Quantitative measurements of the proton-motive force and its relation to steady state lactose accumulation in *Escherichia coli*

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(Received 7 July 1981/Accepted 28 August 1981)

The magnitude of $\Delta\psi$ (membrane potential), ΔpH (pH gradient), lactose accumulation and cytoplasmic volume have been determined over a range of experimental conditions. A study of two probes of ΔpH , benzoate and dimethyloxazolidene-2,4-dione (DMO), and four probes of $\Delta\psi$, Rb^+ , K^+ , tetraphenylphosphonium (TPP^+) and 3,3'-dipropylthiodicarbocyanine iodide, has been carried out. Benzoate and DMO are shown to be equivalent at pH values above the pK of DMO, but the latter may be less accurate below this pH. The cations TPP^+ and Rb^+ were found, by a number of criteria, to be equivalent, and TPP^+ may be used in cells not pretreated with EDTA. These studies are an essential prerequisite to the use of TPP^+ as a quantitative probe in untreated cells.

The chemiosmotic model of energy coupling places the proton in a central position in bacterial energetics. In a respiring bacterium, such as *Escherichia coli*, electron flow to oxygen is associated with proton expulsion generating an electrochemical gradient of protons across the membrane, the proton-motive force. Since the advent of the model (Mitchell, 1963) the general principles of chemiosmotic coupling have been verified for a wide variety of membrane-bound energy transducing systems (Hamilton, 1975; Harold, 1976). Subsequently, the attention of bioenergeticists has been focused on the adequacy of the bulk phase proton-motive force, Δp , to account for the work done either in ATP synthesis or in solute transport (Kell, 1979; Booth & Hamilton, 1980a; Mitchell, 1981). At the present time there is considerable controversy surrounding the finer detail of the chemiosmotic model (Ramos & Kaback, 1977; Kell, 1979; Kell *et al.*, 1981).

Any systematic study of the chemiosmotic model of transport requires an accurate assessment of the magnitude of the components of Δp and of transport itself. Δp consists of a charge gradient, the membrane potential ($\Delta\psi$), and a pH gradient, ΔpH :

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

Abbreviations used: pH_i , internal pH; pH_o , external pH; $\Delta\psi$, membrane potential; ΔpH , pH gradient; Δp , proton-motive force; $\Delta\text{p}_{\text{lac}}$, the lactose accumulation ratio expressed in mV; TPP^+ , tetraphenylphosphonium cation; DMO, dimethyloxazolidene-2,4-dione; Mes, 4-morpholine-ethanesulphonic acid; cyanine diS-C₃(5), 3,3'-dipropylthiodicarbocyanine iodide.

where Z is equal to 59 at 25°C and converts logarithmic concentration gradients to mV. This description of the bulk phase Δp as the driving force for solute uptake has been criticized (Kell, 1979) but none the less remains the only working model (Mitchell, 1981; Kell *et al.*, 1981).

The magnitude of $\Delta\psi$ is usually determined by the transmembrane distribution of a lipophilic cation. The accuracy of the method relies on the passive permeation of the cation in response to the bulk phase $\Delta\psi$ with no contribution to its accumulation from either binding or transport systems. In reality it has proved difficult to find ideal probes and as a consequence each has its own limitation. In the past, K^+ or Rb^+ in the presence of saturating concentrations of the ionophore valinomycin have been the probes of choice because of the simplicity of their assay. There is no evidence for their binding to cell components, but the presence of transport systems in bacteria for K^+ and the major physiological role of the ion has provoked criticism of the method (Maloney *et al.*, 1975). The choice of Rb^+ over K^+ in *E. coli* removes some of these criticisms, since the cation is discriminated against by the K^+ transport systems (Rhoads *et al.*, 1977). In the last few years, lipophilic cations have achieved prominence, since they combine many features of the valinomycin/ Rb^+ couple with the added advantage that they are non-physiological cations and thus are less likely to be actively transported. However, their lipophilic nature leads to great non-specific binding of the cations which may complicate quantification (Zaritsky *et al.*, 1981). The assay of these cations

requires the use of relatively dense cell suspensions, which may in itself cause problems with aeration. Cationic fluorescent dyes on the other hand have the advantage that through their optical properties a continuous monitor of $\Delta\psi$ can be obtained at relatively low densities of cells. Thus although no one probe is ideal, a combination of several with different limitations should provide corroborative data with which to test the chemiosmotic model.

Measurement of ΔpH has presented fewer problems and there is general agreement on its magnitude (Padan *et al.*, 1976; Booth *et al.*, 1979). However, for accurate quantification there is no single probe which can span a wide range of experimental pH values. Benzoic acid and DMO are routinely used probes, but the former is a well known food preservative and can interfere with cellular metabolism (Eklund, 1980), while the latter has a high pK (pK 6.32, Addanki *et al.*, 1968) which may lead to inaccuracy at acid pH. Recent measurements of ΔpH by ^{31}P n.m.r. have generally corroborated the data obtained with weak acids. However, the conditions necessary for n.m.r. are far removed from the ideal for transport experiments (Navon *et al.*, 1977; Slonczewski *et al.*, 1981).

A further critical point in terms of quantification is that of the magnitude of the intracellular volume. Since it was discovered that the periplasm constituted a significant component of the total cell volume in *E. coli* and other Gram-negative bacteria (Stock *et al.*, 1977) more care has been exercised in the choice of probe to measure the true cytoplasmic volume (Booth *et al.*, 1979; Kashket, 1981). However, few studies adequately determine the effects of variations in the experimental conditions on cytoplasmic volume, with consequent errors in the measured parameters, $\Delta\psi$ and ΔpH . Accurate quantification requires that all the potential variables should be measured under identical conditions.

The present communication concerns a detailed study of the magnitude of $\Delta\psi$, ΔpH , and cytoplasmic volume under a variety of experimental conditions. The data presented indicate the limitations of some of the probes of $\Delta\psi$ and ΔpH , and are discussed in the context of lactose accumulation in *E. coli* (Booth *et al.*, 1979).

Materials and methods

Materials

All materials were as previously specified (Booth *et al.*, 1979; Kroll & Booth, 1981) with the following additions: $^{86}RbCl$ and $[^{14}C]TPP^+$ were purchased from The Radiochemical Centre, Amersham; $[^3H]TPP^+$ was generously donated by Dr. R. Kaback (Roche, Nutley, NJ, U.S.A.) and Dr. P. Overath

(MPI für Biologie, Tübingen, Germany). Cyanine dye diS-C₃-(5) was a generous gift from Dr. A. S. Waggoner, Amherst College, Amherst, MA 01002, U.S.A.

Bacterial strains

Experiments were carried out using *E. coli* ML308-225 (*lac*⁻*i*⁻*z*⁻*y*⁺*a*⁺) supplied by Dr. I. C. West, University of Newcastle, Newcastle-upon-Tyne, U.K. and *E. coli* TK2240 (*F*⁻, *trk* *D1*, *trk* *A405*, *nag* *A*, *lac* *Z*, *rha*, *thi*) supplied by Dr. W. Epstein, University of Chicago, Chicago, IL, U.S.A.

Growth conditions and EDTA treatment

E. coli ML308-225 was grown and maintained as previously described (Booth *et al.*, 1979). *E. coli* TK2240 was grown on K20 glucose minimal medium, to repress the Kdp K⁺ transport system, as previously described (Kroll & Booth, 1981). EDTA treatment was as previously described (Booth *et al.*, 1979).

Centrifugation based assays

In order to quantify accurately cytoplasmic volumes, $\Delta\psi$, ΔpH , and Δp_{lac} , the concentration of radioactively labelled probe inside the cell must be estimated. This can be done either by monitoring changes in the external concentration using flow dialysis, or by separating the cells from the incubation medium using Millipore filtration or centrifugation. Each of these methods has its advantages and disadvantages, the ultimate choice being determined by the type of experiments to be done. Centrifugation was the main method chosen in this study for the following reasons. It avoids the possible binding of probe either to the filter or to silicone oil. Unlike with flow dialysis, a potential of 0–30 mV (1–3.2-fold accumulation of probe) can be estimated accurately. Finally all parameters can be measured under identical conditions and in parallel.

Lactose transport

Cells (2 mg dry wt./ml) were equilibrated for 5 min at 25°C in a stirred glass vessel with a large surface-to-volume ratio to ensure continuous aeration. [*glucono*- ^{14}C]Lactose (5 μM final concn.; 5.77 Ci/mol) was then added to the cell suspension together with catalase (250 μg /ml final concn.) and 3H_2O (1–2 μCi /ml) as a water marker. After a further 20 min incubation (steady state accumulation under all experimental conditions was achieved by 20 min) 500 μl samples were transferred to Eppendorf centrifuge tubes (1.6 ml capacity) containing 10 μl of 2 vol. H_2O_2 . These were then spun in an Eppendorf centrifuge (model S412) at 12000 g for 20 s. A sample of supernatant (100 μl) was removed and transferred to a centrifuge tube containing a similar pellet of non-radioactive cells. The remaining supernatant was aspirated off with a vacuum line

(the total time taken from sampling to removal of the supernatant was approx. 1 min). The cell pellets were resuspended to a final volume of 300 μ l with the appropriate buffer and then transferred to a vial containing 2 ml of Triton X-100/toluene/PPO/POPOP scintillant (333 ml of Triton X-100, 667 ml of toluene, 4 g of PPO and 100 mg of POPOP). The samples of supernatant and pellet were counted on the preset $^3\text{H}/^{14}\text{C}$ program of a Packard 3255 liquid-scintillation counter. Calculation of the lactose accumulation ratio requires a knowledge of the external water space. Since $^3\text{H}_2\text{O}$ distributes equally throughout all phases, it overestimates the external water space, leading to an underestimation of internal probe concentration. This inaccuracy can be corrected for by a knowledge of c.p.m. of $^3\text{H}_2\text{O}/\mu\text{l}$ of supernatant and $\mu\text{l}/\text{mg}$ dry wt. (cytoplasmic volume).

After correction for contaminating ^{14}C in the cell pellet, the lactose accumulation ratio can be calculated by dividing the internal radioactivity by the external radioactivity and then adjusting for the relevant volume differences.

Measurement of ΔpH

The magnitude of the pH gradient was estimated from the distribution of a weak acid as previously described (Booth *et al.*, 1979). Either [2- ^{14}C]DMO (5 μM final concn.; 28.6 Ci/mol) or [7- ^{14}C]benzoic acid (2 μM final concn.; 24.4 Ci/mol) were used. Assay conditions were identical to those described for lactose transport. ΔpH was calculated as described by Booth *et al.* (1979).

Measurement of $\Delta\psi$

The magnitude of the membrane potential was estimated in the following ways.

(i) *Uptake of radioactive cations.* $^{86}\text{Rb}^+$ or [^{14}C]TPP $^+$ (5 μM and 4 μM final concn.; $\sim 0.2 \mu\text{Ci}/\text{ml}$ and 8.7 Ci/mol respectively) were used. Assay conditions were as described above except for experiments with *E. coli* TK 2240, where cells were incubated with the probes for 10 min before sampling.

(ii) *K^+ distribution by flame photometry.* This was as previously described (Booth *et al.*, 1979) except cells were incubated for 15 min before sampling. A correction based on the uncoupler-insensitive cation binding to cells was applied to (i) and (ii). The membrane potential was calculated by substitution of concentration ratios into the Nernst equation. An activity coefficient for ^{86}Rb , [^{14}C]TPP and K^+ of 1 was assumed.

(iii) *Cyanine diS-C₃-(5) fluorescence.* Measurements were made on a Perkin-Elmer 3000 fluorimeter, with excitation at 638 nm and emission at 670 nm. Assays were carried out with 20 μg dry wt. of cells/ml at 25°C in a 3 ml quartz cuvette. The

cyanine dye was present at a final concentration of 0.166 μM . All additions were made with Hamilton microsyringes and followed by vigorous stirring. To ensure that the dye was responding to $\Delta\psi$ alone, K^+ was added to 150 mM, final concn., at the end of each assay, causing reversal of fluorescence quenching. This concentration of K^+ collapsed $\Delta\psi$ as measured by $^{86}\text{Rb}^+$ distribution under similar conditions.

Cytoplasmic volumes

Cytoplasmic volumes were estimated under all experimental conditions with *E. coli* ML 308–225 as previously described (Booth *et al.*, 1979). In experiments with *E. coli* TK 2240 cytoplasmic volumes were assumed to be 1 $\mu\text{l}/\text{mg}$ dry wt., as only a comparison of $\Delta\psi_{\text{Rb}}$ and $\Delta\psi_{\text{TPP}}$ was being made, rather than absolute values determined. The protocol for measurement of cytoplasmic volumes was as previously described (Booth *et al.*, 1979). Lactose, Rb^+ , TPP $^+$, DMO, and benzoic acid at the concentrations used in this study had no effect on cytoplasmic volumes.

Triple labelling

The simultaneous measurement of $\Delta\psi$ along with ΔpH or lactose accumulation, or $\Delta\psi$ by both [^{14}C]TPP $^+$ and Rb^+ was facilitated by triple labelling. $^3\text{H}_2\text{O}$ was present as the water marker, $^{86}\text{Rb}^+$ was used for $\Delta\psi$ and an appropriate ^{14}C -labelled probe for the third parameter. The Packard 3255 liquid-scintillation counter was set up such that channels counted $^{86}\text{Rb}^+$ alone, $^{86}\text{Rb}^+$ plus ^{14}C and ^{14}C plus ^3H . A small, 1–2%, spillover of $^{86}\text{Rb}^+$ into the ^3H channel was unavoidable, but could be eliminated as a source of error by increasing the ratio of ^3H c.p.m. to ^{86}Rb c.p.m. to approx. 10 to 1. Standard samples of $^{86}\text{Rb}^+$ and ^{14}C were used to calculate the spillover into the lower energy channel. Thus calculation of the accumulation ratio of ^{14}C and ^{86}Rb probes involved a correction of the ^{14}C channel for Rb^{2+} overlap and a subsequent correction of the ^3H channel for ^{14}C overlap using the corrected ^{14}C data. An independent validation of the method was carried out using $^3\text{H}/^{14}\text{C}$ couples and $^3\text{H}/^{86}\text{Rb}^+$ couples for estimation of $\Delta\psi$.

Oxygen generating system

To ensure aerobic conditions were maintained throughout centrifugation-based assays a catalase/ H_2O_2 mixture was used to generate oxygen. The concentrations of catalase and H_2O_2 required to maintain aerobic conditions for 1 min was calculated from anaerobic oxygen pulse experiments. This precaution of maintaining aeration was found to reduce the variability of ΔpH measurements and prevented a 40 mV depolarization of $\Delta\psi$ which was observed in centrifuge tubes lacking H_2O_2 .

Results

Accurate measurements of the parameters of Δp require a detailed knowledge of the cytoplasmic volume and any changes in this volume brought about by manipulation of the cells. Recently we reported that the volume of *E. coli* cells is varied by conditions of growth and experimental protocol (Kroll & Booth, 1981). In the present study the cytoplasmic volume was dependent upon the concentration of the respiratory substrate, succinate, and also upon the external potassium concentration, but independent of the external pH (pH_o). In the absence of succinate the cytoplasmic volume was $1.1 \pm 0.13 \mu\text{l}$ of water/mg dry wt. of cells which was close to the previously reported value (Booth *et al.*, 1979). In the presence of 2mM- and 16mM-succinate, however, the volume increased to 1.39 ± 0.21 and $1.6 \pm 0.19 \mu\text{l}$ of water/mg dry wt., respectively. When cells were treated with EDTA or EDTA/valinomycin the cell volume increased from 1.1 ± 0.13 to 1.8 ± 0.27 and $2.2 \pm 0.25 \mu\text{l}$ of water/mg dry wt., respectively, as the K^+ concentration was raised to 150mM at constant osmolarity. Account must be taken of these changes during measurements of $\Delta\psi$ and ΔpH . Failure to correct for the increase in volume of EDTA/valinomycin-treated cells, for example, would cause $\Delta\psi$ to be overestimated by 20–25mV at high external K^+ concentrations.

Measurement of ΔpH

We compared two acids, DMO and benzoic acid, as indicators of the internal pH as a function of pH_o and in the presence and absence of succinate. Endogenously-respiring cells were found to have a cytoplasmic pH (pH_i) between 6.5 and 8.25 when the external pH was varied from 5.0 to 8.5 (results not shown). DMO indicated a higher value of the intracellular pH than benzoate when pH_o was below 6.5. This we interpret to be due to the sensitivity of the estimation of ΔpH by DMO to small errors in the accumulation ratio at pH_o values below its pK . The equation derived by Waddell & Butler (1959) for ΔpH is as follows:

$$\Delta pH = \log_{10} \left[\left(\frac{C_i}{C_o} \cdot 10^{pK - pH_o} + 1 \right) - 10^{pK - pH_o} \right] \quad (2)$$

where C_i/C_o is the transmembrane concentration ratio of the weak acid. At values of pH_o below the pK of the acid the expression ($10^{pK - pH_o}$) becomes large and any small error in C_i/C_o is magnified. When ΔpH was reduced at $pH_o 5.5$ by either addition of acetylsalicylic acid or the uncoupler tetrachlorosalicylanilide a similar discrepancy was observed between the magnitude of ΔpH as indicated by benzoate and DMO (Fig. 1). At low values of ΔpH the difference increased, as does the effect of

Fig. 1. Comparison of ΔpH as measured by benzoate and DMO at $pH_o 5.5$ in the presence of agents that reduce ΔpH

EDTA/valinomycin-treated cells were incubated in a Tris (5mM)/Mes (5mM)/choline chloride (150mM) buffer system containing 0.2mM-KCl ($pH_o 5.5$) at 2mg dry wt./ml (Booth *et al.*, 1979). The pH gradient was reduced either by tetrachlorosalicylanilide (0–0.8 μM , final concn.) or by acetylsalicylic acid (0–9mM, final concn. at $pH_o 5.5$). The cells were respiring endogenous reserves and were incubated with the probe under the appropriate conditions for 5 min. (●) Tetrachlorosalicylanilide present; (△) acetylsalicylic acid present.

small errors in the measurement of the accumulation ratio (results not shown). Thus, it may be that benzoate more accurately reflects the magnitude of ΔpH at acid values of pH_o .

When 2mM-succinate was added to cell suspensions, the respiratory rate increased 4-fold; however, the effect on ΔpH was small but significant (results not shown). Above $pH_o 5.5$ both benzoate and DMO indicated the same value of ΔpH . This reflected a doubling of the internal concentration of benzoate while that of DMO remained essentially constant. A possible explanation for this observation might be that due to the high concentration of the undissociated form of DMO, this weak acid is relatively insensitive to small changes in intracellular pH. In the presence of succinate, pH_i was relatively constant at $pH_i 7.1$ over the range $pH_o 5.0$ –7.0, but increased rapidly above $pH_o 7.0$ (results not shown). Above $pH_o 7.5$ the pH gradient was inverted and was estimated by weak acid exclusion to be more acid by 0.1 pH units. When the succinate concentration was increased to

16 mM both pH_i and the pH_o at which inversion of the pH gradient occurred were found to move to the more acidic values of 6.5 and 7.0, respectively (results not shown). This may reflect a depression of the internal pH due to significant accumulation of the acidic respiratory substrate. An important feature of this result is that failure to correct for the change in the cytoplasmic volume entirely compensates for the decreased DMO accumulation and pH_i then appears not to be affected by 16 mM-succinate.

Measurement of $\Delta\psi$

The cations, K^+ , Rb^+ and TPP^+ performed almost identically in EDTA/valinomycin-treated cells of *E. coli* ML308-225. Using Rb^+ and TPP^+ , the measurement of $\Delta\psi$ was found to be time-dependent at all values of pH_o . At pH_o 6.0, $\Delta\psi$ increased rapidly to 120 mV and then rose slowly to a steady value of around 140 mV after 15 min (Fig. 2a). On the other hand, at pH_o 8.0 $\Delta\psi$ was initially measured as around 180 mV but then declined so that after 20 min $\Delta\psi$ was only 150–160 mV (Fig. 2b). These characteristics were not a function of EDTA/valinomycin treatment of the cells since similar results were observed in untreated cells with TPP^+ as $\Delta\psi$ probe (Fig. 2a,b). In such cells TPP^+ responded more slowly. The time dependency could not be attributed to properties of either probe individually. At pH_o 6.0 or 8.0 addition of the probe to cells after a period of incubation led immediately to an indicated value of $\Delta\psi$ equal to that which would have been observed had the probe been present from zero time (results not shown). However, the time-dependency could be abolished by increasing the external K^+ concentration to 1 mM

(results not shown) and thus it may relate to some feature of potassium transport in these cells (Kroll & Booth, 1981).

Titration of the membrane potential with K^+ in EDTA/valinomycin-treated cells at either pH_o 6.0 or 8.0 produced a linear relationship between Rb^+ and TPP^+ accumulation between $\Delta\psi$ values of 50 mV and 170 mV (Fig. 3). The slope of the line is 1, but is

Fig. 3. Titration of $\Delta\psi$ with valinomycin/ K^+ using either TPP^+ or Rb^+ as indicator of $\Delta\psi$

EDTA/valinomycin treated cells were incubated at either pH 8.0 (Δ) or pH 6.0 ($\circ$) in a Tris/Mes buffer system in which choline chloride was replaced with KCl to give an external K^+ concentration range from 0.2–150 mM at constant osmolarity.

Fig. 2. Time-dependency of $\Delta\psi$ at (a) pH 6.0 and (b) pH 8.0

($\blacktriangle$) Rb^+ as $\Delta\psi$ probe, ($\circ$), ($\bullet$) TPP^+ as $\Delta\psi$ probe; open symbols denote untreated cells; closed symbols denote EDTA/valinomycin-treated cells. Incubation conditions were as described in the Materials and methods section and in the legend to Fig. 1 except that the incubation pH was adjusted to the appropriate value.

displaced in favour of TPP^+ measurements by approx. 9 mV. In *Bacillus subtilis* TPP^+ is known to bind to the cells (Zaritsky *et al.*, 1981) and this may also be the case in *E. coli* cells. We have calculated that if $\Delta\psi$ measured by Rb^+ is used as a base line then in the range of $\Delta\psi$ from 50 to 170 mV approx. 30% of the TPP^+ in cells would represent binding. This binding appears to be concentration-dependent since increasing the external TPP^+ concentration to 100 μM did not influence the $\Delta\psi$ value (nor did it affect the time course of its accumulation) at pH_o 6.0. When $\Delta\psi$ fell below 50 mV the relationship between Rb^+ and TPP^+ broke down due to high residual TPP^+ in the cells (Fig. 3). This may represent a second component of binding within the cells, which is concentration-independent (Zaritsky *et al.*, 1981).

Even though some binding of TPP^+ may occur, the cation responds very rapidly to changes in $\Delta\psi$. This is indicated in Fig. 4 in which $\Delta\psi$ was rapidly reduced after 10 min incubation by the addition of 5 mM- K^+ to EDTA/valinomycin-treated cells. The value of $\Delta\psi$ indicated by TPP^+ within 1 min of K^+ addition was almost identical with that in cells preincubated with 5 mM- K^+ (Fig. 4). As a control, Rb^+ was included in the same incubation, and it was observed that the two probes were equivalent in their response to the addition of K^+ (results not shown).

We have previously reported that $\Delta\psi$ was pH_o -dependent (Booth *et al.*, 1979) and this was

Fig. 4. Response of TPP^+ to a rapid reduction of $\Delta\psi$ provoked by the addition of 5 mM- K^+ to EDTA/valinomycin-treated cells

EDTA/valinomycin-treated cells were incubated as in Fig. 1, pH_o 7.0, and $\Delta\psi$ was measured at timed intervals. Rb^+ and TPP^+ were present simultaneously, although only data pertaining to TPP^+ are shown. (●) Control, 5 mM-choline chloride added at 10 min (arrow); (○) 5 mM- K^+ added at 10 min; (▲) 5 mM- K^+ present from time zero.

confirmed by the present study with Rb^+ and TPP^+ . There was a slight discrepancy between TPP^+ and Rb^+ accumulation at all values of pH_o , which was abolished by the addition of succinate at either 2 mM or 16 mM final concn. (results not shown). This was brought about by a slightly larger increase in Rb^+ accumulation than that of TPP^+ in the presence of succinate (results not shown). Similarly in a comparison of valinomycin/ K^+ and valinomycin/ Rb^+ in separate experiments, $\Delta\psi$ measured by Rb^+ increased more than that measured by K^+ (results not shown). However, despite these small discrepancies, $\Delta\psi$ measured by the three cations was within a 10 mV span and there was a day-to-day variation of a similar magnitude.

In order to assess the possible involvement of potassium-transport systems in creating the discrepancy between Rb^+ and TPP^+ we investigated a potassium-transport deficient mutant of *E. coli* K-12, TK2240 (Epstein *et al.*, 1978). The mutant strain was treated with EDTA and valinomycin as for ML308-225 and incubated simultaneously with Rb^+ and TPP^+ at a range of pH_o values. The agreement between Rb^+ and TPP^+ was similar to that in *E. coli* ML308-225 when pH_o was used to vary $\Delta\psi$ (Fig. 5). However, $\Delta\psi$ was somewhat lower

Fig. 5. Correlation of $\Delta\psi$ measured by TPP^+ and Rb^+ in *E. coli* TK 2240

E. coli TK2240 was treated with EDTA and valinomycin as for *E. coli* ML308-225 (Booth *et al.*, 1979) and incubated in Tris/Mes/choline chloride/KCl at pH values between pH_o 5.5 and pH_o 8.5 in the presence (Δ) and absence (○) of 2 mM-succinate. Thus the incubation pH was used to vary $\Delta\psi$ (Booth *et al.*, 1979).

in this strain possibly due to errors in the volume, but also due to strain differences (Kroll & Booth, 1981). Thus, the presence of potassium transport systems does not unduly influence the relationship between Rb^+ and TPP^+ as membrane potential probes.

In conclusion, these three cations, which are subject to different limitations of applicability, suggest similar values of $\Delta\psi$ in *E. coli* ML308-225. This study also reveals that other factors may contribute to the magnitude of the indicated $\Delta\psi$.

Cyanine-dye fluorescence

The fluorescent dye cyanine-diS- C_3 -(5) can be used as a membrane potential indicator in *E. coli* cells (Letellier & Schechter, 1979; Ghazi *et al.*, 1981). We studied this probe to ascertain its usefulness as a continuous $\Delta\psi$ indicator. However, while this work was in progress a similar study has been published which is in essence identical with our own (Ghazi *et al.*, 1981) and thus we will only summarize our observations.

We calibrated the quenching of dye fluorescence at 670 nm by valinomycin/ K^+ titration at pH_0 8.0 and found that the relationship was non-linear over the whole range of $\Delta\psi$ from 100 mV to 170 mV. Below 100 mV changes in fluorescence quenching were not evident. In a time course there was no time dependency of $\Delta\psi$, but the dye did respond in the predicted manner to addition of 5 mM- K^+ (results not shown). On the other hand, addition of 4 mM-lactose did not depolarize the cells, which was observed to occur with either TPP^+ or Rb^+ as $\Delta\psi$ probe. Thus, the dye responded rapidly to large changes in $\Delta\psi$ but did not appear to be sensitive to smaller slow changes. The quenching of dye fluorescence showed a similar dependence on pH_0 as $\Delta\psi$ measured by other probes, but below pH_0 7.0 the values indicated on the basis of calibration at pH_0 8.0 were some 20 mV lower. Thus, although the cyanine dye indicates broadly similar characteristics of $\Delta\psi$ its quantification appears to be of very limited validity.

Lactose transport

The uptake of lactose was measured under the same conditions as $\Delta\psi$, ΔpH and cytoplasmic volume and was found to reach a steady state at around 20 min. The accumulation, Δp_{lac} , was similar to that observed previously (Booth *et al.*, 1979) and was only significantly stimulated by succinate between pH_0 5.0 and 6.5 (results not shown). The relationship of the lactose accumulation to Δp ($\Delta p_{\text{lac}}/\Delta p$) has been defined as the 'effective or apparent stoichiometry' (Booth, 1981). As can be seen, the value of the apparent stoichiometry depends to a significant extent on the experimental conditions (Fig. 6) and upon the probes used. In the

Fig. 6. Variation of the apparent stoichiometry ($\Delta p_{\text{lac}}/\Delta p$) as a function of pH_0 .

The apparent stoichiometry was calculated from the lactose accumulation (results not shown) and the magnitude of $\Delta\psi$ (Rb^+) and ΔpH (benzoate). The incubation system was as described in Fig. 1 with the pH of the buffer system adjusted to the appropriate pH value. (O) endogenously-respiring cells, ($\blacktriangle$) cells respiring succinate (2 mM). All measurements were made at the steady state, 20 min after the addition of lactose.

presence of 2 mM-succinate, the least equivocal results were obtained since benzoate and DMO were in agreement on ΔpH , and Rb^+ and TPP^+ similarly agreed on $\Delta\psi$. Thus in this instance the apparent stoichiometry is markedly pH_0 -dependent, rising from 0.68 at pH_0 5.0 to 0.94 at pH_0 8.0. In endogenously-respiring cells the apparent stoichiometry is dependent upon which probes are used for ΔpH and $\Delta\psi$. Using the lowest values (benzoate and Rb^+ , respectively) the apparent stoichiometry was largely pH_0 -independent between pH_0 5.0 and pH_0 7.0 at around 0.82; above pH_0 7.0 the apparent stoichiometry increased to 0.96 at pH_0 8.0 (Fig. 6). Notwithstanding these differences the conclusion is that the bulk phase Δp is adequate to account for the lactose accumulation observed in cells.

Discussion

The data presented in this paper confirm and extend our earlier observations on the relationship between Δp and lactose accumulation (Booth *et al.*, 1979; Booth & Hamilton, 1980b; Booth, 1980). However, the importance of this study is that it establishes that independent measurements of $\Delta\psi$ and ΔpH using different probes corroborate each other. With respect to $\Delta\psi$ probes this has been lacking from previous studies (Ghazi *et al.*, 1981) which also fall down particularly on assumptions relating to the cytoplasmic volume. In the present study $\Delta\psi$, ΔpH , cytoplasmic volume and lactose

transport have been examined under the same conditions, using centrifugation-based assays.

The cytoplasmic volume of cells was found to be a significant experimental variable. This was shown most markedly when $\Delta\psi$ was titrated to below 50 mV, where underestimation of the cell volume leads to overestimation of $\Delta\psi$ by up to 30 mV. Similarly, if the volume is overestimated this may lead to an underestimate of the measured parameter. With regard to ΔpH , the effect of volume changes is most dramatic when the accumulation of the weak acid is small, as the case of DMO at $\text{pH}_0 6.5$ in the presence of 16 mM-succinate. Failure to correct for the volume changes raise ΔpH from 0.31 units to 0.53 units. Thus, it is essential in quantitative studies of bioenergetics that the cytoplasmic volume be treated as an experimental variable.

In an earlier study of ΔpH in endogenously-respiring cells we used DMO and a membrane-filtration technique to separate the cells from the incubation medium. However, a subsequent study revealed that DMO bound in a pH-dependent fashion to membrane filters (C. V. Salmond, W. A. Hamilton & I. R. Booth, unpublished results). The present study confirms the general trend of our earlier results and indeed emphasizes the lack of a constant internal pH in endogenously-respiring cells. Two weak acids of dissimilar structure and different pK values gave close agreement at pH_0 values above their pK 's. DMO at an incubation pH below its pK gave slightly higher values than did benzoic acid and this may be related to the sensitivity of the assay to small errors under these conditions. Benzoic acid is a food preservative; however, at the concentration used it did not lower ΔpH as measured by DMO (C. V. Salmond, W. A. Hamilton & I. R. Booth, unpublished results). The failure of DMO to show an increase in ΔpH when succinate was added to the cells may reflect its insensitivity to small changes in ΔpH when used at pH_0 values below its pK . When ΔpH was titrated with uncoupler or acetylsalicylic acid at $\text{pH}_0 5.5$, the discrepancy between ΔpH measured by DMO and that indicated by benzoic acid increased as ΔpH decreased (Fig. 1). This appears to confirm the suggestion that DMO is not wholly reliable at such low values of pH_0 .

With cells respiring succinate (2 mM), pH_i was maintained relatively constant and above $\text{pH}_0 7.5$ ΔpH was inverted to become acid inside. This explains one of the significant differences between our previous work (Booth *et al.*, 1979) and that of other groups (Padan *et al.*, 1976; Zilberstein *et al.*, 1979). Our earlier failure to find ΔpH inversion was with endogenously-respiring cells, whereas the other groups provided succinate as a respiratory substrate. However, there is still a slight discrepancy in the actual value of pH_i .

The two probes of $\Delta\psi$ which were investigated in detail, Rb^+ and TPP^+ , gave reasonable agreement. There may be a significant binding of TPP^+ since it registered higher values of $\Delta\psi$ in endogenously-respiring cells and its accumulation was not completely abolished by 150 mM- K^+ /valinomycin (Fig. 3). This, of course, presupposes the validity of the valinomycin/ Rb^+ measurements. Certainly any error in the Rb^+ measurement does not appear to be due to K^+ transport systems, since in the mutant lacking such activities a similar discrepancy between TPP^+ and Rb^+ was observed. However, one disturbing feature of Rb^+ in *E. coli* ML308-225 was that its accumulation increased relative to TPP^+ or K^+ in the presence of succinate (results not shown). We do not have an adequate explanation for this at present.

On the whole Rb^+ and TPP^+ gave similar values of $\Delta\psi$, showed similar time dependencies and responded identically to sudden partial depolarization of the membrane. Thus, it seems probable that TPP^+ may be a quantitatively useful $\Delta\psi$ indicator in whole cells that have not been treated with EDTA and valinomycin. Most importantly, the $\Delta\psi$ values observed with TPP^+ in untreated cells were very similar (within 11 mV) to the magnitude of $\Delta\psi$ in treated cells. In the succeeding paper we will illustrate the usefulness of this in further examining energy coupling to lactose accumulation in *E. coli*.

The authors thank Professor W. A. Hamilton for his generosity and encouragement throughout this work. We also acknowledge our co-workers Dr. R. G. Kroll, Mrs. C. V. Salmond and Miss F. Thomson for their helpful advice, and Dr. D. Burke, Department of Pharmacology, for the use of the fluorimeter. Thanks are also due to Dr. R. M. Macnab, Dr. R. Kaback, Dr. P. Overath, Dr. D. Nicholls for preprints of their papers, chemicals and advice. S. A. is in receipt of financial support from the Science Research Council (grant GR/A.99299) to whom we express our thanks.

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