Use of Dyes to Estimate the Electrical Potential of the Mitochondrial Membrane

Kathleen Walsh Kinnally,* Henry Tedeschi, and Bruce L. Maloff

ABSTRACT: A number of cationic or anionic fluorescent dyes were investigated as possible monitors of the membrane potential of rat liver mitochondria, and giant mitochondria isolated from the liver of mice maintained on a diet containing cuprizone. The fluorescence of four dyes (8-anilino-1-naphthalenesulfonic acid, merocyanine 540, 3,3′-dipropylthiocarbocyanine, and bis[1,3-dibutylbarbituric acid-(5)]-pentamethine oxonol) was found to respond appropriately to changes in an apparent K^+ diffusion potential. Generally, valinomycin-induced K^+ diffusion potentials as calculated using the Nernst equation were used to calibrate the dependence of the fluorescence on the membrane potential. The appropriateness of this approach was verified for two dyes using microelectrodes in giant mitochondria. The apparent membrane potential change induced by the addition of succinate was variable but was very low and generally less than 60 mV in magnitude. The results are consistent with the notion that a large membrane potential is not established upon the initiation of metabolism and that the membrane potential does not play a significant role in the observed ADP phosphorylation.

The chemiosmotic hypothesis proposed that the energy used in the phosphorylation of ADP or in the transport of ions is furnished by the flow of protons in the direction of their electrochemical gradient (the *protonmotive force*). Mitchell proposed that the protonmotive force results from the asymmetric ejection of protons across the mitochondrial membrane as a result of the flow of electrons through the respiratory chain (Mitchell, 1966a,b, 1967, 1968; Mitchell and Moyle, 1969). In mitochondria, the electrical potential is generally assumed to be a major component of the protonmotive force.

Several investigators have estimated the membrane potential in metabolizing mitochondria from the distribution of ions, generally in the presence of the appropriate ionophores (e.g., Mitchell and Moyle, 1969; Rottenberg, 1973; Rottenberg and Scarpa, 1974; Nicholls, 1974), or alternatively from the distribution of synthetic lipophilic ions (e.g., Skulachev, 1971; Grinius et al., 1970, 1971; Bakeeva et al., 1970). We feel that the results of these experiments can be explained readily by a H^+/K^+ exchange or similar model in the absence of a membrane potential (Tedeschi, 1975; Maloff et al., 1978).

We approached the problem with two distinct techniques. We have carried out experiments measuring the membrane potential of giant mitochondria with microelectrodes (Tupper and Tedeschi, 1969a,b,c; Maloff et al., 1977, 1978). In addition, we have made use of electrofluorimetric dyes (Tedeschi, 1974; Walsh Kinnally and Tedeschi, 1976). We have found no evidence for a significant metabolically dependent membrane potential in mitochondria. The present study constitutes a more thorough effort to evaluate the effect of metabolism on the membrane potential with four dyes.

In many biological systems it is not possible to estimate membrane potentials directly by means of microelectrodes. Changes in the fluorescence or the light absorption of dyes in response to membrane potential variations provide alternative, indirect techniques which have been used successfully in the study of transmembrane potentials in a number of biological systems, including mitochondria. The responses of the fluorescent dye diO-C_6-(3)[1] have been used also to estimate Donnan potentials in the absence of membranes (Scordilis et al., 1975). Electrofluorimetric dyes have been used to estimate potential changes in a number of systems, e.g., axons (Cohen et al., 1974), bilayers (Conti and Malerba, 1972), red blood cells (Laris and Hoffmann, 1973; Hoffman and Laris, 1974; Sims et al., 1974), bacteria (Grinius and Bazenaite, 1976; Griniuviene et al., 1975; Laris and Pershadsingh, 1974), liposomes (Bakker and Van Dam, 1974), and mitochondria (Azzi et al., 1971; Tedeschi, 1974; Laris et al., 1975; Laris, 1977; Walsh Kinnally and Tedeschi, 1976).

Several studies have employed electrofluorimetric dyes to estimate the membrane potential change induced by metabolism in mitochondria. Potentials measured with microelectrodes at different acetate concentrations were used to calibrate the fluorimetric responses of diO-C_6-(3) in *Drosophila virilis* mitochondria (Tedeschi, 1974). In these mitochondria a systematic reduction of potential with increasing acetate concentration had been previously demonstrated (Tupper and Tedeschi, 1969). The fluorimetric measurements indicated that no significant change in the potential occurred upon energization. Four studies have used the magnitude of apparent K^+ diffusion potentials, induced by the addition of valinomycin, to calibrate fluorescence or absorption responses to substrate introduction. Laris et al. (1975) with diS-C_3-(5), Azzi et al. (1971) using ANS, and Åkerman and Wikström (1976) with the dye safranine obtained fluorescence or absorption changes consistent with a metabolically induced change in the membrane potential of about −180 mV. In our hands, further investigation of the fluorescence response of diS-C_3-(5) yielded results compatible with only a relatively low negative change in the potential (Walsh Kinnally and Tedeschi, 1976). In the same study, the total maximal protonmotive force was estimated to be only a small fraction of that necessary for ADP

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[1] Abbreviations used are: diBa-C_4-(5), bis[1,3-dibutylbarituric acid-(5)]-pentamethineoxonol; diS-C_3-(5), 3,3′-dipropylthiocarbocyanine; diO-C_6-(3), 3,3′-dihexyl-2,2′-oxacarbocyanine; ANS, 8-anilino-1-naphthalenesulfonic acid; MI, merocyanine 540; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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phosphorylation, as calculated from phosphate potentials (Walsh Kinnally and Tedeschi, 1976). A later study of Laris (1977) with diS-C_3-(5) using ATP-induced energization appears to be in agreement with the lower values reported in this study [see Figures 1 and 5 of Laris (1977)].

The basis for the fluorescent responses to membrane potential variations is largely unknown (for a review, see Sims et al., 1974; Waggoner, 1976). We feel that several dyes should be used, since they may differ in response mechanisms and differ in their sensitivity to phenomena unrelated to potential changes.

The present study is an attempt to evaluate more thoroughly the use of electrofluorimetric dyes to estimate variations in the membrane potential of mitochondria. Since the light absorption of safranine changes when it combines with polyanions (Colonna et al., 1973), and internal anions are likely to be made available by a proton pump (Tedeschi, 1975), this dye was not considered suitable for these studies. For each of the four dyes used, diS-C_3-(5), diBa-C_4-(5), ANS, and MI, the fluorescence varied linearly over a wide range of calculated K^+ diffusion potentials in the presence of valinomycin. That the fluorescence of ANS and diS-C_3-(5) actually reflects variations in K^+ diffusion potentials was verified with mouse giant mitochondria by means of microelectrodes.

Materials and Methods

Isolation of Mitochondria. Mitochondria were isolated from the liver of Holzmann white rats essentially as previously described (Tedeschi, 1959). Giant mitochondria were isolated from the liver of white albino mice obtained from Blue Spruce Farms (Altamont, N.Y.), which were fed a Cuprizone [oxalic acid bis(cyclohexylidene hydrazide) obtained from Aldrich Chemical Co., Milwaukee, Wis.] supplemented diet as described by the method of Suchy and Cooper (1974). The giant mitochondria were isolated as already described (Maloff et al., 1977, 1978).

Fluorescence Measurements. The fluorescence of the dyes diS-C_3(5), ANS, MI, and diBa-C_4-(5) was used to estimate membrane potentials. With the exception of ANS, the dyes were the generous gift of Alan Waggoner (Department of Chemistry, Amherst College). The fluorescence was monitored continually with a Perkin-Elmer-Hitachi spectrofluorimeter Model MPF-3, except during mixing after additions were made. The excitation and emission wavelengths and the dye concentrations used are shown in Table I. Fluorescence is expressed in arbitrary units and is reported as deviations from the baseline fluorescence. The background was negligible when compared to the dye signal, except with ANS where the signal to noise ratio was 2.4 ± 0.2 (eight determinations). In the absence of dye, no significant changes in fluorescence were observed relative to those observed in the presence of the dye.

Mitochondria were suspended in 3 mL of a variety of incubation mediums. Rotenone, antimycin A, and FCCP were used in a final concentration of 0.85, 0.61, and 0.06 μM, respectively. Each of these were added in 5–10 μL aliquots of ethyl alcohol. Rotenone was present in all incubations, except when the medium of Laris et al. (1975) was used. To initiate metabolism, succinate in 10 mM Tris (pH 7.4) was added in a final concentration of 3.3 mM to a suspension of mitochondria and dye. ADP was at a concentration of 1.3 mM. After equilibration, antimycin A or FCCP was added to block metabolism. In the controls, the following serial additions were made to a suspension of mitochondria and dye in parallel determinations: medium, antimycin A or FCCP, succinate and ADP. The change in fluorescence corresponding to a given magnitude of K^+ diffusion potential was estimated with mitochondria and dye after the addition of an appropriate aliquot of stock KCl solution in the presence of valinomycin (0.151 μM, except in Figure 5). All experiments were carried out at 25 °C.

TABLE I: Dye Concentrations.[a]

dye	excitation λ(nm)	emission λ(nm)	concn (μM)	mg of protein/mL
diS-C_3-(5)	622	670	3.0	0.2
MI	540	580	1.5	0.9
ANS	380	480	42	0.2
diBa-C_4-(5)	560	620	3.0	0.2

[a] Dye was added in 5–10-μL aliquots of alcohol to 3 mL of medium for the final concentration shown. Excitation and emission wavelengths were used with slits of 6 nm. The listed mg of protein/mL is approximate.

For rat liver mitochondria, the membrane potential change, ΔE_a, induced by metabolism was calculated by determining the fluorescence change (relative units) and dividing this value by the slope of the linear portion of the calibration ($\Delta F/\Delta E_a$) for that experiment. See the text for details. For mice giant mitochondria the method just described was used but, in addition, the membrane potential was measured directly with microelectrodes (see Maloff et al., 1977, 1978) in single mitochondria. The fluorescence of the suspensions was then related to these potentials directly.

Microelectrode Measurements. The techniques used with microelectrodes were previously described (Maloff et al., 1977, 1978).

Phosphorylative Ability. The phosphorylative ability of each preparation was estimated in parallel determinations by monitoring the disappearance of orthophosphate by the method of Hurst (1964) as previously described (Walsh Kinnally and Tedeschi, 1976). Except in those experiments in which MI was used, the assay for phosphorylation was carried out at protein concentrations which differed from the fluorescence determinations. However, the phosphorylative ability in the absence of dyes was found to be independent of the protein concentration in the range used. In one experiment, the protein concentration was varied from 0.068 to 0.68 mg/mL and the phosphorylative ability corresponded to the esterification of 96 ± 9 nmol of ADP min^{-1} (mg of protein)$^{-1}$ (mean ± SD, including the determinations at all mitochondrial concentrations $n = 4$). In typical experiments, the P:O ratios with succinate were 1.4 ± 0.1 (m ± SD: $n = 5$). In a previous series (Walsh Kinnally and Tedeschi, 1976) it was 1.7 ± 0.3. We have found no correlation between degree of coupling and fluorescence responses.

Results and Discussion

The fluorescence of many dyes has been shown to reflect variations in the membrane potential of several biological preparations. We applied a variety of these probes to an evaluation of the existence and magnitude of membrane potential changes induced by metabolism in mitochondria. Each dye was tested for its metabolic effect (Walsh Kinnahly and Tedeschi, 1978). The dyes ANS and MI were considered the most suitable, since they appear to have no effect on metabolism at the concentrations used. diS-C_3-(5) had no effect on respiration (although it partially blocked phosphorylation) and hence may not affect the membrane potential in the context of the chemiosmotic hypothesis. diBa-C_4-(5) partially inhibited succinate oxidation.

Comparison of Fluorescence to Direct Measurements with Microelectrodes. The fluorescence of certain dyes can be used to estimate membrane potentials, but this technique requires a method of quantitatively correlating these two parameters. A direct approach is to correlate the fluorescence measurements to the potentials measured directly by means of microelectrodes. This method was used by Tedeschi (1974) with *Drosophila* mitochondria and the dye diO-C_6-(3). Figure 1 is an illustration of this approach with mouse giant mitochondria using ANS. (In all our presentation of data the deviations indicated correspond to SD from the mean indicated.) As shown in Figure 1, the fluorescence of a suspension of the same preparation of mitochondria and dye varied linearly with the measured potential. Hence, the relationship between potential and fluorescence can be simply described by the slope of the curves such as that shown in Figure 1, i.e., the change in fluorescence per mV change in membrane potential ($\Delta F/\Delta E$). These results indicate that the fluorescence of ANS may serve as an indicator of the potential across the mitochondrial membrane, at least over the range tested. Similar results were obtained with diS-C_3-(5). This method of calibrating fluorescence with the membrane potential depends on the assumption that the impaled mitochondria are typical representatives of the population. However, this assumption is probably reasonable, since the standard deviations of the measurements are very small (see Figure 1) and no correlation was observed between mitochondrial size and membrane potential (Maloff et al., 1978).

In the experiment of Figure 1, the membrane potential (measured with microelectrodes) plotted as a function of $-\log[K^+]_0$ (not shown; the subscript corresponds to the external concentration) had a slope of 50 rather than the 59 predicted from the Nernst equation. This slope is comparable to the results previously obtained (Maloff et al., 1978). This close agreement supports the use of the Nernst equation in predicting the potential when microelectrode data is not available. However, we have found that at very low external K^+ concentrations neither the membrane potential (Maloff et al., 1978) nor the fluorescence (Walsh Kinnally and Tedeschi, 1976) vary with the external K^+ concentration in a manner consistent with the Nernst equation. Therefore, the data supports the use of the Nernst equation only over a limited range of K^+ concentrations. This nonlinear relationship between membrane potential and te log of the external K^+ concentration has been observed in a number of biological systems which exhibit predominantly a K^+ diffusion potential, e.g., squid axons (Hodgkin and Keynes, 1955). Most probably, under conditions of low $[K^+]_0$, the diffusion of other ions contributes significantly to the membrane potential. Nevertheless, the direct determination of the linear dependence of ANS and diS-C_3-(5) fluorescence on the potential validates the indirect method of imposing K^+ diffusion potentials to calibrate fluorescence. The microelectrode data indicate that, at least in the linear range of response and under the conditions used, the Nernst equation can provide a reasonably accurate measure of potential changes.

The potentials estimated using the dyes and microelectrodes in the absence of metabolism, i.e., the so-called resting potentials, were not equivalent and therefore the microelectrode data was used solely to determine the fluorescence change/mV change in potential. The resting potential was generally found to be about +15 when determined with microelectrodes. In the experiments shown in Figures 1 and 2, this potential as estimated with ANS fluorescence was about −15 mV (when Figure 1 was used as a calibration curve to relate the fluorescence to potential). However, the −15 mV may not be meaningful, since dye fluorescence has been shown to reflect events other than membrane potentials (e.g., see Sackman and Träuble, 1972; Birkett et al., 1971; Azzi et al., 1975). For this reason, we chose to express the results obtained in the presence of metabolism as the difference in potential from the resting level (ΔE_a). In the experiments using rat liver mitochondria, the apparent resting potentials ranged, approximately, from −57 to −68 mV, depending on the dye used. However, in individual experiments, the values could deviate significantly from these. The resting values were obtained using calibration curves similar to that shown in Figure 3 (see below). In these calculations, the assumption is made that the internal concentration of K^+ is 100 mM. In at least some studies, the internal concentration has been found to be much lower (Amoore and Bartley, 1958), and an argument can also be made that the values reported by others are much lower (see Tedeschi,

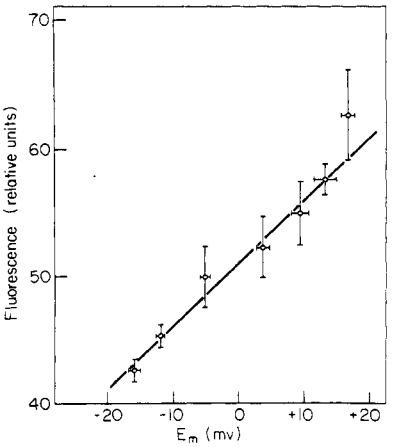

FIGURE 1: The membrane potential of individual giant mitochondria measured with microelectrodes in the presence of valinomycin and ANS compared to the fluorescence of the suspension under identical conditions. The mitochondria were suspended in 0.3 osM solutions with 10 mM Tris, 0.5 mM NaP_i (pH 7.4) in which sucrose was varied to maintain the osmotic pressure constant as the KCl concentration was varied. The deviations indicated correspond to standard deviations from the mean indicated. See the text.

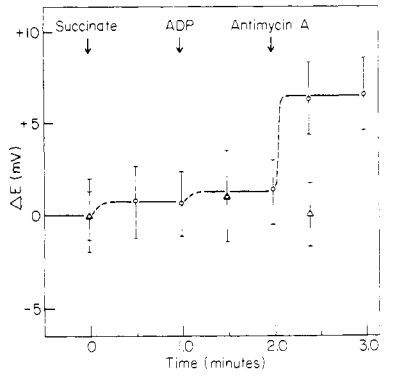

FIGURE 2: The membrane potential during the alteration of the metabolic state; estimation with microelectrodes and from fluorescence. The membrane potential was measured with microelectrodes in parallel determinations and is expressed as Δ. The time course of the microelectrode measurements is only approximate. The apparent membrane potential change ΔE_a is expressed as O. Figure 1 was used as the calibration. Zero ΔE corresponds to the fluorescence level of rotenone-treated mitochondria plus ANS and corresponds to 42.7 ± 0.9 relative units ($n = 4$). After the addition of succinate, ADP, and antimycin A, the fluorescence was 43.0 ± 1.0, 43.3 ± 0.9, and 46 ± 1.0 relative units, respectively ($n = 4$). The giant mitochondria were in 0.3 osM sucrose, 10 mM Tris, 0.5 mM NaP_i, 5 mM KCl (pH 7.4), plus ANS. The changes in potential and fluorescence were monitored continuously.

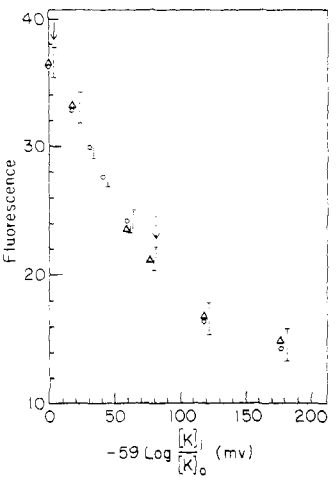

FIGURE 3: The relationship between the fluorescence of diS-C_3-(5) and the apparent K^+ diffusion potential; its independence from the inhibitor present. The fluoresence of a suspension of rat liver mitochondria plus diS-C_3-(5) in the medium of Figure 2 was monitored after the serial additions of KCl from a stock solution in the presence of valinomycin and either rotenone (O) or rotenone plus antimycin A (Δ). The arrows delineate the linear portion of the curve used to calibrate the metabolically induced fluorescence changes. The values reported are about 1 min after additions.

1971). In our hands, in preliminary experiments using atomic absorption, the K^+ concentration was found to be in the range of 30 to 50 mM. The lower $[K^+]_i$ would reduce the estimated resting potential to −20 to −30 mV. Because of these uncertainties, the results are expressed as the difference in potential from the resting level as done in Figure 1.

Independence of the Potential Changes from Metabolism in Giant Mitochondria. Altering the metabolic state of a suspension of giant mitochondria plus dye did not produce a significant change in potential (see Figure 2), either when the microelectrode measurements (i.e., Figure 1) or when the Nernst calculated K^+ diffusion potentials (assuming $[K^+]_i$ is 100 mM) were used to calibrate the fluorescence response. The results are the same whether the potential was measured directly with microelectrodes or calculated through the fluorescence change. The small change in fluorescence observed upon the addition of antimycin A may be attributed to an effect of antimycin a which is unrelated to metabolism, since we have also observed it in nonmetabolizing mitochondria. The results summarized in Figure 2 present convincing evidence that there is no significant change in the membrane potential of mouse giant mitochondria when the metabolic state is altered.

Indirect Determinations of Potentials Using the Nernst Equation. Since microelectrode studies can only be readily done with giant mitochondria and these can only be obtained in low yield, further validation of the potential dependence of the dyes' fluorescence was carried out for ANS, MI, and diS-C_4-(5) using rat liver mitochondria. In these experiments, valinomycin-induced K^+ diffusion potentials were used to calibrate the observed fluorescence changes. Figure 3 illustrates the use of this technique with a calibration curve, i.e., the fluorimetric response as a function of external K^+ concentration (expressed here as $-59 \log [K]_i/[K]_o = \Delta E_a$), where $[K]_i$ and $[K]_o$ correspond to the internal and external K^+ concentration, respectively. This technique assumes a constant volume and a constant internal $[K^+]$ of 100 mM. This value was chosen since this is the concentration assumed by others doing comparable work (e.g., Laris et al., 1975) and it has been calculated from recent data (e.g., of Rottenberg, 1973). However, a case can be made for much lower concentrations based on different assumptions about the size of the inner space (Tedeschi, 1971) (see also previous discussion). In agreement with Laris (1975), the results obtained at varied osmotic pressure were comparable to those obtained when osmotic pressure (and hence volume) was kept constant except for a small deviation in fluorescence at high K^+ concentrations (i.e., 100 mM). The fluorescence changes induced by varying the external K^+ concentration in the presence of valinomycin occurred over a range of dye concentrations, at least in the cases of ANS and diS-C_3-(5) (see Figure 4). For both dyes the general shape of the curves is about the same, and the apparent membrane potential changes induced by energization are approximately the same (see legend of Figure 4 and discussion below) at different dye concentrations. The measurement of fluorescence at various external K^+ concentrations in the presence of valinomycin assumes that the fluorescence changes reflect a K^+ diffusion potential as they do in other systems, e.g., mouse giant mitochondria.

The plausibility of the fluorescence reflecting a K^+ diffusion potential in rat liver mitochondria was examined with three dyes: diS-C_3-(5), ANS, and MI. Figure 3 shows the relationship between the fluorescence (relative units) of diS-C_3-(5) plus mitochondria and the apparent diffusion potential (in the presence of inhibitors) obtained through the Nernst equation. It is typical of the calibration curves used to estimate the apparent membrane potential induced by metabolism. The relationship between the fluorescence and the apparent membrane potential is linear over a wide range of external K^+ concentrations (see Figure 3, the region of the curve between the two arrows). The lack of linearity found at very low external K^+ concentrations is probably the result of the inapplicability of the Nernst equation in this range, as suggested by the mouse mitochondria microelectrode data and the considerations discussed below. For these reasons, only the linear portion of the calibration curve was used to determine the potential change upon energization. In the vast majority of the energization experiments, the fluorescence levels fell in this linear range [all 10 of our ANS experiments, 14 out of 15 experiments with MI, and 21 of 33 experiments with diS-C_3-(5)].

The nonlinearity in the calibration curves observed at low external K^+ concentrations can be expected from our present understanding of biological membrane potentials. There is much information supporting the notion that the Nernst equation cannot be applied at low external K^+ concentrations and the nonlinearity is not the result of some other effect (e.g., a saturation effect resulting from the partition of the dye between the medium and the mitochondria). (a) At low K^+ concentrations, the membrane potential of mitochondria measured with microelectrodes is much less than that calculated from the Nernst equation (Maloff et al., 1978). (b) The fluorescence of two of the dyes used, ANS and MI, has been shown to respond linearly to the membrane potential over a very wide range when the potential is actually measured with microelectrodes in other biological systems (e.g., Conti and Malerba, 1972; Cohen et al., 1974). (c) Two dyes closely related to diS-C_3-(5), diO-C_6(3), and 3,3′-dipentyloxacarbocyanine also show linear relationships between fluorescence and membrane potentials (see Cohen et al., 1974; Davila et al., 1974). (d) The initial deviation from linearity in fluorescence vs. Nernst calculated potential plots corresponds closely in K^+ concentrations for all the four dyes used in this study and in a wide range of dye concentrations. (e) In Ehrlich ascites cells, the nonlinearity of the relationship is not accounted for by the measured partition of the dye between the cells and the me-

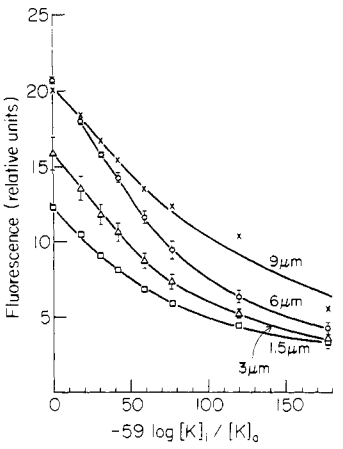

FIGURE 4: The dye concentration dependence of the fluorescence response of diS-C_3-(5). The fluorescence of a suspension of rat liver mitochondria plus dye in 0.3 osM sucrose, 10 mM Tris (pH 7.4) was monitored as the apparent K^+ diffusion potential ΔE_a was varied in the presence of valinomycin and the dye concentration shown. The apparent membrane potential change induced by the addition of succinate was −43 (8.5, 4.6, n = 2), −40 ± 6 (9.5 ± 0.6, 5.0 ± 0.4, n = 4), −58 ± 4 (17.1 ± 0.6, 8.0 ± 0.1, n = 4), and −45 mV (20.3, 15.0) at 1.5, 3.0, 6.0, and 9.0 μM diS-C_3-(5). The fluorescence (relative units) of rotenone-treated mitochondria plus dye before and after succinate addition is shown in parentheses.

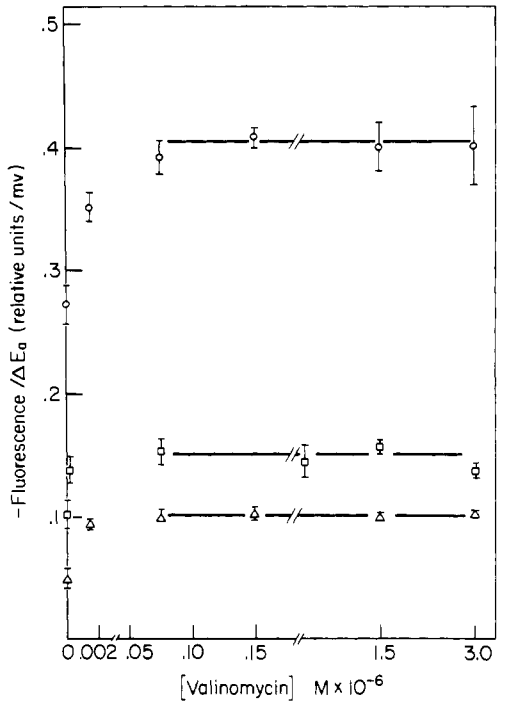

FIGURE 5: Independence of the calibration curve slope from the valinomycin concentration above a critical level. The fluorescence of ANS (○), MI (△), or diS-C_3-(5) (□) plus rat liver mitochondria in 0.3 osM sucrose, 10 mM Tris, 0.5 mM NaP_i (pH 7.4) was measured at varied KCl concentrations using the method of Figure 3, except that the valinomycin concentration present was varied as indicated. Fluorescence/ΔE_a represents the slope of the linear portion of the calibration curve obtained at each valinomycin concentration and is the fluorescence change per millivolt change in the apparent K^+ diffusion potential calculated using the Nernst equation.

TABLE II: Effect of Inhibitors on the Calibration Curve Slope.[a]

expt	dye	metabolic reagent	slope $\Delta F/\Delta E_a$ (rel units/mV)
1a	ANS	rotenone	0.213 ± 0.011
b		FCCP	0.180 ± 0.003
c		FCCP & rotenone	0.159 ± 0.011
2a	ANS	rotenone	0.144 ± 0.007
b		antimycin A	0.156 ± 0.011
c		FCCP	0.147 ± 0.025
3a	MI	rotenone	0.109 ± 0.002
b		antimycin A	0.110 ± 0.007
c		FCCP	0.057 ± 0.004
4a	diS-C_3(5)	rotenone	0.185 ± 0.011
b		FCCP	0.117 ± 0.009

[a] Rat liver mitochondria were suspended as in Figure 2, except for the addition of valinomycin. The uncoupler or inhibitor was present initially. The slope of the calibration curve, i.e., the fluorescence change per millivolt change in potential or $\Delta F/\Delta E_a$, was determined as in Figure 3.

dium (Brockhardt, 1977) and, in fact, a nonlinear relationship between membrane potential and K^+ concentration is indicated by direct studies with microelectrodes at low K^+ concentrations (Sekiya, 1962).

A diffusion potential should be relatively independent of metabolic state as long as the internal K^+ concentration is not critically affected. Figure 3 shows the fluorescence changes in the absence of metabolism, as expected for a true diffusion potential. The circles indicate the presence of rotenone and the triangles that of antimycin A plus rotenone. Several experiments using metabolic inhibitors and uncouplers are summarized in Table II. The slope of the linear portion of the curves ($\Delta F/\Delta E_a$, relative units/mV) was found to be independent of the inhibitor (e.g., antimycin A or rotenone) as shown in Figure 3 and Table II with any of the three dyes. On the other hand, FCCP decreased the slope of the calibration curve with ANS and diS-C_3-(5) (Table II, experiments 1 and 4). This reduction was generally small and did not always take place (e.g., experiment 2). The slope of the MI calibration curve was more greatly reduced by FCCP. However, no significant difference in the giant mitochondrial potential measured with microelectrodes was observed in the presence and absence of FCCP with and without valinomycin (Maloff et al., 1978). This decrease may be an effect of FCCP on dye fluorescence under the special conditions used and may be unrelated to the potential. It should be noted that under some conditions FCCP appears to have no effect on fluorescence (see Table II, experiment 2).

A K^+ diffusion potential should be independent of the valinomycin concentration after surpassing a critical minimum level. The crucial effect of valinomycin would be to increase the permeability of mitochondria to K^+, such that K^+ becomes the predominant mobile ion, thus permitting the use of the Nernst equation over a range of external K^+ concentrations. The dependence of the fluorescence on the valinomycin concentration is shown in Figure 5 for the three dyes. The results are expressed as the slope, $\Delta F/\Delta E_a$ (ordinate), as a function of the valinomycin concentration (abscissa). $\Delta F/\Delta E_a$ was found to be independent of the valinomycin concentration with ANS, MI, and diS-C_3-(5), except at very low concentrations. The slope was approximately the same even at concentrations of valinomycin so low that the uptake of K^+ in metabolizing mitochondria is decreased (Massari et al., 1972). In some preparations fluorescence changes did occur in the absence of valinomycin with all three dyes but generally over a narrow range of K^+ concentrations, and the observed slope of the calibration curve was generally much less than that obtained in the presence of valinomycin (see Figure 5). The results obtained with MI and ANS are comparable. There is some evidence from the microelectrode work with mouse mitochondria (Maloff et al., 1978) that minor changes in potential do take

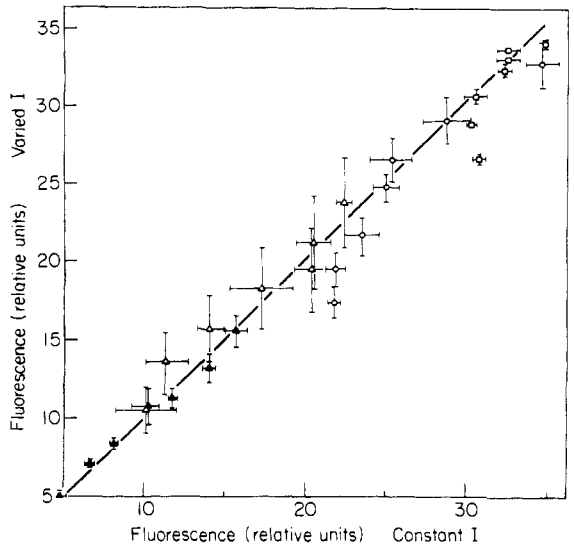

FIGURE 6: The effect of ionic strength on the fluorescence changes. The fluorescence values obtained when ionic strength was varied are plotted as a function of those obtained when it was maintained constant. Rat liver mitochondria were suspended in 0.30 osM sucrose, 10 mM Tris, and 0.5 mM sodium phosphate. The ionic strength was varied by the addition of KCl from 0 to 100 mM (ordinate). In parallel determinations (abscissa), the ionic strength was kept constant by mixing KCl with choline chloride (open symbols) or with NaCl (closed symbols) such that the sum of the concentrations was 100 mM. The dyes are indicated as follows: ANS (○), MI (□), diS-C_3-(5) (△). When high concentrations of Na^+ or choline were present, the fluorescence levels decayed with time (suggesting some collapse of the apparent diffusion potential had occurred), and the values reported are initial levels (about 5 s).

place even in the absence of valinomycin at high KCl concentrations.

Fluorescence changes obtained by varying the concentration of external K^+ do not necessarily reflect solely a diffusion potential (e.g., see Scordilis et al., 1975), even in the presence of valinomycin. However, the changes in surface charge which could produce changes in fluorescence should be independent of the nature of the salt used and should not occur when the ionic strength is kept constant. We tested whether fluorescence is affected by ionic strength (Figure 6). In one set of determinations, the ionic strength was kept constant (corresponding to 0.1 M K^+) with either choline chloride or sodium chloride (ordinate); in another set, the ionic strength at various KCl concentrations was allowed to vary (ordinate). The two sets of data are approximately in agreement. Similarly, the serial addition of choline chloride, which would result in about the same changes in ionic strength as the addition of KCl resulted in small changes in the fluorescence of ANS, MI, and diS-C_3-(5). The fluorescence of the dyes in the presence of valinomycin was stable when K^+ was the predominant ion (see, however, the legend of Figure 6), a finding consistent with the microelectrode data reported by Maloff et al. (1978) with mouse giant mitochondria.

The results of Figures 3-6 indicate that the fluorescence changes obtained by varying the K^+ concentration in the presence of valinomycin do reflect changes in a K^+ diffusion potential in rat liver mitochondria. However, this technique (which assumes K^+ is the only diffusible ion as implied by the use of the Nernst equation) will yield maximal estimates of membrane potential changes. As suggested previously, this notion is supported by microelectrode data with giant mitochondria. Additionally, fluorescence changes observed upon the initiation of metabolism may be the result of mechanisms other than one dependent on membrane potentials (see discussion above).

The Effect of Metabolism on Fluorescence and the Apparent Membrane Potentials. The fluorescence change induced by metabolism was measured with two different designs within the same experiments. The fluorescence was monitored while metabolism was initiated by introducing succinate to rotenone treated mitochondria and then interrupted by the addition of antimycin A or one of the uncouplers FCCP or CCCP. The fluorescence changes obtained with the three dyes ANS, MI, and diS-C_3-(5) suggest that the membrane potential change induced by the addition of succinate is between −8 and −62 mV, depending on the experiment (see Table III, column 2). For the reasons outlined above, the values of the lower magnitude are more likely to be meaningful. The fluorescence changes induced by succinate cannot be attributed to some nonmetabolic effect. Not only was the change reversible by adding either antimycin A or an uncoupler, but the addition of succinate to mitochondria treated with antimycin A or FCCP did not change the fluorescence significantly. For example, in an experiment using ANS in the presence of FCCP, the ΔE_a before the addition of succinate was +36 mV and after was +39 mV (not shown). Similar results were obtained with diS-C_3-(5). Also, the apparent membrane potential change induced by the addition of succinate to rotenone treated mitochondria did not appear to depend on the diS-C_3-(5) (see the legend of Figure 4) or the ANS concentration.

The results are approximately the same when two different dyes are used with the same mitochondrial preparation in the same experiment (Table III, compare part a and b, columns 2 and 3 for experiments 3 to 6). There is one notable discrepancy when diS-C_3-(5) and diBa-C_4-(5) were used (experiment 6). With diS-C_3-(5) the addition of succinate produced an apparent change in potential of −55 mV (experiment 6a), whereas with diBa-C_4-(5) the apparent change was about +65 (experiment 6b). This finding emphasizes the need for caution in interpreting results obtained with electrofluorimetric dyes. However, the remaining results suggest that some of the dyes can be useful for approximate delineation of the order of magnitude of the change induced by metabolism.

When antimycin A or uncouplers are added after succinate, the apparent membrane potentials generally returned to the resting values (before succinate was added), confirming the low potential values estimated after the addition of succinate. There are, however, some significant exceptions. Using the dye MI, FCCP produced apparent positive potentials corresponding to 25 and 59 mV (where the resting level has been set at zero), as shown in Table IV, experiments 1b and 2b. These values correspond to an increase in fluorescence, possibly from an interaction between FCCP and MI. As shown in Figure 7, the effect was not related to the uncoupling activity. With increasing FCCP concentration (up to 2.6×10^{-9} M) the phosphorylative rate declined to zero with minimal deviations of the fluorescence from the resting level. An increase in ΔE_a took place if the FCCP concentration added was in excess of this value. Obviously the ΔE_a estimated in the presence of FCCP and MI is not related to the phosphorylative ability. Similarly, a lack of correlation between the apparent potentials, as indicated by ANS fluorescence, and phosphorylative activity was observed when the coupling was varied by changing the pH (see Figure 8). Comparable results were obtained with MI. This lack of correlation between phosphorylative ability and ΔE_a agrees with our previous observation (Walsh Kinnally and Tedeschi, 1976).

The Effect of Mg^{2+} on Fluorescence. The low ΔE_a induced by succinate additions found in these experiments are comparable to those previously reported by us with the dye diS-C_3-(5) under similar conditions (Walsh Kinnally and Tedeschi,

TABLE III: The Apparent Membrane Potential Changes Observed upon Alteration of Metabolic State.[a]

expt	col 1, dye	col 2, ΔE_a (mV) + succinate	col 3, ΔE_a (mV) + ADP	col 4, ΔE_a (mV) metab reag	col 5, metab reag used
1a	diS-C_3-(5)	−60 ± 11	−80 ± 9	−5 ± 3	FCCP
b		−60 ± 6	−80 ± 7	0 ± 1	antimycin A
c		−62 ± 9	−81 ± 21	−6 ± 5	CCCP
2	MI	−45 ± 13	−47 ± 13	+14 ± 13	FCCP
3a	MI	−29 ± 8	−27 ± 9	−5 ±7	antimycin A
b	diS-C_3-(5)	−19 ± 3	−22 ± 4	+22 ± 8	antimycin A
4a	diS-C_3-(5)	−8 ± 4	−16 ± 5	+26 ± 3	FCCP
b	ANS	−8 ± 3	−6 ± 3	−3 ± 3	FCCP
5a	diS-C_3-(5)	−19 ± 3	−22 ± 4	+22 ± 8	antimycin A
b	MI	−29 ± 8	−27 ± 9	−5 ± 7	antimycin A
6a	diS-C_3-(5)	−55 ± 4	−60 ± 7	+12 ± 8	antimycin A
b	diBa-C_4-(5)	+65 ± 8	+80 ± 8	+33 ± 8	antimycin A

[a] Rat liver mitochondria were suspended as in Figure 2 with rotenone and dye. The apparent membrane potential change, ΔE_a, was estimated about 1 min after each of the serial additions of succinate (column 2), ADP (column 3), and a metabolic inhibitor (column 4) as indicated in column 5. These apparent changes were calculated as described in the text from the fluorescence changes and indicate the deviation of the fluorescence from the level of rotenone-treated mitochondria plus dye.

TABLE IV: Comparison of the Apparent Membrane Potential Change Obtained under Different Conditions.[a]

expt	dye	[K] (mM)	[Mg] (mM)	[P_i] (mM)	ΔE_a (mV) + succinate	ΔE_a (mV) + ADP	ΔE_a (mV) + FCCP
1a	diS-C_3-(5)	5	0	0.5	−26 ± 3	−34 ± 6	+13 ± 4
b	MI	5	0	0.5	−40	−32	+25
2a	diS-C_3-(5)	5	0	0.5	−60 ± 8	−61 ± 11	+14 ± 10
b	MI	5	0	0.5	−24 ± 2	−19 ± 1	+59 ± 7
c	diS-C_3-(5)	5	5	10	−205	−203	−10
d	MI	5	5	10	−54	−44	+51
3[b]a	diS-C_3-(5)	20	5	10	−138 ± 10	−130 ± 10	−6 ± 9
b	MI	20	5	10	−43 ± 7	−27 ± 5	+44 ± 11
4[c]a	MI	5	0	0.5	−13 ± 1		+15 ± 1
b	ANS	5	0	0.5	−3 ± 1		+13 ± 2
c	diS-C_3-(5)	5	0	0.5	−93		−4
d	MI	5	5	0.5	−21 ± 3		+7 ± 3
e	ANS	5	5	0.5	−12 ± 3		+1 ± 2
f	diS-C_3-(5)	5	5	0.5	−141		+18

[a] Rat liver mitochondria were suspended in 0.3 osM sucrose, 10 mM Tris (pH 7.4), plus the indicated concentrations of K^+, Mg^{2+}, and P_i. The apparent membrane potential changes ΔE_a were determined as in Table III. Except for experiment 4, the ΔE_a were calculated using the calibration curves obtained under conditions identical to the energization conditions (without substrate addition). [b] Conditions of Laris et al. (1975). [c] Antimycin A was used in place of FCCP, and the values obtained in the presence of Mg^{2+} were calculated using the calibration curve obtained in the absence of Mg^{2+}. The values obtained using the Mg^{2+} calibration curves for the succinate response were: diS-C_3-(5), −353 mV; ANS, −36 ± 8 mV; MI, −52 ± 6 mV.

1976). In the previous experiments, the values ranged from 0 to −70 mV. However, Laris et al. (1975) have presented evidence for changes consistent with very large membrane potential changes (as high as −180 mV) induced by succinate oxidation in hamster liver mitochondria using diS-C_3-(5). Using a similar procedure, we have found fluorescence changes as high as −300 mV (Walsh Kinnally and Tedeschi, 1976). However, the changes did not correlate with the phosphorylative capacity and depended on the presence of Mg^{2+}. For these reasons and others discussed below, we concluded that the fluorescence changes obtained under these conditions did not correspond to true membrane potentials.

Since large apparent potentials were observed with diS-C_3-(5) in the presence of Mg^{2+} and P_i, we examined the question of whether the ΔE_a values obtained under these conditions are meaningful by comparing the fluorescence changes of several dyes. We chose to use MI most extensively in a comparative study with diS-C_3-(5), since it had no apparent metabolic effect. The results are summarized in Table IV. Invariably, the high potential changes were evident only with diS-C_3-(5) and with no other dyes (for example, experiments 2c, 2d, and 3). These results suggest that the diS-C_3-(5) fluorescence changes induced by metabolism in the presence of Mg^{2+} and P_i are not likely to reflect true membrane potential changes. Additionally, more recent estimates obtained by Laris (1977) with ATP are more in agreement with our lower values obtained in the absence of Mg^{2+} and P_i. Although they are not specifically discussed in the text, the potential changes seem to range from −50 to −70 mV.

Other experiments support the view that metabolically induced diS-C_3-(5) fluorescence changes observed in the presence of Mg^{2+} and P_i do not reflect true changes in the potential. Figure 9 represents an experiment which examines the effect of Mg^{2+} on the fluorescence changes of diS-C_3-(5). Curve 1 (closed circles) represents a suspension in the presence of Mg^{2+}. Curve 2 represents the control in the absence of Mg^{2+}. As shown in curve 1, the presence of Mg^{2+} produced a much greater change in fluorescence. Paradoxically, addition of Mg^{2+} to energized suspensions had no immediate effect on fluorescence (curve 3) until antimycin A was added (however,

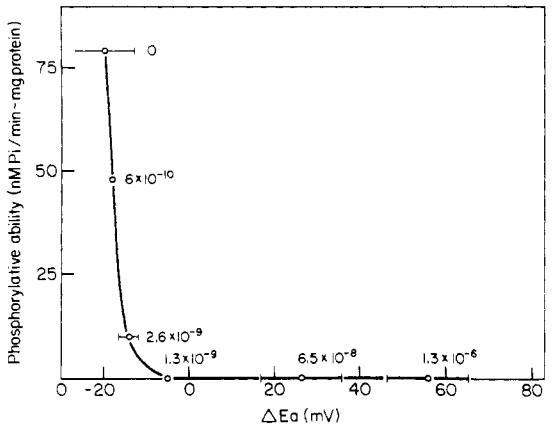

FIGURE 7: The fluorescence changes of MI in the presence of FCCP; independence from phosphorylative ability. Rat liver mitochondria were suspended in the medium of Figure 3 plus MI and 5 mM KCl. The phosphorylative ability was measured in the presence of the concentration of FCCP indicated next to each point. The ΔE_a was determined from the fluorescence change 1 min after the addition of FCCP to metabolizing mitochondria as described in the text. The slope of the calibration curve was 0.084 relative unit/mV change in the apparent K^+ diffusion potential. The fluorescence level of rotenone-treated mitochondria plus dye was 33.8 and the level of the state 3 (rotenone and succinate plus ADP) suspension was 32.2 relative units (n = 14).

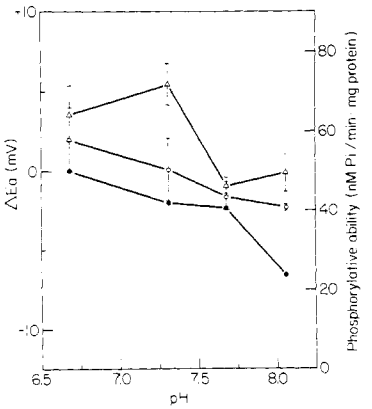

FIGURE 8: pH dependence of the apparent membrane potential changes as indicated by ANS. Rat liver mitochondria were suspended in 0.3 osM sucrose, 10 mM Hepes, 0.5 mM NaP_i, 5 mM KCl, plus ANS. The pH as indicated was measured after the addition of mitochondria. The ΔE_a were estimated by serial addition of succinate (○) and then ADP (△). The phosphorylative ability (●) was determined in parallel.

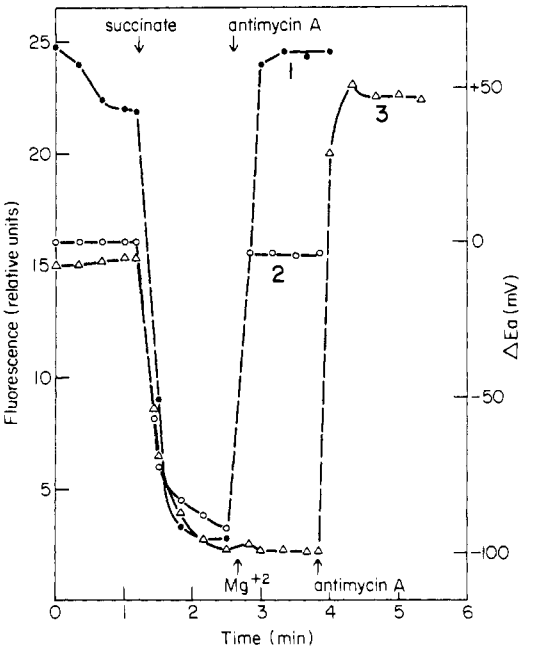

FIGURE 9: The effect of Mg^{2+} on the fluorescence of diS-C_3-(5). Rat liver mitochondria were suspended in 0.3 osM sucrose, 10 mM Tris, 0.5 mM NaP_i, 5 mM KCl (pH 7.4) for curves 2 (●) and 3 (△). In curve 1 (○), $MgCl_2$ was added to the medium to a final concentration of 5 mM. Fluorescence changes were monitored after the serial additions of succinate and antimycin A in curves 1 and 2 and in curve 3, after the addition of succinate, an aliquot of $MgCl_2$ (for a final concentration of 5 mM), and antimycin A. The potential changes were calculated using the calibration curve obtained in the absence of Mg^{2+} (see text). The succinate-induced potential change for curve 1, calculated using the Mg calibration curve, was −353 mV.

in the case of ANS and MI, the addition of Mg^{2+} to energized suspensions did result in the expected increase in fluorescence). In the presence of antimycin A, the fluorescence was about the same as when Mg^{2+} was present from the beginning. Similarly, the addition of Mg^{2+} to suspensions blocked with antimycin A also returned the fluorescence to this level (not shown). Hence, the effect of Mg^{2+} appears to be only on nonenergized mitochondria and is not related to metabolism. Higher apparent potentials also can be calculated by using the apparent diffusion potentials in the presence of Mg^{2+} on the assumption that only K^+ contributes to the potential as previously done (Walsh Kinnally and Tedeschi, 1976) and as shown in Table IV, experiment 2 and 3. Compare these values with experiment 4 where the Mg^{2+} calibration curve was not used.

The effect of Mg^{2+} on the relationship between fluorescence and K^+ concentration is shown in Figure 10. The kinetics of the fluorescence changes (part A) and the change in the dependence of the fluorescence on the external K^+ concentration in the presence of Mg^{2+} (Part B) suggest that the mitochondrial membrane is permeable to Mg^{2+}. Mg^{2+} appears to affect the diffusion potential as one could expect when additional mobile ions are introduced into the system (as predictable, for example, by the Goldman equation [e.g., see Goldman (1943); Hodgkin and Katz (1949)]. In confirmation of these observations, kinetic experiments indicate that the fluorescence levels in the presence of Mg^{2+} (curve 2, Figure 10) and valinomycin were initially about the same as without Mg^{2+} (curve 4, Figure 10). However, when Mg^{2+} was present, a partial collapse rapidly occurred with time. This is shown in parts A (which represents typical tracings) and in B of the figure (compare curve 1 where the fluorescence was recorded 1 min later to curve 3 in the absence of Mg^{2+}). We have observed similar effects at high concentrations of Na^+ or choline. An alternative explanation is suggested by a report that Mg^{2+} decreases the permeability of mitochondria to K^+ in the presence of valinomycin (Ligeti and Fonýo, 1977), hence, decreasing the diffusion potential and invalidating the use of the Nernst equation. As shown in Table V, the presence of Mg^{2+} results in similar slope reductions with each of the dyes. However, with ANS and MI the effect of Mg^{2+} on the ΔE_a induced by metabolism is very small (e.g., see Table IV, experiment 4).

The results of these experiments show that the effect of Mg^{2+} on the ΔE_a in mitochondria is not related to metabolism.

Significance of the Metabolically Induced Potential Changes. The present study cannot exclude the possibility that in some experiments relatively low potential changes are generated by metabolism. However, it is entirely possible that these represent independent events unrelated to membrane potential. For example, a proton pump could produce a re-

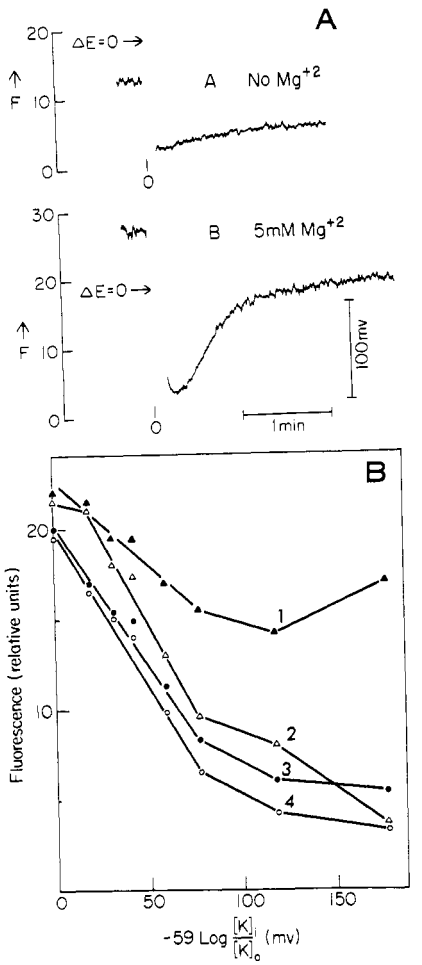

FIGURE 10: The effect of Mg^{2+} on the fluorescence changes of diS-C_3-(5) induced by K^+ diffusion potentials. The fluorescence corresponds to that of a suspension of rat liver mitochondria in 0.3 osM sucrose, 10 mM Tris, 0.5 mM NaP_i (pH 7.4) in the presence of diS-C_3-(5). (a) Typical kinetics in the presence of 0.1 mM KCl with and without 5 mM $MgCl_2$. (B) Initial fluorescence (about 5 s, after the addition of valinomycin) (open figures) and after 1 min (solid figures). Circles (curves 3 and 4) correspond to the fluorescence in the absence of Mg^{2+}, triangles (curve 1 and 2) correspond to results in the presence of Mg^{2+}.

TABLE V: The Effect of Mg^{+2} on the Calibration Curve Slope.[a]

expt	dye	slope + 0 mM $MgCl_2$, $\Delta F/\Delta E_a$	slope + 5 mM $MgCl_2$, $\Delta F/\Delta E_a$
1	diS-C_3-(5)	0.144	0.064
2	diS-C_3-(5)	0.138	0.055
	MI	0.071	0.029
	ANS	0.172	0.058
3	diS-C_3-(5)	0.140	0.065

[a] Rat liver mitochondria were suspended in 0.3 osM sucrose, 10 mM Tris, 0.5 mM NaP_i, pH 7.4, with and without 5 mM $MgCl_2$. The slope of the calibration curve, $\Delta F/\Delta E_a$, was determined as in Figure 3. See Table IV, experiment 4, for the metabolically induced apparent potential changes observed during experiment 2 of this table.

distribution of ionic species (such as dyes). Other studies with ANS have presented evidence for independent events playing such a role in fluorescence levels (e.g., Ferguson et al., 1976; Aiuchi et al., 1977).

Conclusion

In summary, we have used a total of four electrofluorimetric dyes to estimate the membrane potential change induced by energization in two different mitochondrial preparations (i.e., mouse and rat liver). In one technique, fluorescence changes were correlated to membrane potential variations using microelectrodes and giant mitochondria. In another technique, the changes in fluorescence were correlated to apparent K^+ diffusion potentials. The data obtained are consistent with the notion that the membrane potential changes are too low to play a significant role in oxidative phosphorylation. These data are consistent with the microelectrode work of Tupper et al. (1969) and Maloff et al. (1977, 1978).

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References

Aiuchi, T., Kamo, N., Kurihara, K., and Kobatake, Y. (1977), *Biochemistry 16,* 1626.

Åkerman, K. E. O., and Wikström, M. K. F. (1976), *FEBS Lett. 68,* 191.

Amoore, J. E., and Bartley, W. (1958), *Biochem. J. 69,* 223.

Azzi, A. (1975), *Q. Rev. Biophys. 8,* 237.

Azzi, A., Gherardini, P., and Santato, M. (1971), *J. Biol. Chem. 246,* 2035.

Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kulsens, V. V., Levitsky, D. O., Liberman, E. A., Severina, I. I., and Skulachev, V. P. (1970), *Biochim. Biophys. Acta 216,* 13.

Bakker, E., and Van Dam, K. (1974), *Biochim. Biophys. Acta 339,* 157.

Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E., and Salmon, A. G. (1971), *Eur. J. Biochem. 20,* 494.

Brockhardt, G. (1977), *Biochim. Biophys. Acta 468,* 227.

Cohen, L., Salzberg, B., Davila, H., Ross, W., Landowne, D., Waggoner, A., and Wang, C. H. (1974), *J. Membr. Biol. 19,* 1.

Colonna, R., Massari, S., and Azzone, G. F. (1973), *Eur. J. Biochem. 34,* 577.

Conti, F., and Malerba, F. (1972) *Biophysics 8,* 326.

Davila, H., Cohen, L., Salzberg, B., and Chirvastav, B. (1974), *J. Membr. Biol. 15,* 29.

Estabrook, R. W. (1967), *Methods Enzymol. 10,* 41.

Ferguson, S. J., Lloyd, W. J., and Radda, G. K. (1976), *Biochim. Biophys. Acta 423,* 174.

Goldman, D. E. (1943), *J. Gen. Physiol. 27,* 37.

Grinius, L., and Bazenaite, J. (1976), *FEBS Lett. 62,* 186.

Grinius, L. L., Jasaitis, A. A., Kadziauskas, Y. P., Liberman, E. A., Skulachev, V. P., Topall, V. P., Tsofina, L. M., and Vladimirova, M. A. (1970), *Biochim. Biophys. Acta 216,* 1.

Grinius, L. L., Guds, T. I., and Skulachev, V. P. (1971), *J. Bioenerg. 2,* 101.

Griniuviene, B., Dzheia, P., and Grinius, L. (1975), *Biochem. Biophys. Res. Commun. 64,* 790.

Hodgkin, A., and Katz, B. (1949), *J. Physiol. 108,* 37.

Hodgkin, A., and Keynes, R. (1955), *J. Physiol. 128,* 61.

Hoffman, J. F., and Laris, P. C. (1974), *J. Physiol. (London) 239,* 519.

Hurst, R. O. (1964), *Can. J. Biochem. 42,* 287.

Laris, P. C. (1977), *Biochim. Biophys. Acta 459,* 110.

Laris, P. C., and Hoffman, J. F. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 271.

Laris, P. C., and Pershadsingh, H. A. (1974), *Biochem. Biophys. Res. Commun. 57,* 620.

Laris, P. C., Bahr, D. P., and Chaffee, R. R. J. (1975), *Biochim. Biophys. Acta 376,* 415.

Ligeti, E., and Fonýo, A. (1977), *FEBS Lett. 79,* 33.
Maloff, B. L., Scordilis, S. P., and Tedeschi, H. (1977), *Science 195,* 898.
Maloff, B. L., Scordilis, S. P., Reynolds, C., and Tedeschi, H. (1978a), *J. Cell Biol. 78,* 199.
Maloff, B. L., Scordilis, S. P., and Tedeschi, H. (1978b), *J. Cell Biol. 78,* 214.
Massari, S., Balboni, E., and Azzone, G. F. (1972), *Biochem. Biophys. Acta 283,* 16.
Mitchell, P. (1966a), *Biol. Rev. 41,* 445.
Mitchell, P. (1966b), Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Res. Ltd., Bodmin, Cornwall, England.
Mitchell, P. (1967), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26,* 1370.
Mitchell, P. (1968), Chemiosmotic Coupling and Energy Transduction, Glynn Res. Ltd., Bodmin, Cornwall, England.
Mitchell, P., and Moyle, J. (1969), *Eur. J. Biochem. 7,* 471.
Nichols, D. G. (1974), *Eur. J. Biochem. 50,* 305.
Rottenberg, H. (1973), *J. Membr. Biol. 11,* 117.
Rottenberg, H., and Scarpa, A. (1974), *Biochemistry 13,* 4811.
Sackman, E., and Träuble, N. (1972), *J. Am. Chem. Soc. 94,* 4482.
Scordilis, S. P., Tedeschi, H., and Edwards, C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1325.
Sekiya, T. (1962), *Gann 53,* 41.
Sims, P. Y., Waggoner, A. S., Wang, C. H., and Hoffman, J. F. (1974), *Biochemistry 13,* 3315.
Skulachev, V. P. (1971), *Curr. Top. Bioenerg. 4,* 127.
Suchy, J., and Cooper, C. (1974), *Exp. Cell Res. 88,* 198.
Tedeschi, H. (1959), *J. Cell Biol. 6,* 241.
Tedeschi, H. (1971), *Curr. Top. Membr. Transp. 2,* 207.
Tedeschi, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 583.
Tedeschi, H. (1975), *FEBS Lett. 59,* 1.
Tupper, J. T., and Tedeschi, H. (1969a), *Science 166,* 1539.
Tupper, J., and Tedeschi, H. (1969b), *Proc. Natl. Acad. Sci. U.S.A. 63,* 370.
Tupper, J., and Tedeschi, H. (1969c), *Proc. Natl. Acad. Sci. U.S.A. 63,* 713.
Waggoner, A. (1976), *J. Membr. Biol. 27,* 317.
Walsh Kinnally, K., and Tedeschi, H. (1976), *FEBS Lett. 32,* 271.
Walsh Kinnally, K., and Tedeschi, H. (1978), *Biochim. Biophys. Acta* (in press).