The use of valinomycin, nigericin and trichlorocarbanilide in control of the protonmotive force in *Escherichia coli* cells

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Valinomycin, nigericin and trichlorocarbanilide were assessed for their ability to control the protonmotive force in *Escherichia coli* cells. Valinomycin, at high K^+ concentrations, was found to decrease the membrane potential $\Delta\psi$ and indirectly to decrease the pH gradient ΔpH . Nigericin was found to have two modes of action. At low concentrations (0.05–2 μM) it carried out K^+/H^+ exchange and decreased ΔpH . At higher concentrations (50 μM) it carried out a K^+ -dependent transfer of H^+ , decreasing both $\Delta\psi$ and ΔpH . In EDTA-treated cells only the latter mode of action was evident, whereas in a mutant sensitive to deoxycholate both types of effect were observed. Trichlorocarbanilide is proposed as an alternative to nigericin for the specific control of ΔpH , and it can be used in cells not treated with EDTA.

The driving force for endergonic membrane-linked processes such as oxidative phosphorylation remained ill-defined until Mitchell's chemiosmotic model was put forward in the early 1960s (Mitchell, 1961, 1963). Chemiosmosis classifies energy-conserving membrane-linked reactions into two types, Δp -generating reactions and Δp -consuming reactions. Δp -generating reactions (e.g. electron flow via the respiratory chain) transport H^+ across membranes of very low conductance and capacitance, leading to the generation of a membrane potential ($\Delta\psi$). If other ion fluxes partially compensate for this H^+ flux, a pH gradient (ΔpH) is also generated. Together the $\Delta\psi$ and ΔpH constitute the protonmotive force (Δp):

$$\Delta p = \Delta\psi - Z\Delta pH \quad (1)$$

where $Z = 2.3RT/F$ and is equal to 59 at 25°C, and converts logarithmic concentration gradients into mV. Δp can be related to the electrochemical gradient of H^+ ($\Delta\bar{\mu}_{H^+}$) through the Faraday constant F :

$$\Delta p = \Delta\bar{\mu}_{H^+}/F \quad (2)$$

Δp -consuming reactions transduce the free energy available in the protonmotive force for the performance of useful work. Examples of work done by

Δp -consuming reactions include: osmotic work by transport proteins, chemical work by the ATP synthase and kinetic work by the flagellar motor (Hamilton, 1975; Harold, 1976; Mitchell, 1981). More recently, other cellular functions have also been associated with Δp , such as protein secretion (Date *et al.*, 1980), nitrogen fixation (Veeger *et al.*, 1980) and bacteriophage infection (Labedan & Goldberg, 1979). To define quantitatively the relationship between Δp and Δp -consuming reactions it is essential to measure and control accurately the components, the membrane potential and pH gradient, of the protonmotive force. In a previous study (Ahmed & Booth, 1981a) we presented a detailed analysis of the methodology used for assessing the magnitude of these two components, establishing that independent measurements made with different probes corroborate each other.

Ionophores, compounds that increase the ionic permeability of membranes, have often been used to control the protonmotive force (Ramos *et al.*, 1976; Ramos & Kaback, 1977; Khan & Macnab, 1980; Booth *et al.*, 1981; Ahmed & Booth, 1981b). Two classical examples are: (i) valinomycin, a neutral carrier-type ionophore that allows cations, with high specificity for K^+ , to permeate the membrane freely, and that can be used to control the membrane potential; (ii) nigericin, a carboxylic carrier-type ionophore that allows electroneutral cation/ H^+ exchange, again with specificity for K^+ (Pressman, 1976; Reed, 1979), and that can be used to control ΔpH .

Abbreviations used: $[K^+]_o$, external K^+ concentration; Δp , protonmotive force; $\Delta\psi$, membrane potential; ΔpH , pH gradient; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of H^+ .

Since *Escherichia coli* cells possess an outer membrane that excludes high-molecular-weight compounds such as the above ionophores, Leive (1968) developed a method involving the use of EDTA for disrupting this permeability barrier. However, a major problem with EDTA treatment is the possibility of obtaining a heterogeneous population of cells with respect to the degree of disruption of the outer membrane. This heterogeneity will lead to a preparation containing a proportion of cells that still exclude the added ionophore. We have overcome this problem by using an *E. coli* mutant (Doc-S) that has a defective outer membrane (and thus does not possess a permeability barrier to these ionophores) in parallel with EDTA-treated *E. coli* ML308-225.

In the present study we have critically examined the use of valinomycin and nigericin in *E. coli* cells for the control of the protonmotive force, and show the following. Firstly, we show that valinomycin at high K^+ concentrations indirectly decreases ΔpH . Secondly, we show that low ($0.05\text{--}2\ \mu M$) concentrations of nigericin collapse ΔpH in *E. coli* Doc-S (a mutant sensitive to deoxycholate) but fail to affect ΔpH in EDTA-treated *E. coli* ML308-225. However, higher ($50\ \mu M$) concentrations of nigericin decrease $\Delta\psi$ and ΔpH in a K^+ -dependent fashion in both strains. Thirdly, we characterize an ionophore, trichlorocarbanilide, that carries out electroneutral Cl^-/OH^- exchange (Hamilton & Jeacocke, 1972) as an alternative that can be used instead of nigericin. Finally, we show that trichlorocarbanilide can be used in cells not treated with EDTA.

Materials and methods

Materials

All materials were as previously specified (Booth *et al.*, 1979; Ahmed & Booth, 1981a), with the following additions. Trichlorocarbanilide was generously given by Professor W. A. Hamilton (University of Aberdeen, Aberdeen, Scotland, U.K.). Nigericin was generously given by Dr. R. L. Davis (Eli Lilly International Corp., Indianapolis, IN, U.S.A.). Sodium deoxycholate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tryptone, yeast extract and agar were purchased from Oxoid (London E.C.4, U.K.).

Bacterial strains and growth conditions

E. coli ML308-225 (*lacI^- z^- y^+ a^+*) was supplied by Dr. I. C. West (University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.). It was maintained and grown for experimental work as previously described (Booth *et al.*, 1979). *E. coli* K12 Doc-S (*lacI^- z^+ y^+ a^+ pro^- trp^- his^- met^-*) was supplied by Dr. B. A. Haddock (Biogen, Geneva, Switzerland). *E. coli* Doc-S was maintained at $4^\circ C$ after overnight growth on 1.5% (w/v) agar slopes of

LB broth (see below). For experimental studies *E. coli* Doc-S was grown in 100 ml volumes of media in 250 ml Erlenmeyer flasks. The cultures were shaken at $30^\circ C$ on a New Brunswick Gyrotary shaker at setting 8. LB broth contained, per litre, NaCl (10 g), tryptone (10 g) and yeast extract (5 g). The medium was adjusted to pH 7.0 with KOH before being autoclaved. Overnight cultures were diluted to A_{650} 0.4 (A_{650} 2.0 was assumed to indicate 1 mg dry wt./ml) with fresh growth medium, and the cells were re-incubated as above until A_{650} reached 0.8 (approx. 2½ h).

Experimental protocols

(i) *Cell harvest and preparation.* After doubling, cells were harvested at 12000 g for 10 min at $4^\circ C$. Washing was performed by resuspension in an appropriate buffer, as detailed below, followed by re-centrifugation at 16000 g for 5 min at $4^\circ C$. EDTA treatment was performed as previously described (Booth *et al.*, 1979). A harsher treatment involving a 10 min incubation with EDTA did not affect the results (Bakker, 1982). Cells not treated with EDTA were washed once in 120 mM-Tris/HCl buffer, pH 8.0, harvested, washed and resuspended in 5 mM-Tris/5 mM-Mes (4-morpholine-ethanesulphonic acid)/HCl buffer, pH 7.0, containing 150 mM-choline chloride and 0.2 mM-KCl. For experiments in which the ion specificity of trichlorocarbanilide was assessed, cells were harvested, washed and resuspended in 5 mM-Tris/5 mM-Mes buffer, pH 7.0, containing 100 mM of the appropriate salts (K^+ , choline or Na^+ ; Cl^- , Br^- , I^- , NO_3^- , SO_4^{2-} or HPO_4^{2-}). All cells were resuspended to 8–10 mg dry wt./ml final concentration. The ionophores valinomycin and nigericin were added at the appropriate concentrations immediately after the final resuspension. When used, trichlorocarbanilide was added to the cell suspension (1 mg dry wt./ml) 5 min before the addition of radioactive probes.

(ii) *Volume measurements.* These were performed by the method of Stock *et al.* (1977). A 4 ml portion of cells (at 4 mg dry wt./ml) was incubated at $25^\circ C$ for 25 min in a stirred glass vessel in appropriate buffers. Radioactive probes were added 1 min before sampling to assess the volume of the following compartments: the whole cell (hydroxy[^{14}C]methyl-inulin/ 3H_2O couple), the periplasm ([^{14}C]sucrose/[3H]inulin couple) and the cytoplasm ([^{14}C]sucrose/ 3H_2O couple) for *E. coli* Doc-S. Samples were taken and processed and volumes were calculated as previously described (Booth *et al.*, 1979). We have published similar measurements for *E. coli* ML308-225 (Booth *et al.*, 1979). The radioactive sucrose was purified by incubation with 6 mg dry wt. of glucose-grown cells/ml for 5 min. The sucrose was then separated from the cells by centrifugation at 12000 g in an Eppendorf centrifuge for 2 min. Values

for *E. coli* ML308-225 cytoplasmic volumes were as previously described (Ahmed & Booth, 1981a). Nigericin and trichlorocarbanilide, at the concentrations used, did not affect the cytoplasmic volume.

(iii) *Measurement of $\Delta\psi$ and ΔpH .* $\Delta\psi$ and ΔpH were measured by using centrifugation-based assays. Cells at 1 mg dry wt./ml were equilibrated for 5 min at 25°C in a stirred glass vessel. Radioactive probes [^{14}C]tetraphenylphosphonium cation (4 μM ; 8.7 Ci/mol) or $^{86}\text{Rb}^+$ (5 μM ; approx. 0.2 $\mu\text{Ci}/\text{mol}$) for $\Delta\psi$ and [^{14}C]benzoic acid (2 μM ; 24.4 Ci/mol) for ΔpH were then added, along with catalase (250 $\mu\text{g}/\text{ml}$) and $^3\text{H}_2\text{O}$ (1–2 $\mu\text{Ci}/\text{ml}$). After the cells had been incubated for a further 20 min, 500 μl samples were transferred into Eppendorf centrifuge tubes (1.6 ml capacity) containing 10 μl of 2-volume H_2O_2 and centrifuged at 12000 g for 20 s. The radioactivity in the supernatants and pellets was assayed as previously described (Ahmed & Booth, 1981a). $\Delta\psi$ was calculated by substitution of concentration ratios into the Nernst equation. An activity coefficient of 1 was assumed for $^{86}\text{Rb}^+$ and [^{14}C]tetraphenylphosphonium cation. ΔpH was calculated by using the Waddell and Butler equation (Ahmed & Booth, 1981a). The measurements for both parameters were made in triplicate and represent averages for three or four experiments. The maximum variations in measurements of $\Delta\psi$ and ΔpH were $\pm 6\text{ mV}$ and $\pm 10\text{ mV}$ respectively.

(iv) *Sensitivity to deoxycholate.* A sample (100 μl) of an overnight culture of *E. coli* Doc-S was pipetted into 3.5 ml of LB medium (Miller, 1972) containing 0.6% molten agar. The cell suspension was vortex-mixed and immediately poured on to an LB broth/agar plate (1.5% agar). When the upper agar had solidified, sterile filter-paper discs (5 mm diam.) were placed on the surface at regular intervals (about six per plate). To each of these discs was added a small volume (5–20 μl) of a 200 mg/ml solution of sodium deoxycholate. After growth at 30°C a clear zone was evident around the filter discs (approx. 1.4 cm diam. for 10 μl of sodium deoxycholate at 200 mg/ml) *E. coli* ML308-225 was completely resistant to deoxycholate in this concentration range.

(v) *Liquid-scintillation counting of radioactivity.* The Packard Tri-Carb 300C instrument was used for all liquid-scintillation counting of radioactivity. For $^{14}\text{C}/^3\text{H}$ double-labelling the following windows were used: ^3H , 2–12 eV; ^{14}C , 12–156 eV. For $^{86}\text{Rb}^+ / ^{14}\text{C} / ^3\text{H}$ triple-labelling the third window was set at 156–1900 eV. Overlaps of radioactivity counts were estimated by using appropriate standards.

Results

Cell volume

In previous reports we have shown the cytoplasmic volume to be a significant experimental

variable in the measurement of $\Delta\psi$ and ΔpH (Booth *et al.*, 1979; Ahmed & Booth, 1981a). It becomes most relevant when the magnitude of $\Delta\psi$ or ΔpH is small (–30 mV to +30 mV) (Ahmed & Booth, 1981a). So the volume of *E. coli* Doc-S was measured. Table 1 shows the values obtained for the whole cell, the cytoplasm and the periplasm of this *E. coli* strain. The data reveal that *E. coli* Doc-S does not possess a periplasmic space, as the volumes obtained with the [^{14}C]sucrose/ $^3\text{H}_2\text{O}$ couple and the hydroxy[^{14}C]methyl-inulin/ $^3\text{H}_2\text{O}$ couple are very similar. This is confirmed by the [^{14}C]sucrose/[^3H]inulin couple, and demonstrates the effect of the mutation on the outer membrane of this strain. The value obtained for the cytoplasmic volume is similar to that for other K12 strains of *E. coli* (D. Strickland, I. R. Booth & W. A. Hamilton, unpublished work; Kashket, 1981). These measurements are a prerequisite to any quantitative study of the proton-motive force.

*Effect of external K^+ on $\Delta\psi$ and ΔpH in EDTA/valinomycin-treated *E. coli* ML308-225 cells*

The valinomycin/ K^+ couple is frequently used to control $\Delta\psi$ (Padan *et al.*, 1976; Reed, 1979), and it is assumed that, as $\Delta\psi$ is decreased, ΔpH remains relatively unaffected. The method involves exchanging a cation such as choline for K^+ , with maintenance of constant osmolarity. Increasing the external K^+ concentration ($[K^+]_o$) decreases $\Delta\psi$ and clamps it at this new value. To ascertain the exact effect of increasing $[K^+]_o$ on $\Delta\psi$ and ΔpH in this system, we measured the cytoplasmic volume as a function of $[K^+]_o$. Fig. 1 shows that the cytoplasmic volume increases linearly with $\log([K^+]_o)$ and reaches a value of 2.2 $\mu\text{l}/\text{mg}$ dry wt. at $[K^+]_o$ 150 mM, as reported previously (Ahmed & Booth, 1981a). This represents a doubling of the cyto-

Table 1. *Volume measurements for *E. coli* Doc-S*
Volumes were measured by the method of Stock *et al.* (1977), with the use of [^{14}C]sucrose (8.6 Ci/mol), [^3H]inulin (1.8 Ci/mmol), hydroxy[^{14}C]methyl-inulin (10.3 Ci/mol) and $^3\text{H}_2\text{O}$ (10 $\mu\text{Ci}/\text{ml}$). The protocol for measurements was as detailed in the legend to Fig. 1. Standard deviations were calculated, the numbers of observations being indicated in parentheses. ^{14}C -labelled compounds were used at 0.4 $\mu\text{Ci}/\text{ml}$.

Probes	Volume ($\mu\text{l}/\text{mg}$ dry wt.)	Compartment
Hydroxy[^{14}C]methyl-inulin/ $^3\text{H}_2\text{O}$	1.5 ± 0.16 (15)	Whole cell
[^{14}C]Sucrose/ $^3\text{H}_2\text{O}$	1.7 ± 0.29 (7)	Cytoplasm
[^{14}C]Sucrose/[^3H]inulin	0.05 ± 0.31 (4)	Periplasm

Fig. 1. Cytoplasmic volume of *E. coli* ML308-225, EDTA-treated and EDTA/valinomycin-treated, as a function of $[K^+]_0$.

EDTA-treated (■) and EDTA/valinomycin-treated (▲) cells were prepared and the cytoplasmic volume was measured. The cells were prepared and assayed as described in the text and previously (Ahmed & Booth, 1981a). The treated cells were incubated at 4 mg dry wt./ml in 5 mM-Tris/5 mM-Mes buffer, pH_o 5.5, containing 150 mM-choline chloride and 0.2 mM-KCl. The $[K^+]_0$ was increased by exchanging KCl for choline chloride at constant osmolarity.

plasmic volume over the $[K^+]_0$ range 0.2–150 mM. By taking these measurements into account, the $\Delta\psi$ and ΔpH parameters were estimated over the same $[K^+]_0$ range. As $[K^+]_0$ was increased from 1 mM to 25 mM, $\Delta\psi$ decreased by 70 mV from 130 mV to 60 mV, and ΔpH partially compensated by increasing by 36 mV from 90 mV to 126 mV (Fig. 2) (pH_o 6.0; pH_i increased from 7.5 to 8.1). Strikingly, as $[K^+]_0$ was increased further, ΔpH decreased, until at $[K^+]_0$ 150 mM it had reached a value of 36 mV with $\Delta\psi$ close to zero (Fig. 2). EDTA-treated cells in the absence of valinomycin also swell as a function of $[K^+]_0$ (Fig. 1); when $\Delta\psi$ and ΔpH were measured as a function of $[K^+]_0$ in these cells the decrease in ΔpH was not observed (Ahmed & Booth, 1981b). These results indicate that the valinomycin/ K^+ couple may indirectly effect ΔpH .

Effect of nigericin on ΔpH and $\Delta\psi$ in *E. coli* Doc-S and ML308-225

In order to estimate the concentration range of nigericin needed to control ΔpH in EDTA-treated *E. coli* ML308-225, we examined the effect of

increasing nigericin concentration on ΔpH in *E. coli* Doc-S. In addition, we also measured $\Delta\psi$ to ensure that nigericin was acting electroneutrally. Low concentrations of nigericin (2 μM) were sufficient to decrease ΔpH to near zero with a slight increase in $\Delta\psi$ (Table 2). This electroneutral ΔpH collapse was independent of $[K^+]_0$ (results not shown). However, $[K^+]_0$ does effect the magnitude of the $\Delta\psi$ and ΔpH before nigericin treatment and the increase in $\Delta\psi$ on treatment with nigericin. When similar experiments were performed with EDTA-treated *E. coli* ML308-225, 2 μM -nigericin did not affect the magnitude of $\Delta\psi$ or ΔpH (results not shown).

Table 2. Effect of nigericin on ΔpH and $\Delta\psi$ in *E. coli* Doc-S

Measurements were made in 150 mM-choline chloride in 5 mM-Tris/5 mM-Mes buffer at pH_o 5.5. $\Delta\psi$ was measured with [¹⁴C]tetraphenylphosphonium ion and ΔpH with [¹⁴C]benzoic acid as described previously (Ahmed & Booth, 1981a). The concentration of nigericin is that obtained after the concentrated cell suspension had been diluted 10-fold into the incubation buffer.

Concn. of nigericin (μM)	$\Delta\psi$ (mV)	ΔpH (mV)
—	130	52
0.05	133	26
1.0	136	17
2.0	140	6

Fig. 2. Effect of K^+ concentration on $\Delta\psi$ (○) and ΔpH (Δ) in EDTA/valinomycin-treated *E. coli* ML308-225. Experimental conditions were as indicated in the legend to Fig. 1. $\Delta\psi$ was measured with ⁸⁶Rb⁺, and ΔpH with [¹⁴C]benzoic acid, as described previously (Ahmed & Booth, 1981a). $[K^+]_0$ was varied by exchange for choline, with maintenance of constant osmolarity. The incubation pH was pH_o 6.0.

The effect of higher concentrations of nigericin on the $\Delta\psi$ and ΔpH was radically different. At zero $[K^+]_o$, 50 μM -nigericin had no effect on either $\Delta\psi$ or ΔpH in both strains. However, as $[K^+]_o$ was increased both $\Delta\psi$ and ΔpH were decreased. At $[K^+]_o$ 150 mM ΔpH was near zero and $\Delta\psi$ was decreased to approx. 35 mV (Figs. 3a and 3b). These results indicate that under these conditions nigericin is not acting as an electroneutral cation/ H^+ -exchanger, but acting electrogenically. Nigericin cannot therefore be used specifically to control ΔpH in EDTA-treated *E. coli* cells, but can be used to decrease both ΔpH and $\Delta\psi$ in a $[K^+]_o$ -dependent fashion.

Trichlorocarbanilide as an alternative to nigericin for the control of ΔpH

Trichlorocarbanilide has been shown to be an electroneutral anion/ OH^- -exchanger (Hamilton & Jeacocke, 1972). It has a molecular weight of 314 and a structure very similar to that of 3,3',4',5-tetrachlorosalicylanilide, the uncoupler of oxidative

phosphorylation in bacterial and mitochondrial systems (Hamilton, 1968). Thus we expected that the outer membrane would not present a permeability barrier to trichlorocarbanilide. We measured $\Delta\psi$ and ΔpH as a function of the trichlorocarbanilide concentration in *E. coli* Doc-S and *E. coli* ML308-225. The titration curves obtained were very similar in both strains (Figs. 4a and 4b). Trichlorocarbanilide at a concentration of 200 μM decreased ΔpH to near zero with a slight increase in $\Delta\psi$. However, lower concentrations of trichlorocarbanilide had a more dramatic effect on ΔpH in *E. coli* Doc-S than in *E. coli* ML308-225. This may reflect the outer membrane of the latter strain either adsorbing some of the ionophore or hindering its access to the cytoplasmic membrane (Hamilton, 1970). As shown in Fig. 4, ΔpH and hence pH_i can be controlled accurately by choosing a particular trichlorocarbanilide concentration. This makes this ionophore a very useful compound for the study of the effects of ΔpH or pH_i on various cellular functions such as pH_i regulation itself.

Fig. 3. Effect of K^+ concentration on $\Delta\psi$ (O) and ΔpH (Δ) in nigericin-treated (50 μM) *E. coli* (a) Nigericin-treated *E. coli* Doc-S; (b) EDTA/nigericin-treated *E. coli* ML308-225. Experimental conditions were as indicated in the legend to Table 2. The nigericin concentration refers to that obtained after the concentrated cell suspension had been diluted 10-fold into the incubation buffer.

Fig. 4. Effect of external trichlorocarbanilide concentration on $\Delta\psi$ (O) and ΔpH (Δ) in *E. coli* Doc-S and *E. coli* ML308-225

(a) *E. coli* Doc-S; (b) *E. coli* ML308-225. Cells were resuspended at 1 mg/ml in 5 mM-Tris/5 mM-Mes buffer, pH_o 5.5, containing 100 mM- K^+ . $\Delta\psi$ was measured with $[^{14}C]$ tetraphenylphosphonium ion and ΔpH with $[^{14}C]$ benzoic acid as described previously (Ahmed & Booth, 1981a).

Table 3. *Anion-transport-specificity of trichlorocarbanilide in E. coli Doc-S determined by using K⁺-containing buffers*

Cells were washed and resuspended in the appropriate buffers containing 100 mM salts in 5 mM-Tris/5 mM-Mes at pH_o 5.5 (pH_o was adjusted in all cases with Mes). $\Delta\psi$ and Δ pH were measured as described previously (Ahmed & Booth, 1981a). Trichlorocarbanilide was added at 40 μ M final concentration. Similar results were obtained with *E. coli* ML308-225.

Trichlorocarbanilide ...	$\Delta\psi$ (mV)		Δ pH (mV)	
	—	+	—	+
Anion				
HPO ₄ ²⁻	76	110	95	86
SO ₄ ²⁻	79	117	96	70
NO ₃ ⁻	82	114	100	60
I ⁻	92	130	115	39
Br ⁻	87	128	98	22
Cl ⁻	81	126	104	8

External pH did not appear to influence the effectiveness of trichlorocarbanilide. Thus with *E. coli* Doc-S 40 μ M-trichlorocarbanilide was sufficient to collapse Δ pH in the pH range pH_o 5.5–7.7 (results not shown).

Ion-transport-specificity of trichlorocarbanilide

To characterize further the use of trichlorocarbanilide in the control of Δ pH, we investigated its specificity. This was achieved by examining the effect of 40 μ M-trichlorocarbanilide on $\Delta\psi$ and Δ pH as a function of the buffer salt composition. Three cations (choline, K⁺ and Na⁺) and six anions (Cl⁻, Br⁻, I⁻, HPO₄²⁻, SO₄²⁻ and NO₃⁻) were tested. Cells were washed and resuspended in the relevant buffers, and then treated with trichlorocarbanilide for 5 min. The $\Delta\psi$ or Δ pH probes were then added, and the incubation was continued for a further 20 min. From the results obtained (Table 3) it is apparent that trichlorocarbanilide has a strong anion-transport-specificity for halides, and in particular Cl⁻. However, it also transports SO₄²⁻ and NO₃⁻, but to a smaller extent. It has no cation-transport-specificity, as K⁺, Na⁺ and choline salts yield almost identical results (results not shown). The anion-transport-specificity of trichlorocarbanilide as seen by its effect on $\Delta\psi$ and Δ pH is the following: Cl⁻ > Br⁻ > I⁻ > NO₃⁻ > SO₄²⁻ > HPO₄²⁻. Similar ion-transport-specificity has been observed by Hamilton and co-workers in sphaeroplast/protoplast swelling experiments (see Hamilton, 1968, 1970).

Discussion

The data in the present paper represent an analysis of the use of three ionophores in the control

of Δ pH and $\Delta\psi$. We provide additional support for the importance of considering cytoplasmic volume as an experimental variable (Ahmed & Booth, 1981a; Kroll & Booth, 1981).

The use of low sucrose concentrations to measure the cytoplasmic volume has been criticized on the basis that sucrose might penetrate into the cell via the *lac* permease (Heller & Wilson, 1979). However, the present data demonstrate that the method can be used in cells constitutive for the *lac* permease. Thus one observes the swelling of cells as a consequence of K⁺ uptake (Fig. 1), and in *E. coli* strain Doc-S hydroxymethyl-inulin, inulin and sucrose report similar volumes for the cytoplasm (Table 1). These effects would not be expected for a solute that can be transported.

When the valinomycin/K⁺ couple is used to depolarize the cell membrane, compensation must be made for the increase in cell volume (Fig. 1). However, it is clear that above 25 mM-K⁺ other effects begin to arise. Thus Δ pH, having increased over the range 0.2–25 mM-K⁺, starts to decline. One possible cause of this effect could be excessive swelling, leading to an increase in ionic permeability of the cytoplasmic membrane (Bakker & Mangerich, 1981). In the absence of valinomycin, the effect of K⁺ on the cell volume is similar, but the maximum value attained is lower. This may account for the lack of a decline in Δ pH at high [K⁺]_o in the absence of valinomycin (Ahmed & Booth, 1981b). However, since pH_i reaches a very high value in valinomycin/K⁺-treated cells at high [K⁺]_o (approx. pH_i 8.1), it is possible that other, more specific, effects may be involved. In particular, several reports suggest that alkaline pH_i can stimulate H⁺-entry mechanisms (Brey *et al.*, 1978, 1980; Plack & Rosen, 1980; Padan *et al.*, 1981; Zilberstein *et al.*, 1982).

Nigericin has been used to control specifically Δ pH in *E. coli* membrane vesicles (Ramos *et al.*, 1976) by electroneutral exchange of K⁺ for H⁺. In *E. coli* Doc-S 2 μ M-nigericin was sufficient to decrease Δ pH to zero without significant effect on $\Delta\psi$ (Table 2). The same concentration of nigericin had no effect on either Δ pH or $\Delta\psi$ in EDTA-treated *E. coli* ML308-225, although the cells were permeable to valinomycin (results not shown). At higher concentrations (50 μ M) of nigericin the effect was the same with both strains, that is both $\Delta\psi$ and Δ pH were collapsed in a [K⁺]_o-dependent manner (Fig. 3). In a previous study on black lipid membranes dimerization was suggested to occur at high concentrations of nigericin (Toro *et al.*, 1976). This tends to suggest that the dimer might pass through the outer layer of EDTA-treated *E. coli* ML308-225 rather more readily than does the monomer.

The uncoupling effect of high concentrations of nigericin has previously been reported for mito-

chondria (Ferguson *et al.*, 1971). In a subsequent study it was concluded that K^+ transfer by a protonated nigericin dimer could account for the uncoupling (Toro *et al.*, 1976). This would be consistent with the $[K^+]_o$ -dependent collapse of $\Delta\psi$ in our experiments. However, it does not explain why ΔpH was not collapsed at low $[K^+]_o$ but was collapsed at higher $[K^+]_o$ values. Rather, one would suggest either that the uncoupling ion transferred is H^+ or that both K^+ and H^+ are transferred. A more definite statement about the mode of action of nigericin cannot be made, as the data do not allow us to discriminate between the possible K^+-H^+ fluxes due to nigericin and those of cellular transport systems.

The inability of nigericin to control specifically ΔpH in EDTA-treated cells led us to seek an alternative ionophore. Previous work on anti-bacterial agents had shown that the mechanism of action of one of these, trichlorocarbanilide, was by anion/ OH^- exchange (Hamilton & Jeacocke, 1972). In addition, trichlorocarbanilide had a very similar structure and molecular weight to the well-known uncoupler of oxidative phosphorylation 3,3',4',5-tetrachlorosalicylanilide, raising the possibility that this compound could be used without prior EDTA treatment of cells. Indeed, when we performed a titration curve in *E. coli* Doc-S and untreated *E. coli* ML308-225 with trichlorocarbanilide the ΔpH was decreased to near zero in both strains (Figs. 4a and 4b). These data undoubtedly show trichlorocarbanilide to penetrate the outer membrane.

To characterize this compound further we analysed its ion-transport-specificity. This was achieved indirectly by examining the effect of trichlorocarbanilide on $\Delta\psi$ and ΔpH in buffers of various ion concentrations. Trichlorocarbanilide did not discriminate between K^+ , Na^+ or choline cations, and the following anion-transport sequence was found: $Cl^- > Br^- > I^- > NO_3^- > SO_4^{2-} > HPO_4^{2-}$. So, by changing the anion content of the buffer at saturating trichlorocarbanilide concentration or by changing the trichlorocarbanilide concentration in a Cl^- -based buffer, the ΔpH and hence pH_i can be set accurately and precisely at a variety of values, without the need for EDTA treatment.

In conclusion, we have shown in the present work that two classical ionophores may not behave in the expected manner in terms of their effects on $\Delta\psi$ and ΔpH . Therefore we recommend that, when ionophores are used for control of $\Delta\psi$ and ΔpH , the measurement of these two components also be carried out. In addition, we propose trichlorocarbanilide as an agent for the specific control of ΔpH .

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