

Measurements of Membrane Potentials in Plant Mitochondria with the Safranin Method¹

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ABSTRACT

The positively charged dye, safranin, has been used as an indicator of membrane potentials in mung bean (*Phaseolus aureus*) and Voodoo lily (*Sauromatum guttatum*) mitochondria under a variety of metabolic conditions. The spectral response of safranin has been calibrated with respect to a K⁺ diffusion potential and was found to be linearly related to the developed potential within the range of 50 to 160 millivolts. Both respiration and ATP hydrolysis gave rise to a membrane potential of approximately 135 millivolts. Respiratory inhibitors such as cyanide and antimycin depolarized the potential, whereas rotenone has little effect. No potentials were developed during NADH supported cyanide insensitive respiration. It is concluded that safranin may be a useful spectrophotometric probe of the mitochondrial membrane potential.

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria were isolated from etiolated mung bean hypocotyls (*Phaseolus aureus*) or the spadices of Voodoo lily (*Sauromatum guttatum*) by the general method of Bonner (5), with adaptations as previously described (9).

Oxygen Consumption. Mitochondrial respiration was measured polarographically in 2 ml of reaction medium containing 0.3 M mannitol, 5 mM MgCl₂, 10 mM KCl, and 10 mM K-phosphate (pH 7.2) (unless otherwise indicated) using a Rank O₂ electrode (Rank Bros., Cambridge, U.K.) at 25°C.

Spectrophotometric Techniques. A Johnson Research Foundation dual wavelength spectrophotometer was used to monitor changes in the absorption of safranin 0 using the wavelength pair 511 to 533 nm. Safranin 0 was purchased from Sigma, and used without further purification by addition of small aliquots of 2 mM aqueous solution of the dye to give a final concentration of 16 μM or approximately 25 nmol/mg protein. All experiments were performed with glass cuvettes of 1 cm light path, at room temperature, in 2.5 ml medium containing 0.3 M mannitol, 5 mM MgCl₂, and 20 mM Hepes (pH 7.2). Further additions were made as indicated in the figure legends.

Assays and Reagents. Protein was determined by the method of Lowry *et al.* (11) using crystalline BSA as a standard.

Valinomycin, oligomycin, rotenone, and FCCP³ were purchased from Sigma and dissolved in absolute ethanol. Nigericin was a gift from Dr. J. C. Smith (Johnson Research Foundation). All other reagents were of the highest purity commercially available.

RESULTS

Probe Calibration. Figure 1A shows that the spectral response of safranin to an induced membrane potential can be calibrated against a K⁺ diffusion potential and a dye:protein ratio of 25 nmol/mg protein. A K⁺ diffusion potential was induced by suspending de-energized mitochondria in a K⁺-free medium (0.3 M mannitol and 20 mM Hepes [pH 7.2]). The addition of 0.1 μg/ml of valinomycin induced a K⁺ efflux from the mitochondria and resulted in an increase in the absorbance of the dye at the respective wavelength pair. This indicates a stacking of the dye molecules (1, 3, 7, 23) and corresponds to the establishment of a membrane potential (3, 22). At this dye:protein ratio, the time for the response of the safranin probe to reach a steady state was less than 10 s and resulted in much larger spectral changes than observed by Åkerman and Wikström (3). The extent of the membrane potential was measured from the difference in absorption after the addition of valinomycin compared to that following an addition of 1 μM FCCP. The external K⁺ concentration was varied within the range 0 to 10 mM and the valinomycin induced K⁺ diffusion potential calculated according to the Nernst equation on the basis of an intra-mitochondrial K⁺ concentration of 125

It is generally accepted that in the energized state, mitochondria generate a large membrane potential ($\Delta\Psi$) (6, 8). Measurements of such membrane potentials are based mainly on the distribution of permeant ions and are thought to provide reliable indications of $\Delta\Psi$ under steady-state conditions (19). Recently, however, attention has been focused on the use of extrinsic probes as indicators of membrane potential (4) in both cells (2) and organelles (1, 3). The use of such probes has several advantages over ion distribution methods. In particular, experimental procedures are simple, information is obtained rapidly and continuously, and, because such compounds may be used in micromolar concentrations, they cause minimal interference to the system under investigation. In the present study, we have used the positively charged dye, Safranin 0 as an indicator of membrane potential in intact plant mitochondria and investigated its response under a variety of metabolic conditions. It was found that the extent of the spectral shift in safranin absorbance was linearly related to the developed membrane potential and could be calibrated with reference to a K⁺-diffusion potential. The linearity of the shift was, however, affected by variations in dye:protein ratios. The present study indicates a qualitative correlation between $\Delta\Psi$ and safranin response and suggests that the probe may be a useful indicator of changes in membrane potential in intact plant mitochondria.

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³ Abbreviations: FCCP, *p*-trifluoromethoxy carbonylcyanide phenylhydrazine; SHAM, salicylhydroxamic acid.

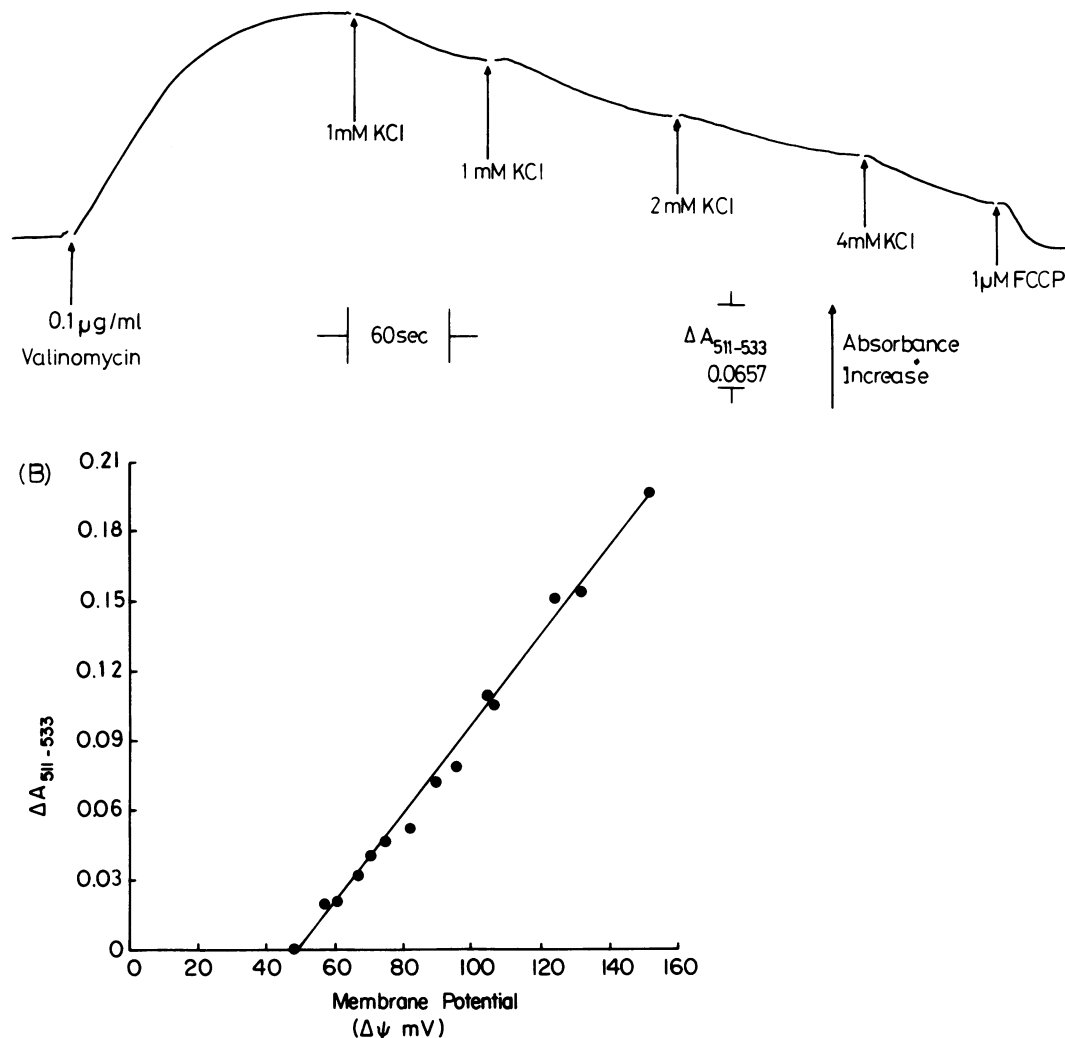


FIG. 1. Absorbance changes in safranin induced by a potassium diffusion potential. A, Mung bean mitochondria (0.64 mg/ml), were suspended in 0.3 M mannitol, 5 mM MgCl₂, and 20 mM Hepes (pH 7.2) containing 16 µM safranin and KCl concentrations as indicated. Further additions were made as indicated in the figure. B, Calibration of the absorbance changes with a K⁺ diffusion potential. Conditions for the potassium potential as described in A. The potential was calculated according to the Nernst equation where

$$\Delta\Psi = 60 \log \frac{[K^+]_{in}}{[K^+]_{out}} \quad \text{and} \quad [K^+]_{in}$$

was equal to 125 mM and [K⁺]_{out} equal to added potassium. The drawn straight line is the best fit to the K⁺ diffusion potential data and is based on a series of four experiments.

mm (measured by atomic absorption spectroscopy). Figure 1B indicates that, at a dye:protein ratio of 25, the extent of the spectral shift was linear, when compared to a K⁺ diffusion potential within the range 50 to 160 mv (interior negative). At potentials above 160 mv, however, the relationship became nonlinear. In agreement with Zanotti and Azzone (23), the deviation from linearity was found to be a function of the dye:protein ratio, low ratios (<10) resulting in large deviations at high potentials. Linearity of the calibration curve was also found to be a function of the dye concentration at a constant dye:protein ratio. High dye concentrations (>30 µM) again resulted in larger deviations from linearity in the high potential region, with 50-100 µM dye concentrations also tending to decrease respiratory control ratios.

Figure 1B shows that the intercept occurs at a potential of approximately 50 mv. This value, corresponding to an energy independent Donnan potential, is in good agreement with our previous data on uncoupled mitochondria using ion-distribution techniques (14).

Probe Response during Substrate Oxidation. The responses of

safranin observed during various metabolic conditions are summarized in Figure 2. The addition of a respiratory substrate such as 1 mM NADH results in a rapid spectral shift, indicating the formation of a membrane potential. On the basis of the calibration plot this is equivalent to a potential of approximately 134 mv. A similar magnitude of shift was observed irrespective of whether the substrate was succinate, malate, or ascorbate (plus, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine). The further addition of Pi to a phosphate-free medium induced a rise in the potential of approximately 10 mv presumably due to the partial interconversion of ΔpH into ΔΨ (see 15). This was confirmed by the finding that *N*-ethylmaleimide, an inhibitor of the phosphate translocator (8) abolished such changes (not shown). The transition to state 3 resulted in a rapid fall (5-10 mv) in the membrane potential which was subsequently reestablished at its initial value on transition to state 4. Such cyclic responses, abolished by 1 µg/ml oligomycin are consistent with the idea that ATP synthesis puts an energetic demand on the protonmotive force. The addition of 1 µM FCCP rapidly collapsed the remaining potential. Figure 2B shows that,

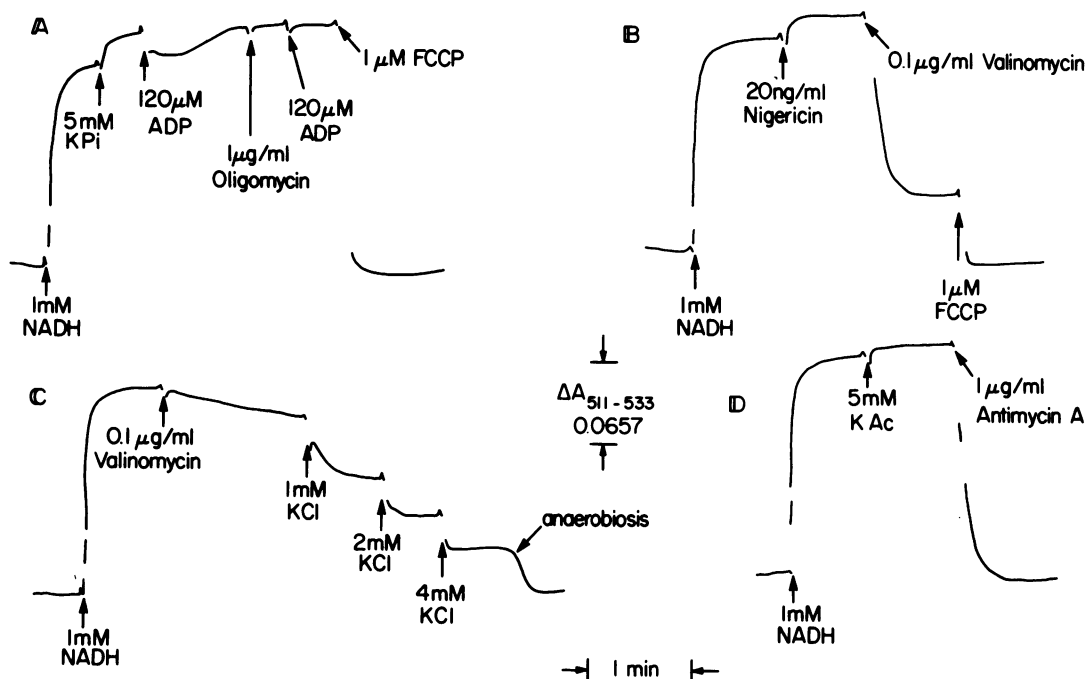


FIG. 2. Probe response during substrate oxidation. Approximately 0.6 mg/ml of mung bean mitochondria were suspended in 0.3 M mannitol, 5 mM $MgCl_2$ and 20 mM Hepes (pH 7.2). A, Effect of ADP. B, Addition of nigericin and valinomycin in the presence of 10 mM KCl. C, Effect of valinomycin and KCl. D, Effect of acetate and antimycin.

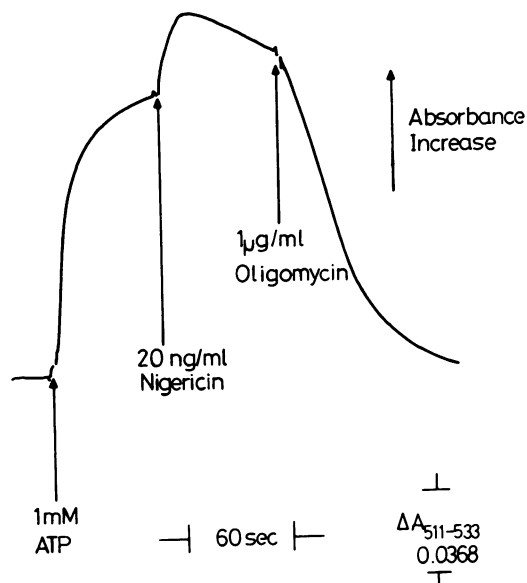


FIG. 3. ATP induced spectral shifts. Conditions as described in Figure 2.

in the presence of 10 mM KCl, the addition of 20 ng/ml nigericin resulted in an approximate 10 mv increase in $\Delta\Psi$ in agreement with the suggestion that this ionophore partially collapses the pH gradient, converting it to a membrane potential. This is substantiated by the finding that the subsequent addition of valinomycin rapidly collapses the membrane potential, presumably due to the electrogenic influx of K^+ . It is interesting to note that the collapse is not complete, requiring the further addition of 1 μM FCCP. Similar observations have been made using ion distribution techniques (14 and *cf.* Ref. 21).

In the absence of added K^+ , valinomycin has little effect on respiring mitochondria (Fig. 2C), confirming that it does not increase H^+ conductance (15). Subsequent addition of K^+ de-

creases the absorbance, due to the electrophoretic movement of K^+ mediated by valinomycin. Under these conditions, respiration is uncoupled and the reaction rapidly goes anaerobic.

The addition of an electroneutral translocatable species such as acetate also results in the partial interconversion of ΔpH to $\Delta\Psi$ (Fig. 2D), although the extent of the change is not as great (2–4 mv) as observed with phosphate or nigericin. Figure 2D also demonstrates that respiratory inhibitors such as antimycin A rapidly collapse an existing potential.

Effect of ATP. The addition of 1 mM ATP to nonrespiring mitochondria in the absence of Pi or ADP induces a spectral shift of similar magnitude to that observed with NADH (Fig. 3). Nigericin results in an increase in membrane potential suggesting that the ΔpH component is large under these conditions. The observed response is sensitive to oligomycin, as would be expected from an inhibitor of the ATPase proton pump. Interestingly, both the magnitude of the ATP-generated potential and the cyclic responses to ADP were not dependent upon the concentration of nucleotide but upon the ratio of $ATP:ADP \times Pi$. A detailed analysis of this behavior has been presented elsewhere (13).

Effect of Rotenone. Figure 4 shows the effect of rotenone on mitochondria that were oxidizing malate as substrate. Several points are relevant from this experiment. First, the addition of a second aliquot of ADP, following an initial state 3–4 transition, induces both a faster and slightly larger absorbance change upon subsequent ADP addition. This observation may be related to the phenomenon of conditioning—a situation characterized by increasing state 3 rates and respiratory control following an initial state 3–4 transition (17). It was also observed that following a pulse of ADP, the subsequent state 4 potential is also slightly higher than its initial value (Fig. 4). Upon addition of 4 μM rotenone, in the absence of ADP, a slight fall in potential was observed, which quickly returns to a state 4 value. It is well established that plant mitochondria are relatively insensitive to Site 1 inhibitors (16), and this result confirms that a membrane potential may still be maintained in the presence of rotenone. A low affinity for binding of rotenone by Site 1 or the presence of a rotenone-insensitive bypass of this site, as suggested by Wiskich

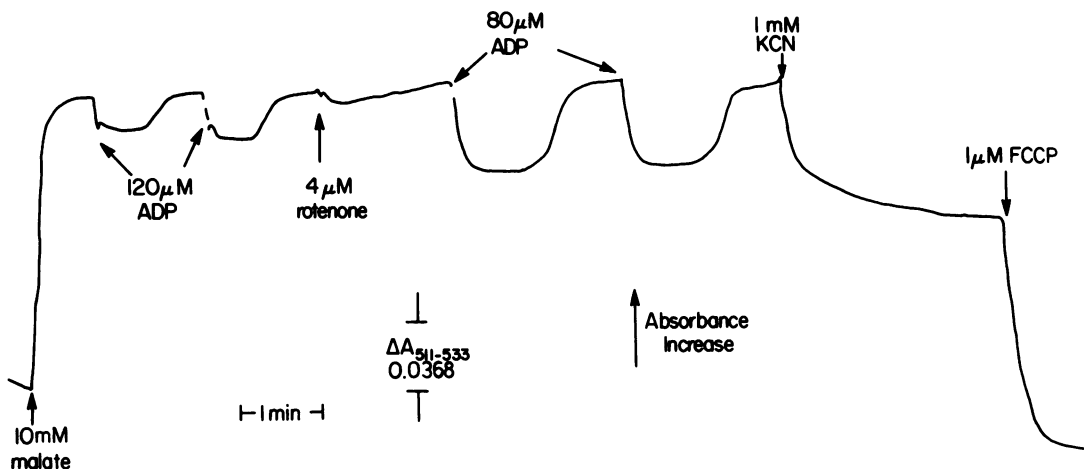


FIG. 4. Effect of rotenone during malate oxidation. Conditions as described in Figure 2 with further additions as indicated, except for the inclusion of 5 mM phosphate.

and Day (22), may be used to explain such data. Whatever the answer, the membrane potential is presumably maintained by Sites 2 and 3, and it is of interest to note that the addition of ADP in the presence of rotenone (Fig. 4) results in a larger fall in potential (from 131–105 mv), for a smaller aliquot of ADP (80 μ M as opposed to 120 μ M). This is consistent with O_2 electrode recordings which suggest that in the presence of rotenone ADP:O ratios are reduced from 3 to 2 (22). Addition of 1 mM cyanide caused a collapse of the measured potential and no subsequent cyclic responses to ADP could be obtained. Under these conditions, however, O_2 consumption is not completely inhibited due to the functioning of a cyanide insensitive alternative oxidase, suggesting that malate oxidation can occur in a rotenone and cyanide insensitive fashion (20). Complete depolarization of the remaining potential was only observed upon the further addition of 1 μ M FCCP. No such decrease was observed if SHAM was used instead of the uncoupling agent, suggesting that the observed potential is not maintained by the alternative oxidase.

Safranin Response during Cyanide-Insensitive Respiration. In order to confirm this result and our previous observations, using other techniques (12, 14), on the lack of H^+ efflux via the alternative oxidase, the response of safranin during substrate oxidation in highly cyanide-insensitive mitochondria was examined. As previously observed for *Arum lily* mitochondria (14), the oxidation of exogenous NADH by *S. guttatum* mitochondria also generates a membrane potential (Fig. 5A). This is unaffected by 1 mM SHAM but is rapidly collapsed by cyanide. If cyanide is added prior to the substrate (Fig. 5B) no absorbance changes are observed. In order to determine the lack of a potential is not due to any uncoupling properties of the inhibitor, the effect of cyanide on malate oxidation was investigated. From Figure 5F, it can be seen that 1 mM cyanide has relatively little effect upon the membrane potential, suggesting that it does not increase H^+ conductance under these conditions. Presumably the membrane potential observed in the presence of cyanide is maintained by proton pumping at Site 1. This is supported by the observation that the cyanide-insensitive malate induced potential is collapsed by 4 μ M rotenone (not shown), and in the absence of rotenone, but in the presence of cyanide, is sensitive to SHAM.

Mitochondria from *S. guttatum* rapidly oxidize menadiol and duroquinol in a cyanide insensitive, SHAM sensitive fashion displaying respiratory rates of 1,149 and 244 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, respectively. Menadiol oxidation was 100% cyanide insensitive, whereas with duroquinol, the rate was 25% cyanide-sensitive, and only duroquinol appeared to generate a membrane potential (Fig. 5C). Why menadiol is an ineffective donor of reducing equivalents to the Cyt-chain remains unclear, at present,

but we have previously observed (18) that this quinol can donate to a number of points within the respiratory chain, in a manner which seems to bypass the H^+ generating loops. Nevertheless, the experiments depicted in Figure 5, C through E clearly show that, in the presence of cyanide, quinol oxidation is not accompanied by the generation of a membrane potential.

DISCUSSION

The results of the present study show that the permeant cation dye, safranin, can be used to monitor membrane potentials in intact plant mitochondria. The calibration plot, which is linear up to 150 mv when compared to a K^+ diffusion potential, indicates that this method could prove useful for quantitative estimations of membrane potentials under different metabolic conditions. Indeed, there is a remarkable qualitative and quantitative similarity between the membrane potentials measured using safranin and those based on ion distribution (14), which confirms that the energized state is associated with a membrane potential of approximately 135 mv (positive extramitochondrially). Caution does, however, need to be exercised in the use of safranin as a quantitative measure of membrane potentials inasmuch as it is very dependent on the dye:protein ratio. At a constant dye concentration, high levels of protein lead to lowered absorbance changes and greater deviation from the calibration curve, particularly in the high potential region. According to Zanotti and Azzone (23), this is probably due to the fact that high protein levels result in an increased probability that dye binding occurs to sites where the nearby site is not occupied by another dye molecule. In this case, the dye remains in a monomeric form and the dye uptake is not accompanied by changes in absorbance. It is apparent, however, that at the dye:protein ratio used in this study not only are large absorbance changes observed, but the values measured are in agreement with ^{86}Rb distribution (14, 15). The advantage of extrinsic probes over ion distribution techniques are rapidity and their ability to enable continuous monitoring of membrane potential changes in response to various applied conditions. That safranin monitors energy-dependent membrane potential changes is demonstrated by the very fast depolarization caused by inhibitor, or ionophore addition. Reagents such as nigericin, acetate, or phosphate, that perturb ΔpH gradients result in an increase in absorbance indicating that ΔpH and $\Delta\Psi$ are interchangeable for retention of a membrane potential. Complete depolarization, either upon addition of inhibitor or upon anaerobiosis is a further indication that absorbance changes are not caused by nonspecific binding. The inhibitor and anaerobiosis experiments also show that in the absence of O_2 or reducing equivalents the membrane potential rapidly depolarizes presumably via the natural conduct-

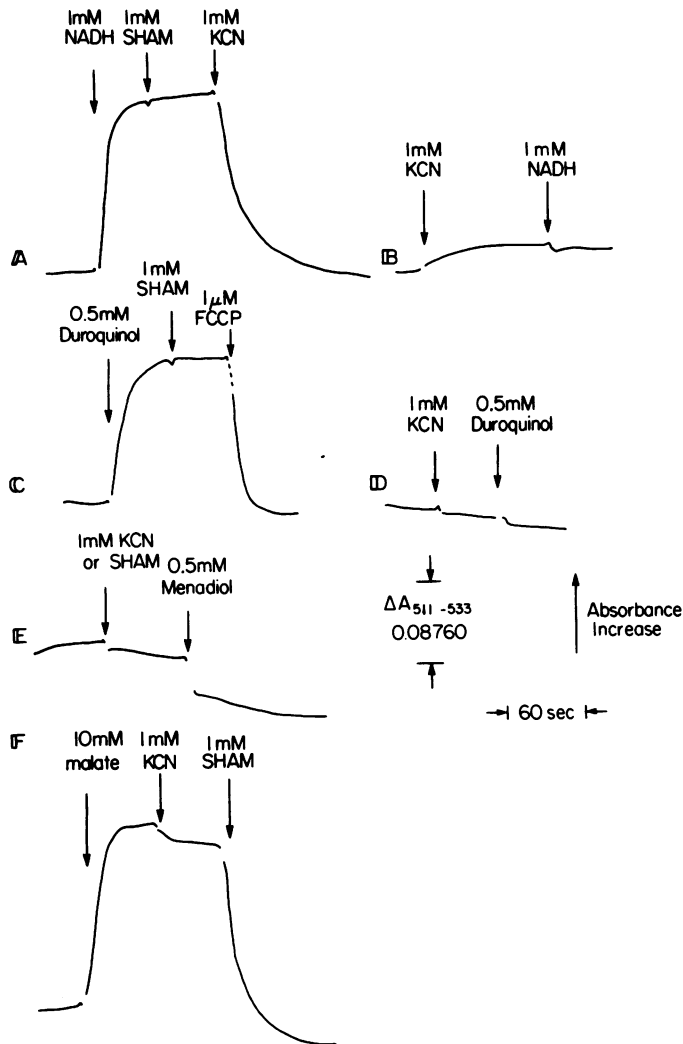


FIG. 5. Effect of cyanide and SHAM on the spectral shift of safranin during substrate oxidation in *S. guttatum* mitochondria. Approx. 0.3 mg/ml of mitochondria were suspended in the mannitol medium containing $22.5 \mu\text{M}$ safranin. A, Effect of SHAM during NADH oxidation. B, Effect of cyanide. C, Effect of SHAM during duroquinol oxidation. D, Effect of cyanide during duroquinol oxidation. E, Effect of cyanide and SHAM during menadiol oxidation. F, Effect of cyanide and SHAM during malate oxidation.

ance (due to H^+ leaks and ancillary ion movements, *i.e.* K^+ - H^+ antiporter) of the membrane. All of these studies would therefore tend to suggest that the technique is suitable for monitoring membrane potentials and may be particularly useful in detecting transient changes. For instance, the addition of small aliquots of ADP results in a decrease in membrane potential which returns to its initial value upon ADP exhaustion. The observation that the membrane potential is higher following an initial ADP pulse suggests that under these conditions the membrane becomes less leaky to protons and, upon subsequent addition of ADP, the decrease in membrane potential is not only quicker but the return to State 4 more abrupt. This correlates with O_2 electrode studies which indicate an increase in respiratory control following a State 3/4 transition (17). The safranin technique has shown to be useful in an investigation of this phenomenon, and we have consistently observed that in preparations which oxidize malate poorly, ADP plays a role other than as a phosphate acceptor. In these situations, its addition results in a rapid increase in membrane potential which is oligomycin and atractyloside insensitive (A. L. Moore, unpublished results). These data are reminiscent of

the potentiating effect of ADP in uncoupling oxidative phosphorylation as observed by Latic (10), and a fuller description of these observations will be presented in detail elsewhere.

The safranin method also demonstrates that, unlike the addition of cyanide or antimycin A, rotenone has relatively little effect upon the membrane potential. Because this inhibitor blocks proton-pumping in Site 1, it suggests that the potential is maintained by Sites 2 and 3. Whether this is due to the NADH being oxidized by the externally located dehydrogenase (16) or by a pathway that bypasses Site 1 is uncertain. It appears that it is not via a pathway essentially linked to the alternative oxidase as suggested by Rustin *et al.* (20), inasmuch as the potential generated, and maintained, is comparable in the presence or absence of rotenone, and in rotenone-treated mitochondria is depolarized only by cyanide. Furthermore, the large changes in potential observed during a State 3/4 transition, in the presence of rotenone, compared with those observed with NADH (*cf.* Fig. 2A with Fig. 4) tend to discount a role of external NADH dehydrogenase in rotenone insensitive malate oxidation. Obviously, further experiments are required to clarify this point.

The experiments depicted in Figure 5 quite categorically indicate that there is no energy conservation by the alternative oxidase, there being no membrane potential generated during cyanide-insensitive respiration. This is the case irrespective of whether the substrate is NADH, a quinone or malate in the presence of rotenone. Similar results are achieved with mitochondria isolated from both highly cyanide insensitive tissues, such as *S. guttatum* spadix, or from those with low alternative oxidase activity (*i.e.* mung bean hypocotyls). Similarly, it is not the concentration of cyanide that is depolarizing the potential, by uncoupling, inasmuch as a membrane potential is maintained in cyanide-inhibited mitochondria when rotenone is omitted (Fig. 5F). Under these conditions, SHAM promotes a rapid depolarization of the membrane potential whereas, in the absence of cyanide, it has no depolarizing effect. This is contrary to the conclusion of Wilson (21) who suggested that energy can be conserved by the activity of the alternative oxidase. His proposal, that SHAM does not cause membrane depolarization (as observed in Fig. 5, A and C) and is merely a reflection of the ability of ATP to support the membrane potential and not to the lack of energy conservation, it is clearly not the case, inasmuch as ATP hydrolysis is not sensitive to cyanide. A potential should, therefore, still be maintained in cyanide and SHAM-treated mitochondria, if it is maintained by ATP hydrolysis. Figure 5A demonstrates a depolarization under these conditions. It is conceivable that the observations reported by Wilson (21) may merely be a reflection of the low proton conductance of the membrane, preventing total dissipation of the membrane potential. This situation is only realized upon subsequent addition of an uncoupler such as FCCP which increases proton permeability (see Fig. 8, ref. 21).

The present work supports our previous conclusion, from ion distribution data (12, 14), that a phosphorylation site is not associated with the cyanide-insensitive alternative oxidase. Results also indicate that monitoring of changes in safranin absorbance can be used, both as a qualitative and as a quantitative monitor of membrane potential in plant mitochondria, particularly in the detection and measurement of transient changes.

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