mitochondria used were from different sources. However, it seems unlikely that organelles of such striking similarity and ubiquity in terms of their structure and function would be so different with respect to membrane potential. Second, different dyes were employed. Although the results we have reported here were obtained with diS-C_3-(5), we have also obtained similar results with CC_6. We have used diS-C_3-(5) routinely in our investigations with mitochondria because larger changes in fluorescent intensity were recorded with this dye (approx. 30% decrease with CC_6 versus 80% with diS-C_3-(5)). Sims et al. [23] have also shown that diS-C_3-(5) is more sensitive to changes in the membrane potential of the erythrocyte. Third, in our experiments greater concentrations of dye and amounts of mitochondria were used. Studies with erythrocytes have shown that the

magnitude of the change in fluorescent intensity (which reflects a change in potential) is highly dependent upon the relative concentrations of dye and cells [13, 23]. In our system we used the same concentration of dye that had been used successfully with erythrocytes. The amounts of mitochondria were varied and the system was tested with valinomycin and succinate. The amount of mitochondria which gave the largest change was used for all experiments. It should be noted that given $2.9 \cdot 10^{-6}$ M dye, and 0.003 mg/ml mitochondrial protein no changes in fluorescent intensity were seen upon the addition of valinomycin, succinate, or ATP. Fourth, in our system we record fluorescent intensity continuously while Tedeschi's measurements are reported at 2–4-min intervals, so that if changes were transient they would not be detected in his system.

Finally, in his most recent communication Tedeschi [31] was unable to detect changes in the fluorescence of anilinonaphthalene sulfonate accompanying changes in metabolic state with an inner membrane preparation capable of oxidative phosphorylation. He suggested that these data might argue against a role of a membrane potential in oxidative phosphorylation. Using the same preparation, however, we were able to measure a change in fluorescent intensity indicative of a hyperpolarization of the mitochondrial membrane potential in State 4.

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