Stoichiometry of the H^+-ATPase of Growing and Resting, Aerobic *Escherichia coli*†

Eva R. Kashket

ABSTRACT: The H^+/ATP stoichiometry of the proton-translocating ATPase was investigated in growing and nongrowing, respiring cells of *Escherichia coli*. The protonmotive force, Δp, was determined by measuring the transmembrane chemical gradient of protons, ΔpH, from the cellular accumulation of benzoate anions, and the electrical gradient, $\Delta\Psi$, from the accumulation of the lipophilic cation tetraphenylphosphonium (TPP^+). The accumulation of lactose was also used to calculate the Δp in this lactose operon constitutive, β-galactosidase negative mutant. The phosphorylation potential, $\Delta G_p'$, was determined by measuring the cellular concentration of ATP, ADP, and inorganic phosphate. According to chemiosmotic principles, at steady state the phosphorylation potential is in thermodynamic equilibrium with the protonmotive force, and thus the ratio $\Delta p/\Delta G_p'$ can be used to determine the H^+/ATP ratio. Respiring *E. coli* cells, in mid-exponential phase of growth or incubated in buffer, at external pHs from 6.25 to 8.25 had a constant $\Delta G_p'$ of about 500 mV. The H^+/ATP ratio was found to be 3 when the Δp value derived from lactose accumulation levels was used. However, when the Δp values derived from ΔpH and $\Delta\Psi$ were used in the calculations, the H^+/ATP ratio varied from about 2.5 at external pH 6.25 to about 4 at pH 8.25. Arguments are presented for the hypothesis that the $\Delta\Psi$ values obtained from the TPP^+ measurements are likely to be inaccurate and that a value of 3 H^+/ATP, independent of the external pH, is likely to be the valid stoichiometry.

In the respiring bacterial cell a reversible H^+-translocating adenosinetriphosphatase (ATPase) catalyzes the phosphorylation of ADP to ATP in a reaction coupled to the flow of protons into the cells down their electrochemical gradient (Mitchell, 1961, 1966; Harold, 1972, 1977). The proton gradient, $\Delta\tilde{\mu}_{H^+}$,[1] generated by the electrogenic pumping of H^+ out of the cell during respiration, consists of a transmembrane electrical potential, $\Delta\Psi$, and a transmembrane pH gradient, ΔpH. These two components of the protonmotive force, PMF or Δp, are added when the interior of the cells is alkaline and negative with respect to the exterior by the relationship Δp (in mV) = $\Delta\Psi - Z\Delta pH = \Delta\tilde{\mu}_{H^+}/F$, where ΔpH is pH_{out} (pH of the bulk medium) minus pH_{in} (pH of the cytosol); the factor Z is $2.303RT/F$ and equals 59 mV at 25 °C; R, T, and F have the usual meanings.

A question of current interest is the H^+/ATP stoichiometry of the ATPase, since this bears on the mechanism by which proton translocation is coupled to phosphorylation in the reaction catalyzed by the ATPase: $nH^+_{out} + ADP + P_i \rightleftharpoons ATP + H_2O + nH^+_{in}$. On the basis of the chemiosmotic theory's tenet that a thermodynamic equilibrium exists under steady-state conditions, the H^+/ATP stoichiometry can be calculated from a comparison of the protonmotive force and the phosphorylation potential. The relationship $n = H^+/ATP = \Delta G_p'/(\Delta pF)$ is used, where $\Delta G_p'$, the phosphorylation potential, is the Gibbs free energy of ATP synthesis and equals $\Delta G_0' + RT \ln [[ATP]/([ADP][P_i])]$; $\Delta G_0'$ is the standard free energy of ATP hydrolysis (reviewed by Slater, 1979).

The H^+/ATP stoichiometry has been estimated in a number of systems and found to vary widely. Reported values for n include 2 or 3 H^+/ATP for mitochondria and 3 for chloroplasts (reviewed by Fillingame, 1980), 2–5 for bacterial chromatophores (reviewed by Baccarini-Melandri et al., 1981), 3 for resting cells of *Paracoccus denitrificans* (McCarty et al., 1981), and up to 8 for the alkalophile *Bacillus alkalophilus* (Guffanti et al., 1981). Direct measurements of H^+ influx and ATP synthesis by *Streptococcus lactis* under an imposed PMF showed a stoichiometry of 2, while steady-state glycolyzing cells extruded 3 H^+/ATP hydrolyzed (Maloney & Saitta, 1982).

In the experiments reported here the PMF and $\Delta G_p'$ were measured in growing and nongrowing, respiring cells of *Escherichia coli*. Strain ML 308-225 cells, which are constitutive for the *lac* operon and deficient in β-galactosidase, were chosen, so that the PMF could be measured from the lactose accumulation at steady state, as well as from the ΔpH and $\Delta\Psi$. The PMF was modulated by varying the pH_{out}. The stoichiometry was found to be 3 H^+/ATP independently of the medium pH when Δp_{lac} was used for calculating n but varied from 2.6 to 4 H^+/ATP between pH_{out} 6 and 8, when Δp ($\Delta\Psi$ plus ΔpH) was used for the calculations.

Materials and Methods

Materials. The radioactive materials were obtained from New England Nuclear Corp., Boston, MA, except for [D-*glucose*-1-^{14}C]lactose ([^{14}C]lactose), which was bought from Amersham Corp., Arlington Heights, IL. All other chemicals were reagent grade and are available commercially.

Growth of Cells. *E. coli* ML 308-225 (*lac* $i^- z^- y^+ a^+$) cells were grown aerobically with rapid shaking at 28 °C at pH 6.25 or pH 7.25 in medium 63 (Cohen & Rickenberg, 1956) and at pH 7.25 in medium Y, which consists of 40 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) adjusted to pH 7.25 with NaOH, 10 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 4 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), 0.5 mM L-arginine, 2 μM thiamin, 2 μM ferrous sulfate, 1.5 mM

† From the Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118. *Received May 18, 1982.* This work was supported by Grant PCM 80-01178 from the National Science Foundation.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; TPP^+, tetraphenylphosphonium ion; $\Delta\Psi$, transmembrane electrical gradient; ΔpH, transmembrane chemical gradient of H^+; pH_{in}, internal pH; pH_{out}, external pH; $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient; Δp, $\Delta\tilde{\mu}_{H^+}/F$ or protonmotive force (PMF); Δp_{lac}, $\Delta\mu_{lac}/F$; $\Delta G_p'$, phosphorylation potential; $\Delta G_0'$, free energy of ATP hydrolysis.

0006-2960/82/0421-5534$01.25/0

sodium phosphate, 5 mM KCl, and the "micronutrient" mixture of trace salts of Neidhardt et al. (1974). For growth at pH 8.25, medium AA was used; this was similar to medium Y but lacked Tricine and instead of Mops contained 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) and 50 mM 3-(cyclohexylamino)propanesulfonic acid (Caps) adjusted to pH 8.2 with NaOH or KOH. The media were supplemented with 0.5% glycerol, and the pH was adjusted as needed with KOH or HCl. Cells were also grown in medium Y with 0.5% succinate instead of glycerol, but no differences were seen in any of the parameters studied.

Measurements of PMF in Growing Cells. E. coli cells, inoculated from overnight cultures grown in the same medium as that used in the experiment, were allowed to double at least twice to mid-exponential phase (density of A_{625nm} = 1.0).

Radioactive probes were added to final concentrations as follows: for ΔpH, [^{14}C]benzoate (0.16 μCi/mL, 6 μM) or [^{14}C]methylamine (0.3 μCi/mL, 5 μM); for ΔΨ, [^{3}H]tetraphenylphosphonium bromide ([^{3}H]TPP$^+$) (0.17 μCi/mL, 1 μM); for Δp_{lac}, [D-*glucose*-1-^{14}C]lactose ([^{14}C]lactose) (0.5 μCi/mL, 12.5 μM); for evaluating trapped medium on the filters, [1,2-$^3H_2$]poly(ethylene glycol) ([^{3}H]PEG) (M_r 4000; 0.83 μCi/mL, 0.34 mM). Fifteen minutes later samples of 0.2–0.4 mL were removed from the cultures (total volume of 6 mL) shaking in 50-mL Erlenmeyer flasks at 28 °C, after steady-state accumulations of the probes had been reached. Samples also were taken for measuring the external pH. Growth continued to be monitored after sampling to check that the addition of the probes had not impaired the cells' metabolism. The samples were filtered through polycarbonate membrane filters (0.45-μm pore size) to separate the cells from the medium and counted for radioactivity as described previously (Kashket, 1981). The volume of contaminating medium left on the filters was measured in parallel cultures to which [^{3}H]PEG had been added.

For [^{3}H]TPP$^+$ uptake measurements, the cells were treated with ethylenediaminetetraacetic acid (EDTA), which was added at the same time as the radioactive probes. The concentrations of EDTA necessary to give maximal [^{3}H]TPP$^+$ uptake depended on the pH of the medium. In a previous publication (Kashket, 1981) it was shown that the addition of 5 mM EDTA at approximately pH 7 did not affect [^{3}H]-TPP$^+$ accumulation but did result in increased sensitivity of the cells to gentamicin, which suggested that the chelator had been effective in increasing the access of the antibiotic to the cells. However, in the current experiments, addition of EDTA to concentrations above 5 mM increased the uptake of [^{3}H]TPP$^+$. Therefore, various concentrations of EDTA were added until maximal [^{3}H]TPP$^+$ uptake was observed. With cultures growing at pH 6.25, EDTA was added to 10–50 mM; cultures growing at pH 7.25 were supplied with 10–25 mM EDTA, and those at pH 8.25, with 5–10 mM chelator. These EDTA additions resulted in decreased growth rates, and further EDTA addition stopped growth and reduced [^{3}H]TPP$^+$ accumulation. Only the cultures still growing exponentially are considered here. The uptakes of [^{14}C]benzoate and [^{14}C]lactose by cells treated with EDTA at concentrations corresponding to those used in the ΔΨ assays were the same as those without added chelator, showing that the treatment with EDTA had not affected the cells' integrity as long as growth continued.

Because relatively dense cultures are needed for detecting ADP in extracts by the HPLC technique, in the present experiments cells were sampled later in exponential phase (at A_{625nm} > 1.0, compared to A_{625nm} = 0.4 in earlier studies), when lower ΔΨ values are seen, compared to the previous studies (Kashket, 1981). Also, binding of TPP$^+$ by polycarbonate filters differed for different lot numbers; thus the filter-bound counts were assayed in each experiment and the counts subtracted from the counts in the filtered cell samples before calculating ΔΨ. Thus, the values in the present studies are lower by about 30 mV than those reported previously for aerobically growing *E. coli* cells (Kashket, 1981).

Resting Cell Preparations. For experiments with nongrowing, respiring cells, *E. coli* were grown to mid-exponential phase, harvested by centrifugation at 4 °C for 10 min at 10000*g*, suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, and treated with 2 mM EDTA for 2 min at 37 °C, based on the method of Leive (1968). The cells were recentrifuged and resuspended in 0.1 M Tris buffer adjusted to pH 6.25 with citric acid (TCB buffer), 0.1 M maleic acid adjusted to pH 6.25 with NaOH, or 0.1 M Mops adjusted to pH 7.25 or 8.25 with KOH, to a cell density of 3.8 mg dry weight/mL (A_{625} = 10). Assays of ΔpH, ΔΨ, and Δp_{lac} were carried out by incubating the cells at 0.38 mg dry weight/mL in buffer, supplemented with 1 mM sodium phosphate, 0.5% glycerol, and the radioactive probes. After a 15-min incubation with rapid shaking at 28 °C, the cells were sampled as with growing cells.

Determination of Cellular Adenine Nucleotide Concentrations. Acid-soluble nucleotides were extracted from 2.5 mL of shaking cultures or incubating resting cells by rapidly adding 0.5 mL of ice-cold 15% trichloroacetic acid (Cl_3CCOOH) which contained 8 nmol of xanthosine diphosphate (XDP) as an external marker. After 30 min at 0 °C the precipitated cell suspension was centrifuged at 4 °C for 10 min at 10000*g*. The Cl_3CCOOH was removed by vigorous extraction of the supernatant fluid with tri-*n*-octylamine in Freon 113 by the method of Khym (1975), and the aqueous extracts were kept at −80 °C. The procedure of Chen et al. (1977) for high-pressure liquid chromatography (HPLC) was followed, using a Whatman Partisil-10 SAX column with a Water Associates instrument, as described by Rapaport et al. (1979). A nonlinear gradient (number 8 on the Model ALC 202 instrument) from 7 mM K_2HPO_4, pH 4.5, to 0.5 M K_2HPO_4, pH 4.0, over 35 min was used at ambient temperature, at a flow rate of 1.5 mL/min. The identity of the peaks was confirmed by cochromatography with standards, and the recovery of ATP added to the cultures just before Cl_3CCOOH addition was over 95%. There was no ADP or ATP in the growth media. It was necessary to extract the entire cultures because extraction of filtered cells resulted in lower ATP and higher ADP levels (not shown). Addition of EDTA to the cultures obscured the ADP peak but had no effect on the ATP peak; hence, the nucleotide levels were measured in cultures not treated with EDTA.

Determination of Cellular Inorganic Phosphate. Cell samples were filtered as for the PMF measurements, the collected filters were extracted with 5% (w/v) ice-cold Cl_3C-COOH, and the precipitated material was removed by centrifugation as for nucleotide extracts. The volume of the trapped medium on the filters was measured with [^{3}H]PEG. The Cl_3CCOOH extracts and the extracellular medium, collected by centrifuging the cells through silicone oil (Kashket et al., 1980), were assayed for inorganic phosphate by the method of Ames & Dubin (1960). The internal phosphate values of cells growing in low phosphate (media Y and AA) were used to calculate the phosphorylation potentials; these intracellular phosphate levels were similar to those of cells growing in medium 63, which contained 100 mM phosphate, but there was less variability in the values because less ex-

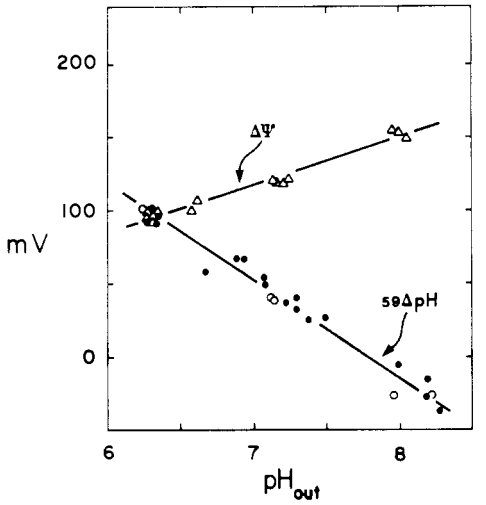

FIGURE 1: Effect of medium pH on ΔpH and ΔΨ of mid-exponential phase *E. coli* ML 308-225 cells growing under aerobic conditions. The experiment was carried out as described under Materials and Methods. Each value is the mean of triplicate determinations from separate cultures. The standard deviations were 0.1–12 mV for 59ΔpH and 0.6–5 mV for ΔΨ. The unfilled symbols are values from cells treated with EDTA, as described under Materials and Methods.

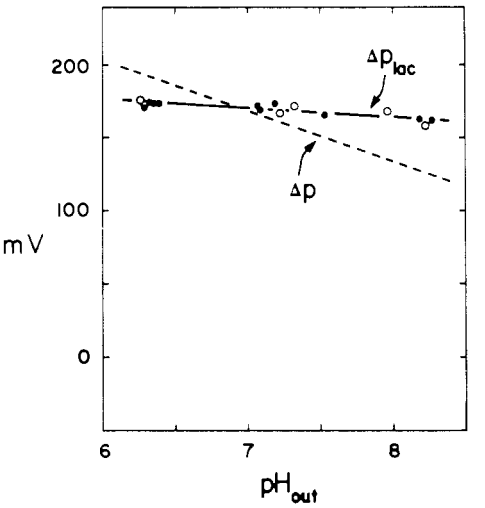

FIGURE 2: Effect of medium pH on the lactose accumulation and Δp of mid-exponential phase aerobic *E. coli*. The experiments were carried out as described under Materials and Methods. The Δp_{lac} values were obtained from EDTA-treated (○) and untreated (●) cultures, and the regression line was calculated. The dashed Δp line was calculated from the regression lines of the ΔΨ and 59ΔpH points in Figure 1.

tracellular inorganic phosphate was present on the filters.

Calculations. The ΔpH, ΔΨ, Δp, and Δp_{lac} values were calculated by using the Nernst equation, as described previously (Maloney et al., 1975; Kashket et al., 1980). The intracellular volume was taken to be 1.63 μL/mg dry weight cells (Kashket, 1981). The radioactive counts on the filters were corrected for counts due to trapped medium, and for ΔΨ measurements, the [^{3}H]TPP counts bound to the filters and nonspecifically bound to cells treated with butanol to disrupt the membrane (Kashket, 1981). The phosphorylation potential, $\Delta G_p'$, was calculated as described by Slater (1979) by using the value 7.2 kcal/mol for the $\Delta G_0'$.

Results

E. coli strain ML 308-225 cells were chosen because the absence of β-galactosidase and the constitutivity of the lactose operon offer the opportunity of using the accumulation of lactose as an index of the protonmotive force during exponential growth. The components of the PMF were varied by varying the pH of the medium. Other methods used to modulate the PMF in resting cells or in membrane vesicles such as inhibition of respiration (Sprott & Usher, 1977) or ionophorous antibiotics cannot be used with growing cells. In the present experiments, as shown previously under different growth conditions (Kashket, 1981), *E. coli* cells growing aerobically maintained a large PMF when growing at pHs from 6.25 to 8.25 (Figures 1 and 2). The contribution of ΔpH to the PMF was dependent on the pH_{out} and was equivalent to 100 mV (alkaline inside) when the pH_{out} was 6.25 and decreased to 0 when the pH_{out} was 7.8 (Figure 1). At higher pHs, the interior of the cells became more acidic, reaching a value of −30 mV at pH_{out} of 8.25. Thus, the growing cells maintained a constant internal pH of 7.8 (SD = 0.15) at all the medium pHs tested. The ΔΨ values also showed a dependence on pH_{out}, increasing from 94 mV (negative inside) at pH 6.25 to 157 mV at pH 8.25 (Figure 1).

The Δp values calculated by adding the ΔpH and the ΔΨ components were compared to the Δp_{lac} values obtained in cells growing under the same conditions (Figure 2). When the medium pH was about 7 or lower, the Δp exceeded Δp_{lac}, but at higher pH the reverse was true.

Table I: Stoichiometry of the H$^+$-ATPase of Mid-Exponential Phase, Aerobically Growing *E. coli*[a]

	external pH		
	6.25	7.25	8.25
$[ATP]_{in}$ (mM)	3.9 ± 0.3	3.3 ± 0.4	2.7 ± 0.4
$[ADP]_{in}$ (mM)	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.2
$[P_i]_{in}$ (mM)	7.9 ± 1.2	5.6 ± 0.5	6.9 ± 4.3
$\Delta G_p'$ (kcal/mol)	11.4	11.6	11.5
(mV)	494	501	499
Δp_{lac} (mV)	175	169	163
n (from Δp_{lac})	2.83	2.97	3.05
Δp (mV)	194	160	127
n (from Δp)	2.55	3.13	3.93

[a] The values for intracellular nucleotides and inorganic phosphate are means ± standard deviations of the means of two to five cultures. The Δp_{lac} and Δp values are from Figure 2.

For determination of the phosphorylation potential, the adenine nucleotides and inorganic phosphate contents were determined in cells growing under similar conditions. There was no significant difference in the cellular concentrations of these molecules in the various cultures (Table I). The values obtained fall within reported ranges for bacterial cells (summarized by Karl, 1980). The $\Delta G_p'$ values calculated from these data were 11.4–11.6 kcal/mol (494–501 mV), showing that there was no significant effect of medium pH on $\Delta G_p'$.

The stoichiometry of the H$^+$-translocating ATPase in growing cells, therefore, was 2.83–3.05 H$^+$/ATP when Δp_{lac} was used to measure the PMF (Table I). The values at pH 6.25–8.25 were not significantly different. However, when Δp values were used, the ATPase apparently translocated 2.55 H$^+$/ATP at pH 6.25, 3.13 H$^+$/ATP at pH 7.25, and 3.93 H$^+$/ATP at pH 8.25.

Similar results were obtained with nongrowing, respiring cells (Table II). Again, n was equal to about 3 when Δp_{lac} values were used for the PMF, while pH-dependent n values varying from 2.70 at pH 6.25 to 3.95 at pH 8.25 were calculated from the Δp measurements. Interestingly, respiring cells had similar PMF and $\Delta G_p'$ values whether growing or not, as is seen by comparing Tables I and II.

It should be noted that the value 1.63 μL/mg of cells was used for the intracellular volume in all the calculations in this report, although it is known that factors such as energization

Table II: Stoichiometry of the H^+-ATPase of Nongrowing, Respiring *E. coli*[a]

	external pH		
	6.4	7.3	8.1
$\Delta G_p'$ (mV)	529	520	521
Δp_{lac} (mV)	173	175	166
n (from Δp_{lac})	3.05	2.97	3.13
$59\Delta pH$ (mV)	56	14	−39
$\Delta\Psi$ (mV)	138	151	171
Δp (mV)	194	165	132
n (from Δp)	2.70	3.15	3.95

[a] The intracellular concentrations in two to nine cultures were 1.9–2.7 mM ATP (SD = 0.5), 0.04–0.11 mM ADP (SD = 0.04), and 4–14 mM P_i (SD = 2.0). The pH_{in} was 7.3–7.5.

affect the degree of plasmolysis in *E. coli* (Robbie & Wilson, 1969; Ahmed & Booth, 1981). However, when the data shown in Table I were recalculated on the basis of 2.2 μL/mg of cells, the cell water value reported by Ahmed & Booth (1981) for the same strain of *E. coli* cells after treatment with EDTA, the n values were not significantly different from those obtained with the 1.63 μL/mg of cells value: at pH_{out} of 6.25, 7.25, and 8.25, the n (from Δp_{lac}) increased to 3.0, 3.1, and 3.2, respectively, while the n (from Δp) increased to 2.8, 3.5, and 4.5, respectively.

Discussion

The discrepancy between the values for the H^+/ATP stoichiometry calculated from lactose accumulation and those from Δp can be explained most simply by one of two possibilities: first, the stoichiometry of the lactose carrier and of the H^+-ATPase may vary depending on the external pH; second, the Δp values measured with the ionic probes may not be accurate.

The conclusion that the H^+/lactose stoichiometry varies with medium pH was first reached by Ramos & Kaback (1977) for *E. coli* membrane vesicles and recently by ten Brink et al. (1981) for intact, respiring cells. Under steady-state conditions the stoichiometry apparently increased from 1 H^+/lactose at about pH 5 to 2 H^+/lactose at about pH 8. However, other workers concluded that the membrane carrier in intact cells translocates 1 H^+/lactose at all external pHs from 5 to 8 (Zilberstein et al., 1979; Booth et al., 1979; Felle et al., 1980) in similar, steady-state conditions. Another approach, the direct measurements of unidirectional H^+ and lactose influxes into deenergized cells, also gave a stoichiometry of 1 H^+ and lactose, at exterior pH values up to pH 7.8 (West & Mitchell, 1973; Zilberstein et al., 1979; Booth et al., 1979). In the present experiments the H^+/lactose stoichiometry in both growing and nongrowing cells apparently varies from 0.9 at pH 6.3 to 1.3 at pH 8.2.

The second possibility, that the measured Δp values are incorrect, at least under some conditions, is based on doubts about the accuracy of the $\Delta\Psi$ values obtained with ionic probes. The ΔpH measurements can be considered as valid because the measured internal pH, which remained constant at 7.4–7.8 at all the external pHs tested, is in agreement with the values measured in respiring resting cells by ^{31}P NMR spectroscopy, a noninvasive technique (Slonczewski et al., 1981).

The $\Delta\Psi$ values are considered by many investigators to be the least accurate parameter measured in PMF evaluations. In bacterial cells $\Delta\Psi$ is measured most commonly from the accumulation of permeant cations such as TPP^+, or with membrane potential sensitive fluorescent cationic dyes [reviewed by Maloney et al. (1975), Rottenberg (1979), and Waggoner (1976)]. The Nerst equation is used to calculate steady-state $\Delta\Psi$ in such experiments, if a number of conditions are met: the cations may not be transported actively, they must not alter the cells' metabolism significantly, and if they are bound nonspecifically to cell components, that fraction of the total cell-associated cations must be known. It is increasingly evident that the last requirement may not be met in many cases, and hence, the values for $\Delta\Psi$ may be sufficiently inaccurate to lead to incorrect stoichiometries.

In most published studies the extent of nonspecific binding of cationic probes has been measured in control cells whose membranes were disrupted by treatment with butanol or toluene or in cells deenergized with ionophores such as carbonyl cyanide (trifluormethoxy)phenylhydrazone plus nigericin. The accumulation of the lipophilic cation is then calibrated with the K^+ diffusion potential in deenergized, valinomycin-treated cells. In cells, such as *Streptococcus lactis*, which are energy depleted without treatment with ionophores, the accumulation of TPP^+ matches that of K^+ in the presence of valinomycin, after corrections have been made for the TPP^+ bound to butanol-treated cells (Kashket et al., 1980). However, when *S. lactis* cells were energized by adding a fermentable sugar, such a $\Delta\Psi$ calibration could not be carried out, since the cellular K^+ was not in equilibrium with the $\Delta\Psi$, in spite of the presence of valinomycin. This was due, presumably, to the pumping of K^+ by a high-energy phosphate bond-driven K^+ transport system (E. R. Kashket, unpublished experiments). Thus, PMF-independent nonspecific binding of cationic probes can be estimated, but these experiments do not measure any PMF-dependent binding of these probes.

When the cells are energized, it is possible that the extent of nonspecific binding of lipophilic cations is different from that found in nonenergized cells. Binding of TPP^+ by deenergized *E. coli* cells has been shown to be influenced by pH and to be concentration dependent (ten Brink et al., 1981). Indeed, these authors concluded that the H^+/lactose stoichiometry is pH_{out} dependent after correcting the TPP^+ taken up by energized cells for nonspecific binding of the probe. Extensive TPP^+ binding in mitochondria has been inferred (Shen et al., 1980), as has $\Delta\Psi$-dependent nonspecific binding in *E. coli* (Ghazi et al., 1981). In *Bacillus subtilis*, but not in *E. coli*, there is potential-dependent binding of lipophilic cations apparently at the cell surface, in addition to the potential-effected uptake into the cytoplasm (Zaritsky et al., 1981). In principle, techniques used to measure $\Delta\Psi$ by following the TPP^+ concentrations in the medium such as flow dialysis (Ramos et al., 1976) or TPP^+-sensitive electrodes (Hirota et al., 1980) also would not discern variable, PMF-sensitive nonspecific binding of lipophilic cations. Nor is there any reason to suppose that cyanine dyes, the $\Delta\Psi$-sensitive fluorescent probes (Waggoner, 1976), would not present similar problems, since they are also lipophilic cations. It should be emphasized, however, that at near neutrality the Δp and Δp_{lac} agree fairly well. Since concentration-dependent binding of TPP^+ by deenergized cells is relatively more significant at low TPP^+ concentrations (ten Brink et al., 1981), the calculated $\Delta\Psi$ values are probably overestimated to a greater degree at low pH_{out} than at high pH_{out}. Such corrections for bound TPP^+ would result in less pH-dependent Δp values, resembling Δp_{lac} more closely.

From the results presented here, I speculate that the Δp_{lac} is the more correct value for PMF in both growing and nongrowing *E. coli* cells and, from this, that the H^+/ATP stoichiometry is 3 and is independent of the external pH. Indeed, since the $\Delta G_p'$ is not affected by the external pH, a variable

H^+/lactose stoichiometry would require that the H^+/ATP stoichiometry vary similarly with external pH. It is difficult to envision that the lactose carrier and the H^+-translocating ATPase, which are such different membrane-associated proteins, would change their mechanism of action in a similar manner when the pH on one side of the membrane (the internal pH is constant) changes.

Flux studies with *S. lactis* (Maloney & Hansen, 1982) showed a 2 H^+/ATP stoichiometry, while glycolyzing cells had a 3 H^+/ATP steady-state value (Maloney & Saitta, 1982). These authors attribute the difference to the net effect of an inward leak of 1 H^+, a 2 H^+/ATP stoichiometry being the basic value for the ATPase per se. It is difficult to envisage a constant net contribution of 1 H^+ to the sum of all the other H^+-linked transport systems operating in the growing and nongrowing *E. coli* cell. One would expect that, at least for the maintenance of a constant internal pH, the transport activities of other ions involved would be different as the medium pH is altered. For example, Na^+/H^+ antiporter activity is required for maintenance of a constant pH_{in} only at pH_{out} >7.6–7.8 (Zilberstein et al., 1982).

Finally, in *E. coli* cells growing aerobically in various minimal salts media over a range of 2 pH units, the internal pH remained constant, as did the concentrations of ATP, ADP, and inorganic phosphate. Thus, the growing cells' metabolism is regulated to maintain homeostasis. Resting, respiring cells maintained a similar proton gradient and phosphorylation potential, which indicates that the energetic demands of growth such as synthesis of macromolecules were met by the growing cells. It appears, therefore, that the transduction of metabolic energy to and from a transmembrane proton gradient is not limiting for bacterial growth under respiratory conditions.

Acknowledgments

I thank Y. P. Luong for excellent technical assistance, E. Rapaport for use of the HPLC apparatus, A. S. Waggoner for stimulating discussion, and T. H. Wilson for *E. coli* ML 308-225.

References

Ahmed, S., & Booth, I. R. (1981) *Biochem. J. 200*, 573–581.
Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.
Baccarini-Melandri, A., Casadio, R., & Melandri, B. A. (1981) *Curr. Top. Bioenerg. 12*, 197–258.
Booth, I. R., Mitchell, W. J., & Hamilton, W. A. (1979) *Biochem. J. 182*, 687–696.
Chen, S.-C., Brown, P. R., & Rosie, D. M. (1977) *J. Chromatogr. Sci. 15*, 218–221.
Cohen, G. N., & Rickenberg, H. V. (1956) *Ann. Inst. Pasteur, Paris 91*, 693–720.
Felle, H., Porter, J. S., Slayman, C. L., & Kaback, H. R. (1980) *Biochemistry 19*, 3585–3590.
Fillingame, R. H. (1980) *Annu. Rev. Biochem. 49*, 1079–1113.
Ghazi, A., Schechter, E., Letellier, L., & Labedan, B. (1981) *FEBS Lett. 125*, 197–200.
Guffanti, A. A., Bornstein, R. F., & Krulwich, T. A. (1981) *Biochim. Biophys. Acta 635*, 619–630.
Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172–230.
Harold, F. M. (1977) *Curr. Top. Bioenerg. 6*, 83–149.
Hirota, N., Matsuura, S., Mochizuki, N., Mutoh, N., & Imae, Y. (1980) *J. Bacteriol. 148*, 399–405.
Karl, D. M. (1980) *Microbiol. Rev. 44*, 739–796.
Kashket, E. R. (1981) *J. Bacteriol. 146*, 377–384.
Kashket, E. R., Blanchard, A. G., & Metzger, W. C. (1980) *J. Bacteriol. 143*, 128–134.
Khym, J. X. (1975) *Clin. Chem. (Winston-Salem, N.C.) 21*, 1245–1252.
Leive, L. (1968) *J. Biol. Chem. 243*, 2373–2380.
Maloney, P. C., & Hansen, F. C., III (1982) *J. Membr. Biol. 66*, 63–75.
Maloney, P. C., & Saitta, M. R. (1982) *Abstr. Annu. Meet., Am. Soc. Microbiol.*, 140.
Maloney, P. C., Kashket, E. R., & Wilson, T. H. (1975) *Methods Membr. Biol. 5*, 1–49.
McCarthy, J. E. G., Ferguson, S. J., & Kell, D. B. (1981) *Biochem. J. 196*, 311–321.
Mitchell, P. (1961) *Nature (London) 191*, 144–148.
Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445–502.
Neidhardt, F. C., Bloch, P. L., & Smith, D. F. (1974) *J. Bacteriol. 119*, 736–747.
Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 854–859.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1892–1896.
Rapaport, E., Garcia-Blanco, M. A., & Zamecnik, P. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1643–1647.
Robbie, J. P., & Wilson, T. H. (1969) *Biochim. Biophys. Acta 173*, 234–244.
Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–569.
Shen, C., Boens, C. C., & Ogawa, S. (1980) *Biochem. Biophys. Res. Commun. 93*, 243–249.
Slater, E. C. (1979) *Methods Enzymol. 55*, 235–245.
Slonczewski, J. L., Rosen, B. P., Alger, J. R., & Macnab, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6271–6275.
Sprott, G. D., & Usher, J. R. (1977) *Can J. Microbiol. 23*, 1683–1688.
ten Brink, B., Lolkema, J. S., Hellingwerf, K. J., & Konings, W. N. (1981) *FEMS Microbiol. Lett. 12*, 237–240.
Waggoner, A. (1976) *J. Membr. Biol. 27*, 317–334.
West, I. C., & Mitchell, P. (1973) *Biochem. J. 132*, 587–592.
Zaritsky, A., Kihara, M., & Macnab, R. M. (1981) *J. Membr. Biol. 63*, 215–231.
Zilberstein, D., Schuldiner, S., & Padan, E. (1979) *Biochemistry 18*, 669–673.
Zilberstein, D., Agmon, V., Schuldiner, S., & Padan, E. (1982) *J. Biol. Chem. 257*, 3687–3691.