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MEASUREMENTS OF MEMBRANE POTENTIALS IN *ESCHERICHIA COLI* K-12 INNER MEMBRANE VESICLES WITH THE SAFRANINE METHOD

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Summary

The use of safranine, a positively-charged dye, as a probe for the determination of membrane potentials in *Escherichia coli* vesicles has been studied.

1. Shifts in the spectrum of safranine were observed during induction of potassium ion diffusion potentials with valinomycin or during oxidation of formate by vesicles prepared from cells of *E. coli* K-12 or ML 308-225 subjected to anaerobic growth with nitrate. The extent of the valinomycin-dependent spectral change correlated linearly with the magnitude of the K^+ equilibrium potential, as calculated from the Nernst equation, from 50 to 160 mV (interior negative). The formate-induced changes could also be calibrated by increasing the concentration of potassium in the presence of valinomycin, after the formation of formate-dependent responses. In this case, results identical to those obtained with the first method were obtained.

2. O_2 or nitrate-dependent oxidation of formate resulted in a membrane potential of the order of 170 mV. The oxidation of ascorbate-reduced *N*-methylphenazonium methosulphate resulted in a potential of similar magnitude, but anaerobically with nitrate only a small but definite potential was formed.

3. The water-soluble quinones, duroquinone and menadione, could produce membrane potentials when used in their oxidized or reduced forms in the presence of formate or nitrate (or oxygen). 2-Hydroxy-1,4-naphthoquinone was not only ineffective but was found to be inhibitory.

4. *N,N'*-dicyclohexylcarbodiimide at suitable concentrations increased the rate of formation and the extent of membrane potentials induced by respiration or by artificial means.

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Introduction

The chemiosmotic hypothesis predicts the formation of a membrane potential and proton gradient upon energization of the bacterial inner membrane [1—3] due to a vectorial translocation of H^+ coupled to the flow of electrons. The membrane potential and proton gradient thus formed control the cytosolic environment of the cell by providing energy for the translocation of substrates and inorganic ions [4] as well as for oxidative phosphorylation [5]. Measurements of the membrane potential have, in the past, been made with ion-distribution techniques [6—11], but these methods are rather slow for detecting fast changes in response to different perturbations. It has recently been shown that the organic cationic dye, safranine, responds with large spectral changes upon energization of the mitochondrial membrane [12] or induction of membrane potentials across the membrane of bacterial vesicles [13], liposomes [14] and mitochondria [15]. In mitochondria, the response of the dye as a function of the magnitude of K^+ or H^+ diffusion potentials is linear up to approx. 200 mV [15]. Thus, fairly good estimates of membrane potentials in this fraction can be obtained by measuring the spectral changes of the dye under various conditions [15,16]. The aim of the present study was to test safranine as a possible probe for membrane potentials in bacterial vesicles.

Materials and Methods

The strains and conditions of cultivation. The *Escherichia coli* strains, ML 308-225 and K-12 Hfr Cavalli K-10 (Coli Genetic Stock Center No. 5023 [17]), were used. The latter strain is devoid of one of the transport systems catalyzing the uptake of inorganic phosphate [18]. Membrane vesicles prepared from cells of this strain do not exhibit phosphate exchange or respiration-dependent phosphate uptake [19]. The conditions of growth and the minimal medium used have been described [20,21]. The carbon source for the ML strain was glucose (0.4%) and for the K-12 strain, glycerol (0.5%). Nitrate was added in both cases as an electron acceptor for anaerobic growth.

The isolation of membrane vesicles. Cells were harvested and converted to spheroplasts as described previously [20,21] except that lysozyme (40 μg/ml) and 30% sucrose were used to obtain spheroplasts from the K-12 strain [22]. Membrane vesicles were prepared by osmotic lysis of the spheroplasts in a small volume of buffer (5—10 ml per g wet wt. of the original cell paste) composed of 50 mM potassium phosphate (pH 6.6) and 2 mM $MgSO_4$. After harvesting by centrifugation (30 000 × *g* for 1 h), the vesicles were resuspended at concentrations of 5—15 mg protein/ml in 50 mM potassium phosphate buffer (pH 6.6). In experiments where safranine responses were to be calibrated, the vesicles were prepared and resuspended in phosphate buffer (pH 6.6) made by mixing 100 mM KH_2PO_4 and 50 mM K_2HPO_4 to give 100 mM K^+. Small aliquots of concentrated preparations (26 mg protein/ml) and standard preparations were frozen in polypropylene vials in liquid N_2 and stored frozen under liquid N_2.

Measurements of membrane potential. The changes in the spectrum of safranine were measured in the phosphate buffer systems used for resuspension

of the preparations, as described above, except sodium replaced potassium and the reaction mixture contained $MgSO_4$, safranine and *N,N'*-dicyclohexylcarbodiimide as indicated in the legends to the figures. An Aminco DW-2 spectrophotometer was used for most of the experiments employing the wavelength pair 524 and 554 nm (see Fig. 2). In addition, a Beckman DU-equipped Gilford Model 2220 spectrophotometer, operated with a Servogor S compensating recorder, was used for measurements at a single wavelength of 524 nm.

Anaerobic conditions in the round-necked cuvette were obtained by bubbling moist N_2 through needles inserted through a rubber sleeve stopper. Additions of N_2-saturated solutions were made with Hamilton syringes and the contents were mixed by inverting the cuvette.

The absorbance changes were related to the valinomycin-induced potassium equilibrium potential given by the Nernst equation, $\Delta\psi = -60$ mV $\log(K_i^+/K_o^+)$, where K_i^+ and K_o^+ denote the potassium concentrations inside and outside of the vesicles, respectively.

Uptake experiments. The methods have been described previously [20,21]. L-[G-^{3}H]glutamic acid (specific activity, 28 Ci/mmol) was diluted to a specific activity of 2.8 Ci/mmol with unlabelled glutamic acid and used at a final concentration of 6.5 μM. The volume was 0.1 ml and the temperature 25 °C. After dilution and filtration, washed vesicles on Millipore cellulose nitrate filters (pore-size, 0.45 μm) were transferred to scintillation vials containing 10% H_2O in Insta-Gel®, mixed vigorously for 30 s in a Vortex mixer and counted for radioactivity.

Other methods. Protein was measured using the method of Lowry et al. [23] using bovine serum albumin, fraction V (Sigma) as standard. Duroquinone and menadione were reduced with $NaBH_4$ in N_2-saturated methanol under a stream of N_2. Unreacted reductant was removed with HCl [24]. The solutions were protected from air and light and used within 6 h after preparation. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and 1799 were kindly donated by Dr. P.G. Heytler (DuPont, Wilmington, DE). Antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, valinomycin and *N*-methylphenazonium methosulphate were obtained from Sigma Chemical Co., St. Louis, MO, safranine from Merck A.G., Darmstadt, F.R.G. Insta-Gel® was obtained from LKB-Wallac, Turku, Finland. L-[G-^{3}H]glutamic acid was obtained from the Radiochemical Centre, Amersham, U.K.

Results

Calibration of the safranine response. Fig. 1A shows that the induction of a K^+ diffusion potential with valinomycin across the membrane of bacterial vesicles suspended in the presence of safranine resulted in a decrease in the absorbance of the dye at the respective wavelength pair (524 and 554 nm). The response indicates a stacking of the dye molecules [12] and is similar to the changes which occur upon induction of membrane potentials in isolated mitochondria [15,16]. For calibration of the response, an aliquot of a concentrated suspension of vesicles was added to the medium lacking potassium to give a 500-fold K^+ concentration gradient. Valinomycin was then added

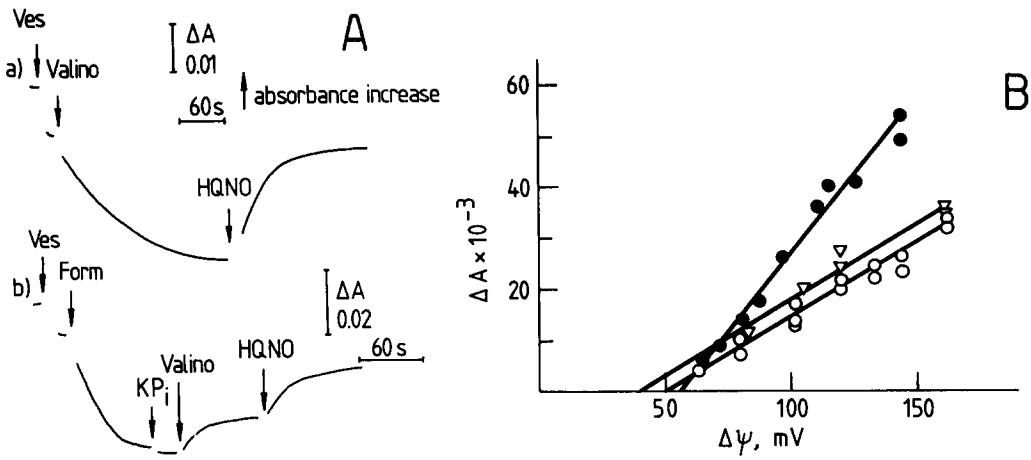

Fig. 1. Kinetics of safranine responses during calibration and calibration plot. (A) The cuvette contained (volume, 2.5 ml, room temperature): sodium phosphate (pH 6.6), prepared as described in Materials and Methods, 19 mM $MgSO_4$, 10 μM safranine and 80 μM *N,N'*-dicyclohexylcarbodiimide. Additions: trace a, concentrated vesicles of *Escherichia coli* K 12 (50 μg protein/ml) (Ves); valinomycin, 0.36 μM (Valino) and 20 μM 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). In trace b, additions as in trace a except sodium formate, 10 mM (Form) and potassium phosphate, 8 mM (KP_i), as shown. (B) The diffusion-potential-induced responses plotted (○, ●) are taken as absorbance differences from the level before the addition of valinomycin to the point of maximum change (compare trace a). The formate-induced responses (△) are taken from the level before the addition of formate to the level obtained after stabilization of the valinomycin-induced change (trace b). The solid lines represent least-squares fits to the experimental points.

and the change recorded (Fig. 1A, trace a). This amounts to a calculated potential of approx. 160 mV (interior negative). With an increase in the outside potassium concentration, a decrease in the response was observed which was linear until no response was obtained at approx. 50 mV (Fig. 1B). This value probably corresponds to an energy-independent Donnan potential as in the case with mitochondria (cf. Refs. 15, 25). By increasing the vesicle concentration 2-fold, the extent of the response was doubled as evident from the plot in Fig. 1B.

An addition of formate to the vesicle suspension caused a spectral change similar to that observed in the presence of valinomycin (Fig. 1A, trace b). Once formed, additions of valinomycin and potassium diminished the response in a manner which was linearly dependent on the potassium concentration gradient (Fig. 1B, triangles). Valinomycin alone had no significant effect on the formate response with the maximal potassium gradient (Fig. 1B). This indicates that the oxidation of formate produces a membrane potential of approx. 160 mV, corresponding to a value of 170 mV obtained by extrapolation from the calibration plot using diffusion potentials.

Fig. 1A also shows, surprisingly, that the electron transfer inhibitor, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, abolished responses to valinomycin or formate. Well-characterized uncouplers like carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (see Fig. 4) or 1799 were equally effective (not shown). Since this inhibitor specifically inhibits the vesicular formate-nitrate reductase at much lower concentrations ([26], Huttunen, T., unpublished results), the dissipation of an artificial potential might occur by uncoupling

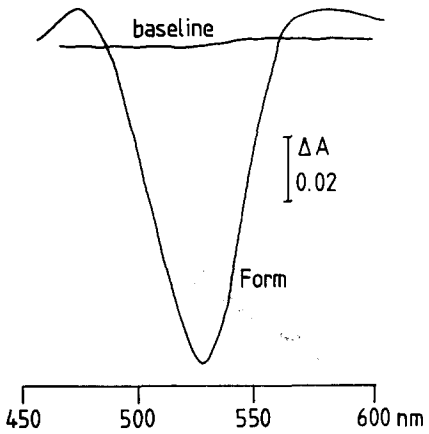

Fig. 2. Energization difference spectrum. The reaction mixture was as described in Fig. 1 except 50 mM sodium phosphate buffer, containing 0.2 mM dicyclohexylcarbodiimide and vesicles at a concentration of 0.15 mg protein/ml, were used. The suspension was divided into two cuvettes and the baseline recorded. Sodium formate (2.5 mM) was then added to one of the cuvettes and the spectrum recorded when no further change was obtained.

in the presence of an excess of the inhibitor (see Ref. 27). A further addition of 20 mM KCl had no significant effect on the signal (not shown).

With some preparations, a transient response was obtained already on addition of the vesicles. To avoid possible leakage and redistribution of potassium ions during this activity, such preparations were not used for calibrations presented here. Without this precaution, calibrations involving vesicles from the strains ML 308-225 or K-12 yielded almost identical results although the potential spans covered were not as extensive as that shown. The membrane potentials obtained with vesicles from the ML 308—225 strain during formate oxidation were always somewhat lower, of the order of 140 mV.

The energization difference spectrum. The difference spectrum of a large formate-induced safranine response is shown in Fig. 2. The spectral change, consisting of a decrease in the absorbance at 526 nm, is similar to the response which occurs upon induction of membrane potentials in liposomes [14] or in isolated mitochondria [15,16]. The observed change of approx. 0.1 A represents a substantial portion of the absolute absorbance of approx. 0.3 A of the safranine solution employed here. Control experiments (not shown) revealed no change in the features of the absolute spectrum of the safranine solution on addition of the vesicles, indicating that the dye is completely unstacked in de-energized conditions.

Glutamate uptake and safranine responses with water-soluble quinones as electron acceptor. Glutamate uptake is known to be coupled to the formation of membrane potentials during anaerobic electron flow in vesicles derived from *E. coli* ML 308-225 [9,26]. Fig. 3 shows the stimulation of anaerobic glutamate uptake by electron acceptors not characterized previously. Duroquinone and menadione stimulated the uptake less efficiently than nitrate and no uptake occurred with 2-hydroxy-1,4-naphthoquinone.

Safranine responses to these acceptors are compared in Fig. 4 in the absence

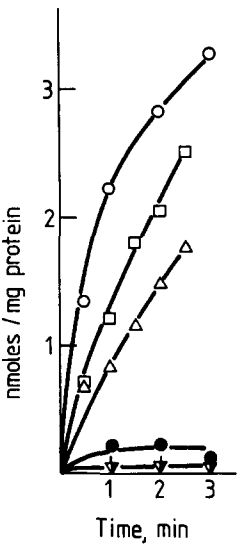

Fig. 3. Stimulation of the anaerobic uptake of L-glutamate by water-soluble quinones and nitrate. Vesicles of *Escherichia coli* ML 308-225 (0.25 mg protein/ml) were used in 50 mM potassium phosphate buffer, containing 10 mM $MgSO_4$, incubated under anaerobic conditions with formate (10 mM). The acceptors (nitrate, 10 mM and ethanolic solutions of the quinones, 0.6 mM) were added 30 s prior to the initiation of the uptake assays with 6.5 μM of tritiated L-glutamate. The concentration of ethanol was 1%. The reactions were terminated at the times indicated. ○———○, formate plus nitrate; □———□, formate plus menadione; △———△, formate plus duroquinone; ●———●, formate alone; ▽———▽, formate plus 2-hydroxy-1,4,-napthoquinone, or acceptors alone, or no addition.

(A) or presence (B) of *N,N'*-dicyclohexylcarbodiimide. Trace a in Fig. 4A shows the transient nature of the responses produced by menadione. Comparison of traces b and c with a shows that the rate of formation of the response was inhibited by 30% with 2-hydroxy-1,4-naphthoquinone and was about the same as that obtained with nitrate.

In the presence of *N,N'*-dicyclohexylcarbodiimide (Fig. 4B), fast responses were obtained with duroquinone and menadione but not with 2-hydroxy-1,4-naphthoquinone. Compared to trace a of Fig. 4A, the rate of formation of the response shown in trace b increased at least 5-fold (note the different time scale). The extents of the responses increased somewhat. Moreover, addition of nitrate (trace b) increased the extent of the menadione-dependent response only marginally, indicating that the differences between these acceptors evident in Fig. 4A might be insignificant in the presence of *N,N'*-dicyclohexylcarbodiimide. Higher concentrations of this compound significantly increased the rate of formation and extent of the responses, but these responses were not stable (not shown). Trace of Fig. 4B further shows the inhibitory effect of 2-hydroxy-1,4-naphthoquinone, since duroquinone and menadione are ineffective in the presence of this compound. Although not shown, 2-hydroxy-1,4-naphthoquinone was not reduced by formate and was found to uncouple an artificially-formed potential. In contrast, the reduced form of this compound is readily oxidized by nitrate [28].

Safranine responses with artificial electron donor system. Aerobic oxidation

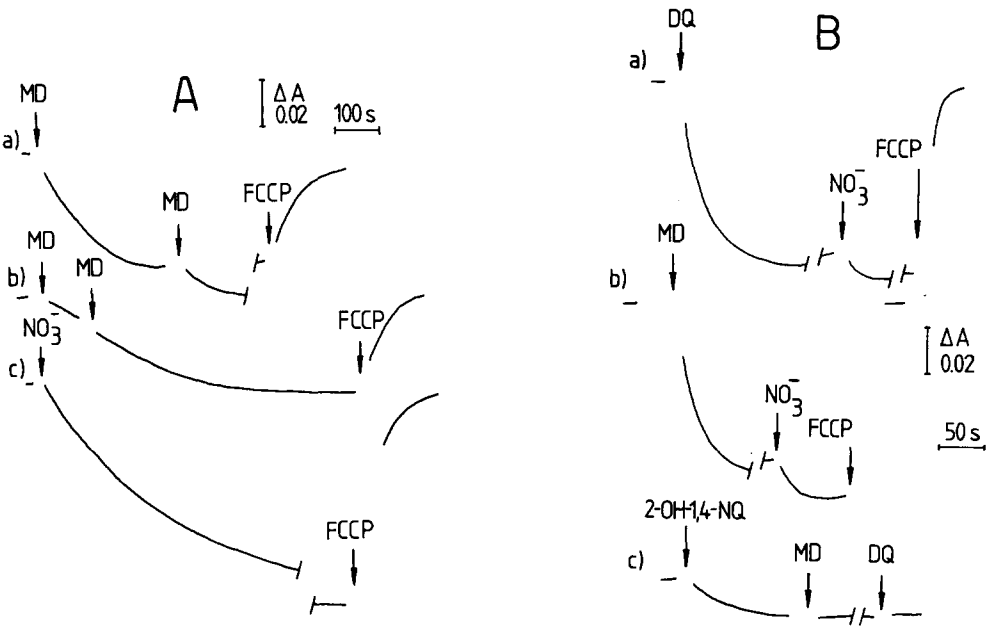

Fig. 4. Safranine responses in the presence and absence of *N,N'*-dicyclohexylcarbodiimide. (A) Anaerobic conditions. The reaction mixture was as described in Fig. 2, except no *N,N'*-dicyclohexylcarbodiimide was added. Vesicles of *Escherichia coli* ML 308-225 were incubated in the presence of formate (10 mM in traces a and b, 0.8 mM in c). Solutions of the electron acceptors were added as indicated. In trace a, menadione, 0.12 mM each (MD) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, 0.8 μM (FCCP). In trace b, 0.05 mM 2-hydroxy-1,4-naphthoquinone was included in the pre-incubation mixture and additions made were menadione, 0.05 mM, then 0.13 mM, followed by FCCP, 0.8 μM, as shown. In trace c, sodium nitrate (0.8 mM NO_3^-) then FCCP, 0.8 μM. (B) Conditions as in A, except 0.2 mM *N,N'*-dicyclohexylcarbodiimide was used. In trace a, duroquinone, 0.36 mM, then 0.05 mM (DQ), followed by sodium nitrate, 10 mM and FCCP, 0.8 μM. In trace c, 2-hydroxy-1,4-naphthoquinone, 0.12 mM (2-OH-1,4-NQ); menadione, 0.12 mM and duroquinone, 0.12 mM.

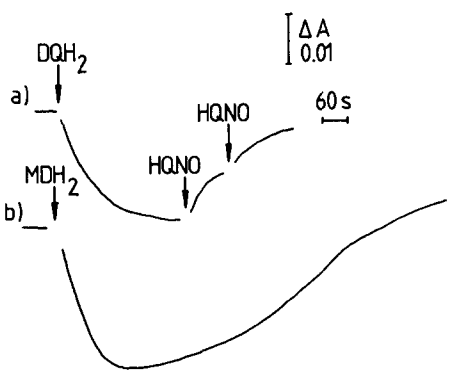

Fig. 5. Safranine responses during oxidation of quinols. The conditions were as those described in Fig. 4, except the measurements were made aerobically using a single-beam apparatus at 524 nm. Additions: trace a, durohydroquinone, 0.32 mM (DQH_2), then HQNO, 1 μM each; trace b, menadiol, 0.32 mM (MDH_2).

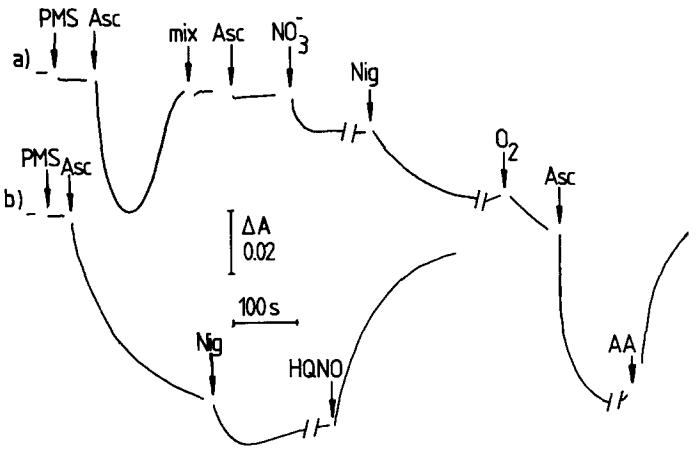

Fig. 6. Safranine responses in the presence of ascorbate-reduced *N*-methylphenazonium methosulphate during nitrate or O_2-dependent respiration. Conditions as in Fig. 2, except in trace a, anaerobic conditions. Additions: trace a, *N*-methylphenazonium methosulphate, 20 μM (PMS); ascorbate, 1 mM; potassium nitrate, 1 mM; nigericin, 80 μg/l (Nig); air (O_2) and antimycin A, 4 μg/ml (AA), as indicated. For the deflection following the addition of ascorbate, see text. In trace b, additions as in a, and 8 μM HQNO subsequently as indicated.

of duroquinol and menadiol produced transient responses (Fig. 5) as recorded at 524 nm with a relatively simple single-bean spectrophotometer. Thus, qualitative results were obtained without the need to use more sophisticated instruments. Similar responses were obtained with the dual-wavelength technique and using nitrate as an acceptor (not shown).

With nitrate as an acceptor, no significant response resulted from the addition of *N*-methylphenazonium methosulphate in the presence of ascorbate (Fig. 6), in contrast to the quinols mentioned above. However, similar weak hyperpolarizations were also seen in other preparations. The increase in the response after addition of nigericin indicates that a significant ΔpH is formed under these conditions. Admission of air to the cuvette resulted in an instantaneous response, as was also obtained at the beginning of the experiment due to inadvertent introduction of air during manipulation of the cuvette. No trace of oxygen was, however, left after the burst of respiration, as shown by the absence of any response after mixing, as indicated. The response increased after the last addition of ascorbate, indicating that this donor was apparently consumed anaerobically in the presence of nitrate. The data in trace b of Fig. 6 confirm that, under aerobic conditions, ascorbate-reduced *N*-methylphenazonium methosulphate readily forms a membrane potential. The effects of antimycin A seen in these experiments are most likely due to uncoupling [27], since electron transfer in preparations from *E. coli* is quite resistant to antimycin A [26].

Discussion

The results of the present study show that the safranine method previously used in studies on membrane potentials in isolated mitochondria [15] can

also be applied to bacterial vesicles. The calibration plot, which was linear up to 150—160 mV as measured with two different experimental approaches, indicates that this method might prove useful for quantitative estimations of membrane potentials under different experimental conditions.

Membrane potentials of bacteria and bacterial vesicles have been previously measured with cyanine dyes [29,30], ion-distribution techniques [6—11] or electrodes [31,32]. The response of cyanines is nonlinear above 80—100 mV [33] and, therefore, quantitation of the response is difficult. Furthermore, these dyes are toxic to bacteria [30,34], mitochondria [35,36] and, under some conditions, isolated cells [36—39]. We have observed no toxic effect of safranine on mitochondria at concentrations employed for measurements of membrane potentials. There is, however, a small decrease in the respiratory control index at safranine concentrations above 100 μM (Åkerman and Wikström, unpublished results). The safranine response is also unaffected by changes in pH [40] or pH gradients [15]. Changes in the spectrum of safranine might occur nonspecifically, however, upon interaction of the dye with some anions such as EGTA or nitriloacetate [16] or polyanions [12]. Furthermore, multivalent cations might interfere with the penetration of safranine across or its binding to, the membrane [14].

With all preparations of both strains studied the responses were qualitatively the same whenever the same activities were compared, in spite of the quantitative variations that occurred. The magnitude of the membrane potential of 160—170 mV found in this work with formate as a substrate, in vesicles derived from the K-12 strain, is in the same range as that obtained with whole bacteria [11]. Our values are somewhat higher (140 mV) than the value reported by Boonstra and Konings [9] on ML strain vesicles. However, these workers did not use *N,N'*-dicyclohexylcarbodiimide in their medium (see below). Furthermore, if the Donnan potential of 40—50 mV is subtracted from our values of the membrane potential, our results would be in complete agreement with theirs. The Donnan potential would not be noticed by flow-dialysis methods, where only changes in membrane potential can be observed.

It is also shown that artificial electron donor/acceptor systems, such as the pair ascorbate/*N*-methylphenazonium methosulphate, and some water-soluble quinones are able to generate membrane potentials. Both duroquinone and menadione stimulated formation of the membrane potential in their oxidized or reduced forms, under appropriate conditions, with formate, nitrate or oxygen. The availability of acceptors with lower redox potentials than ferricyanide [9,26] might help to analyze formate-nitrate reductase by partial reactions. Reactions of duroquinone and menadione with the electron transfer chain of aerobic *E. coli* have been demonstrated [41]. Since 2-hydroxy-1,4-naphthoquinone did not accept electrons from formate it is evident that the quinone-reactive sites in the anaerobic membrane have special requirements. Although the reduced form of this compound is oxidized by nitrate reductase [28], its use in the studies of membrane potentials seems to be limited, due to uncoupling phenomena observed. It is not known why no significant change in potential occurs anaerobically with ascorbate-reduced *N*-methylphenazonium methosulphate and nitrate. This finding is, however, in agreement with amino acid uptake data [20].

2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide, a widely-used respiratory chain inhibitor, abolishes membrane potentials artificially induced by potassium ion gradients, suggesting that this inhibitor may act as an uncoupler as well. This is in agreement with the results of Wikström [27] on isolated mitochondria. The effects of antimycin A and 2-hydroxy-1,4-naphthoquinone observed in this study could be explained on a similar basis.

N,*N*'-dicyclohexylcarbodiimide, an inhibitor of the ATPase proton pump, significantly increases the rate of formation and magnitude of the membrane potential, probably because of its ability to block proton channels unconnected with ATPase molecules (cf. Ref. 42). Such channels might well become unmasked by the vesicle isolation procedure. On the other hand, at high concentrations, *N*,*N*'-dicyclohexylcarbodiimide reduced the membrane potential probably because of its uncoupling action. At high concentrations, this compound indeed increases the proton conductance across the mitochondrial membrane (Wikström, personal communication).

The observed change in the safranine spectrum upon induction of membrane potentials is large enough to be seen with single-beam equipment as well. In this case, however, nonspecific volume changes should be excluded by measurements at a wavelength such as 554 nm or higher. Furthermore, the safranine response can be monitored with fluorimetry [13].

It is concluded that the safranine method offers an alternative to ion-distribution techniques of measuring membrane potentials in bacterial vesicles. The method has an advantage in its rapidity. However, the quantitation of the signal is time consuming since each vesicle preparation has to be calibrated separately. An additional advantage is that with the large optical changes observed, determination of membrane potentials even with rather simple equipment becomes possible.

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