

## Effects of Aerobiosis and Nitrogen Source on the Proton Motive Force in Growing *Escherichia coli* and *Klebsiella pneumoniae* Cells

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The electrochemical gradient of hydrogen ions, or proton motive force (PMF), was measured in growing *Escherichia coli* and *Klebsiella pneumoniae* in batch culture. The electrical component of the PMF ( $\Delta\psi$ ) and the chemical component ( $\Delta\text{pH}$ ) were calculated from the cellular accumulation of radiolabeled tetraphenylphosphonium, thiocyanate, and benzoate ions. In both species, the PMF was constant during exponential phase and decreased as the cells entered stationary phase. Altering the growth rate with different energy substrates had no effect on the PMF. The  $\Delta\text{pH}$  (alkaline inside) varied with the pH of the culture medium, resulting in a constant internal pH. During aerobic growth in media at pH 6 to 7, the  $\Delta\psi$  was constant at 160 mV (negative inside). The PMF, therefore, was 255 mV in cells growing at pH 6.3, and decreased progressively to 210 mV in pH 7.1 cultures. *K. pneumoniae* cells and two *E. coli* strains (K-12 and ML), including a mutant deficient in the  $\text{H}^+$ -translocating ATPase and a pleiotropically energy-uncoupled mutant with a normal ATPase, had the same PMF during aerobic exponential phase. During anaerobic growth, however, both species had  $\Delta\psi$  values equal to 0. Therefore, the PMF in anaerobic cells consisted only of the  $\Delta\text{pH}$  component, which was 75 mV or less in cells growing at pH 6.2 or greater. These data thus met the expectation that cells deriving metabolic energy from respiration have a PMF above a threshold value of about 200 mV when the ATPase functions in the direction of  $\text{H}^+$  influx and ATP synthesis; in fermenting cells, a PMF below a threshold value was expected since the enzyme functions in the direction of  $\text{H}^+$  extrusion and ATP hydrolysis. *K. pneumoniae* cells growing anaerobically had no  $\Delta\psi$  whether the N source added was  $\text{N}_2$ ,  $\text{NH}_4^+$  or one of several amino acids; the  $\Delta\text{pH}$  was unaffected. Therefore, any energy cost incurred by the process of nitrogen fixation could not be detected as an alteration of the proton gradient.

In previous papers (13, 14), my co-workers and I reported the magnitude of the proton motive force (PMF) of two species of exponentially growing gram-positive bacteria. In *Streptococcus lactis* and *Staphylococcus aureus* cells growing anaerobically, the PMF was less than 150 mV. This value is lower than the postulated threshold value of about 200 mV for the electrochemical gradient of  $\text{H}^+$  ions across the cytoplasmic membrane. Below the threshold PMF, the membrane ATPase ( $\text{BF}_0\text{F}_1$ ) functions in the direction of  $\text{H}^+$  efflux and ATP hydrolysis, whereas above this threshold, the direction of  $\text{H}^+$  movement is inward through the enzyme complex at ATP synthesis (for reviews, see references 9, 10, 22, 23). Indeed, the PMF was higher during the aerobic growth of *S. aureus* than during the anaerobic growth of these cells.

I have now undertaken a study of the effects

of aerobiosis on the PMF during growth of two species of gram-negative cells. Various strains of *Escherichia coli* were examined, including two energy-coupling mutants. *Klebsiella pneumoniae* cells were studied to determine whether the energy-consuming process of  $\text{N}_2$  fixation affects the PMF of growing cells and to observe the possible effects of alternate nitrogen sources on the proton gradient.

### MATERIALS AND METHODS

**Growth of cells.** *E. coli* K-12 AN180 (CGSC strain 5547) and its *uncA* mutant AN120 (4) were grown in medium 63 (6) supplemented with arginine (1 mg/ml), thiamine (0.125  $\mu\text{g}/\text{ml}$ ), and 0.5% glucose. The parental strain AN180 was also grown aerobically with 0.2% succinate or 0.2% malate instead of glucose, as indicated. The pH of the medium was adjusted as needed with Tris base or HCl. Cells of *E. coli* K-12 strain MN-

2 and its *ssd* mutant MN-2M (24; E. B. Newman, J. F. Morris, C. Walker, and V. Kapoor, *Mol. Gen. Genet.* in press) were grown in medium 63 supplemented with histidine (25  $\mu\text{g}/\text{ml}$ ) and 0.5% glucose. Cells of *E. coli* ML308 (ATCC 15224) were grown in the same medium as that used for the AN180 cells.

*K. pneumoniae* M5A1 cells, a generous gift from R. C. Valentine, were grown on the medium of Yoch and Pengra (32) as modified by Streicher and co-workers (30); this is a minimal salts medium plus sucrose. The nitrogen source was 0.5% ammonium sulfate,  $\text{N}_2$ , or an amino acid, as indicated. Growth was started by inoculation with cells that were grown overnight at 28°C in the same medium as that used in the experiment. Growth was carried out at 28°C, aerobically by rapid shaking of the flasks and anaerobically by continuous sparging with  $\text{N}_2\text{-CO}_2$ , as described previously (13).

**Measurement of the  $\Delta\psi$  and the  $\Delta\text{pH}$  in growing cells.** After growth of cells for at least two generations, [ $^3\text{H}$ ]tetraphenylphosphonium bromide ([ $^3\text{H}$ ]TPP $^+$ ) or [ $^{14}\text{C}$ ]benzoic acid was added, and samples were removed for assay, as described previously (13). For *E. coli* and *K. pneumoniae*, 5 mM EDTA was added 15 min before the first samplings to chelate  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  and thus "loosen" the outer membrane (18, 19). EDTA did not affect the growth rate of the cells. In resting cells, however, lower uptakes of the  $\Delta\psi$  probe were seen in the absence of EDTA, indicating that without the chelator, there exists a partial permeability barrier to the probe. By treating washed, exponential-phase *K. pneumoniae* suspended in growth medium with EDTA and then testing for sensitivity to gentamicin, I confirmed that EDTA indeed increased the access of small molecules to the cells (18). This inhibitor of protein synthesis was expected to be effective at low concentrations in cells in which the outer membrane did not prevent its free access to the cell proper. I found that in untreated control cells, the minimum inhibitory concentration of gentamicin was 6  $\mu\text{g}/\text{ml}$ , whereas in non-growing cells treated in growth medium for 15 min at 23°C with 5 mM EDTA, the addition of 0.37  $\mu\text{g}$  of the antibiotic per ml resulted in no overnight growth at 34°C. However, in later experiments I found that the addition of EDTA to growing cells was not necessary, since the same uptakes of [ $^3\text{H}$ ]TPP $^+$  and [ $^{14}\text{C}$ ]benzoate were observed with or without the chelating agent.

Sampling of aerobic cultures was carried out by filtration of the cultures through polycarbonate filters, as previously described (13). Sampling of *E. coli* or *K. pneumoniae* had to be done rapidly and with small samples of culture. For example, a respiring, resting suspension of *E. coli* at an absorbance at 625 nm of 1.14 (defined below) gave apparent  $\Delta\psi$  values of (a) 141 mV, (b) 114 mV, or (c) 80 mV, depending on whether (a) 0.1-ml or (b) 1.0-ml samples of the suspensions were filtered through polycarbonate filters, or if (c) a 1.0-ml sample was centrifuged through silicone oil. This suggests that samples (b) and (c) had become sufficiently anaerobic to lower the  $\Delta\psi$ . I have established that filtration (<5 s) of samples containing 0.07 mg of bacteria (dry weight) or less (either larger samples of dilute cultures or smaller samples of more concentrated cells) gave constant and high uptakes of [ $^3\text{H}$ ]TPP $^+$ . Therefore, I routinely removed such sam-

ples from the aerobic cultures, and the low  $\Delta\psi$  values seen in stationary-phase cells (below) are not artifacts of the assay. The flow dialysis technique (26), which avoids these difficulties, requires cell concentrations larger than those of batch cultures at exponential phase.

Anaerobic samples were removed and centrifuged under paraffin oil to exclude air (13). Non-specifically bound [ $^3\text{H}$ ]TPP $^+$  was measured in butanol-treated cells isolated by centrifugation. The intracellular and extracellular aqueous spaces were measured with  $^3\text{H}_2\text{O}$  and [ $^3\text{H}$ ]polyethylene glycol (21).

The calculations of  $\Delta\psi$  and the  $\Delta\text{pH}$  were made by using the Nernst equation and done as previously described (14, 20) after correcting for the extracellular counts of [ $^{14}\text{C}$ ]benzoate and for the non-specifically bound counts of [ $^3\text{H}$ ]TPP $^+$ . For both *E. coli* and *K. pneumoniae*, we used the following relationship: 1.0 ml of cells at an absorbance at 625 nm of 1.0 was equivalent to 0.38 mg of bacteria (dry weight) and 0.62  $\mu\text{l}$  of intracellular water (this is equivalent to 1.63  $\mu\text{l}$  of cellular water per mg [dry weight]), which is similar to the values reported by Bakker [1]. In centrifuged samples (0.38 mg [dry weight]), the extracellular medium occupied 0.32  $\mu\text{l}$ , whereas in filtered cell samples (0.07 mg [dry weight] in 0.1 ml), the contaminating medium was 0.7 to 0.9  $\mu\text{l}$  and was determined in each experiment. For some comparisons, the uptakes of [ $^3\text{H}$ ]TPP $^+$  or  $\text{S}^{14}\text{CN}^-$  by the cells (e.g., see Tables 2 through 4) are expressed in normalized counts per minute, i.e., counts per minute per cell pellet calculated as that derived from 1.0 ml of culture at an absorbance at 625 nm of 1.0 containing 100,000 cpm of radioactivity.

## RESULTS

**PMF of *E. coli* cells during aerobic batch growth.** During balanced growth such as that during the exponential phase in batch culture, all the systems for  $\text{H}^+$  extrusion and influx are expected to change at the same rate (5). Thus, the poise of the  $\text{H}^+$  gradient also would be expected to be constant. This expectation was met in *E. coli* cells growing aerobically in which the PMF was constant at about 210 mV throughout the growth period (Fig. 1). While the pH of the medium decreased from 6.94 to 6.76 in this culture, the  $\Delta\text{pH}$  increased from 45 to 60 mV (alkaline inside). The  $\Delta\psi$  decreased from 164 (negative inside) to 145 mV. This conforms to the pattern observed previously in growing cells (13, 14) in that the medium pH influences the relative contributions of the  $\Delta\text{pH}$  and the  $\Delta\psi$  to the  $\Delta\mu$ .

**Effect of medium pH on the PMF of exponential-phase *E. coli* during aerobic growth.** The response of PMF to external pH was tested in aerobic cultures of *E. coli* cells growing in media ranging in pH from 6.3 to 7.15 (Fig. 2). The  $\Delta\text{pH}$  varied from 95 mV in pH 6.3 cultures to 43 mV in pH 7.15 cultures, which resulted in a constant internal pH of about 7.8.

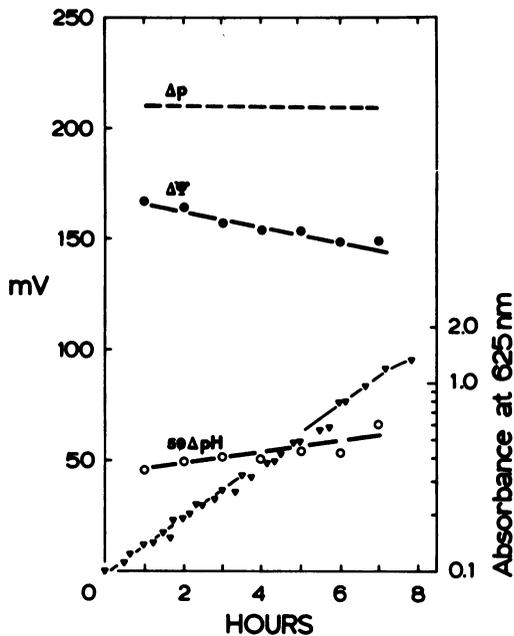


FIG. 1. PMF of *E. coli* cells during batch growth under aerobic conditions. Cells of *E. coli* K-12 AN180 were grown and tested as described in the text. Samples of culture were taken at indicated times. The pH of the medium decreased from pH 6.94 after 1 h of incubation to pH 6.76 after 7 h of growth. Each  $\Delta\psi$  value (●) is the mean of 7 to 11 replicate determinations of four experiments. Each  $59\Delta\text{pH}$  value (○) is the mean of four to seven replicate determinations of three experiments. The standard deviations of the means were  $<2$  mV for  $\Delta\psi$  and  $<10$  mV for  $59\Delta\text{pH}$ . The  $\Delta p$  line, PMF, was calculated from the regression lines of the  $\Delta\psi$  and  $59\Delta\text{pH}$  points. The culture doubling time was 119 min.

The  $\Delta\psi$  remained constant in these cells at 161 mV (negative inside). The PMF, therefore, was higher (255 mV) in cells growing at pH 6.3 than in the cells growing at the higher medium pH of 7.15 (206 mV).

**Effect of energy substrate on the PMF of *E. coli* cells.** Most of the experiments with *E. coli* were carried out in a minimal salts medium with glucose as the energy source. I also tested two citric acid cycle intermediates which cannot be fermented by *E. coli*, and found that the PMF was the same as that in cells growing aerobically with the fermentable sugar (Table 1). For these cultures the doubling times ranged from 80 to 200 min, but no effect on the PMF was detected.

**PMF of various strains of *E. coli* growing anaerobically.** Various strains and energy metabolism mutants of *E. coli* were examined during aerobic growth at constant medium pH (Table 1). I found that exponential-phase cells of both

*E. coli* strains K-12 and ML had the same PMF of about 217 mV. Among the K-12 strains, the ATPase-deficient mutant AN120 (*uncA*) had the same PMF as its parental cell AN180. The mutant MN-2M cells (24; Newman et al., in press) were of interest because they are deficient in their ability to couple metabolic energy to the transport of nutrients and other processes, yet have a normal respiratory chain and  $\text{BF}_0\text{F}_1$ . The present experiments show that growing MN-2M cells had the same PMF as their parent MN-2 and as all of the other *E. coli* strains tested.

**PMF of *E. coli* cells growing anaerobically.** In contrast to cells growing aerobically, *E. coli* cells growing anaerobically had no  $\Delta\psi$  (Table 2, Fig. 3). In both the K-12 and ML strains, the cell-associated counts of  $[^3\text{H}]\text{TPP}^+$  were the same for anaerobic cells before and after butanol treatment to disrupt the membrane (Table 2). Thus, the  $[^3\text{H}]\text{TPP}^+$  taken up by the growing cells was counted for by nonspecifically bound cation and the  $\text{TPP}^+$  in the intracellular and extracellular fluids. The 2,000 to 3,000 normalized cpm found in these pellets should be

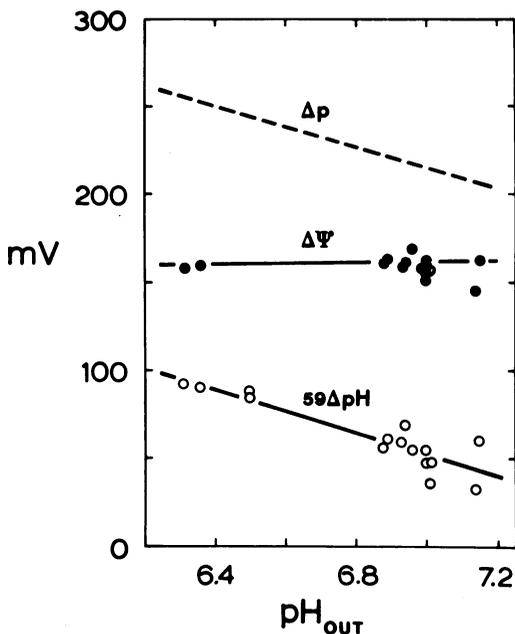


FIG. 2. Effect of medium pH on the PMF of exponential-phase *E. coli* cells growing aerobically. The experiment was carried out as described in the text and in the legend to Fig. 1. Cells were sampled at an absorbance at 625 nm of 0.4. Each value is the mean of five replicate determinations from separate cultures of cells; the standard deviation values were 1 to 8 mV for  $59\Delta\text{pH}$  (○) and these were 1 to 4 mV for  $\Delta\psi$  (●). The PMF ( $\Delta p$ ) was calculated as in Fig. 1.

compared to the more than 40,000 cpm found in aerobically growing cells. To check whether the small difference seen in the uptake of [ $^3\text{H}$ ]TPP $^+$  in *E. coli* K-12 was due to a "reverse" membrane potential (positive inside), I measured the uptake of  $\text{S}^{14}\text{CN}^-$ . This lipophilic anion is used to measure the  $\Delta\psi$  in organelles in which the transmembrane potential has the polarity of inside positive (15). There was no uptake of  $\text{S}^{14}\text{CN}^-$  by anaerobically growing *E. coli* (Table 2). Note that far less of the  $\text{S}^{14}\text{CN}^-$  label was associated with the cell pellet than of the [ $^3\text{H}$ ]TPP $^+$  label, which was also true for the [ $^{14}\text{C}$ ]benzoate anion (not shown). Indeed, the anion present could be accounted for by the intra- and extracellular spaces of the pellet (62.4 normalized cpm would be present within the cells, and 56.1 normalized cpm in the extracellular fluid).

*E. coli* cells growing under anaerobic conditions had no  $\Delta\psi$  during the exponential phase (Fig. 3), although the  $\Delta\text{pH}$  was equivalent to 20

TABLE 1. PMF ( $\Delta p$ ) of various strains of *E. coli* at mid-exponential phase during aerobic growth at pH 6.9 $^a$

Strain and energy source	$\Delta\psi^b$	59 $\Delta\text{pH}^b$	$\Delta p^b$
K-12 AN180			
Glucose	158 $\pm$ 8 (26)	57 $\pm$ 14 (17)	215
Succinate	165 $\pm$ 4 (10)	56 $\pm$ 2 (10)	221
Malate	160 $\pm$ 3 (10)	60 $\pm$ 2 (10)	220
K-12 AN120 ( <i>uncA</i> )			
Glucose	156 $\pm$ 8 (17)	63 $\pm$ 3 (6)	219
K-12 MN-2			
Glucose	156 $\pm$ 5 (7)	56 $\pm$ 4 (6)	212
K-12 MN-2M ( <i>ssd</i> )			
Glucose	158 $\pm$ 5 (6)	48 $\pm$ 12 (8)	206
ML308			
Glucose	151 $\pm$ 1 (5)	73 $\pm$ 2 (5)	224

$^a$  The cells were grown and samples removed for assay at an absorbance at 625 nm of 0.4, as described in the text.

$^b$  Each value is presented in millivolts as the mean  $\pm$  the standard deviation (number of replicates of one to three experiments).

TABLE 2. Uptake of [ $^3\text{H}$ ]TPP $^+$  and  $\text{S}^{14}\text{CN}^-$  by exponential phase *E. coli* during anaerobic growth with (+) or without (-) butanol treatment at pH 6.8 $^a$

Strain	[ $^3\text{H}$ ]TPP $^+$		$\text{S}^{14}\text{CN}^-$	
	-	+	-	+
<i>E. coli</i> K-12 AN180	1,848 $\pm$ 375 (15)	2,809 $\pm$ 555 (15)	26 $\pm$ 4 (4)	60 $\pm$ 5 (4)
<i>E. coli</i> ML308	2,904 $\pm$ 226 (5)	2,944 $\pm$ 349 (2)	ND $^b$	ND

$^a$  The cells were grown under anaerobic conditions and tested as described in Methods. The radioactivity per pellet is expressed as normalized counts per minutes  $\pm$  the standard deviation (number of replicate determinations).

$^b$  ND, Not done.

mV when the medium pH was 6.98, and increased to 50 mV as the medium pH decreased to 6.61. The PMF, therefore, was composed only of the chemical component of the  $\Delta p$ , and was considerably lower than that seen in cells growing aerobically in the same medium at the same external pH. In exponential-phase anaerobic cells growing in media of pH ranging from 6.2 to 7, the internal pH remained constant at 7.6, showing that under anaerobic conditions these cells behaved like the aerobic ones in this respect. Lastly, *E. coli* cells growing anaerobically in the rich medium SB used for *S. aureus* cells (13) had no  $\Delta\psi$ .

**PMF of *K. pneumoniae* cells growing aerobically in batch culture.** During aerobic growth, *K. pneumoniae* cells had a constant PMF in mid-exponential phase (Fig. 4). Both the  $\Delta\psi$  and the  $\Delta\text{pH}$  decreased as the cells entered stationary phase. Therefore, in subsequent experiments, samples were assayed when cultures were at an absorbance at 625 nm of 0.4 or less. In other experiments in which *K. pneumoniae* cells grew aerobically in media of pH 6 to 7 (not shown), the internal pH was maintained at 7.8, as in *E. coli* cells.

**PMF of *K. pneumoniae* cells during anaerobic growth with and without  $\text{NH}_4^+$ .** Since the reason for examining *K. pneumoniae* cells was to determine whether the energy demands of nitrogen fixation are reflected in altered  $\text{H}^+$  gradients, I grew these cells under anaerobic conditions with nitrogen sparged through the suspension. When ammonium ions were added to the medium, the cells grew more rapidly (Fig. 5), as expected of cells using  $\text{NH}_4^+$  rather than  $\text{N}_2$  as the N source. As seen with *E. coli* cells, anaerobically-growing *K. pneumoniae* cells had no  $\Delta\psi$ . The cell pellets contained the same amounts of [ $^3\text{H}$ ]TPP $^+$  whether the membranes were disrupted with butanol or not (Table 3). The failure to take up  $\text{S}^{14}\text{CN}^-$  confirmed that there was no reverse membrane potential. In contrast, aerobically growing cell pellets contained over 40,000 cpm of the TPP $^+$  probe, from which a  $\Delta\psi$  value of 164 mV was calculated.

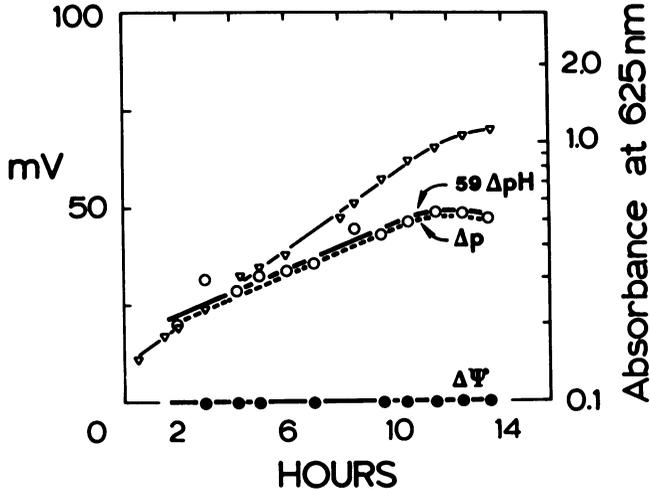


FIG. 3. PMF of *E. coli* cells during batch growth under anaerobic conditions. Cells of *E. coli* K-12 AN180 were grown and tested as described in the text. The pH of the culture decreased from pH 6.98 after 2 h of growth to pH 6.61 after 13.5 h. The  $\Delta p$  line is superimposable on the line through the 59 $\Delta$ pH points (○). The culture doubling time was 247 min.

Anaerobically growing cells showed a pH-dependent  $\Delta$ pH (Fig. 5). As the medium pH decreased during the course of exponential growth, the  $\Delta$ pH increased. Thus, in anaerobic *K. pneumoniae*, the  $\Delta$ pH accounted for all of the PMF, and the PMF value was much lower than that of aerobic cells.

Effects of nitrogen source on the PMF of anaerobically growing *K. pneumoniae*. Since *K. pneumoniae* cells grew more rapidly when ammonium ions were added to the medium than when dinitrogen was the only nitrogen source available, I compared the exponential-phase  $\Delta\psi$  and  $\Delta$ pH at comparable cell densities (Fig. 5). The two cultures were indistinguishable. In addition, the  $\Delta$ pH values of ammonium-supplied cells were the same whether the cultures were sparged with He-CO<sub>2</sub> or N<sub>2</sub>-CO<sub>2</sub>.

Several amino acids, instead of ammonium sulfate, were added to the cultures in order to examine whether supplying a nutrient that is negatively charged, such as aspartic acid, or one which has no net charge, such as serine, might affect the H<sup>+</sup> gradient differently from the positively charged ammonium ions (Table 4). It is clear that there was no difference in any of these cultures and that they were like NH<sub>4</sub><sup>+</sup> or N<sub>2</sub>-grown cells. The  $\Delta\psi$  was 0 and the  $\Delta$ pH was equivalent to 64 to 75 mV when the medium pH was 6.2. In other experiments, glutamine-supplied cells were sparged with He-CO<sub>2</sub> instead of N<sub>2</sub>-CO<sub>2</sub>, and again, no difference in the PMF was seen.

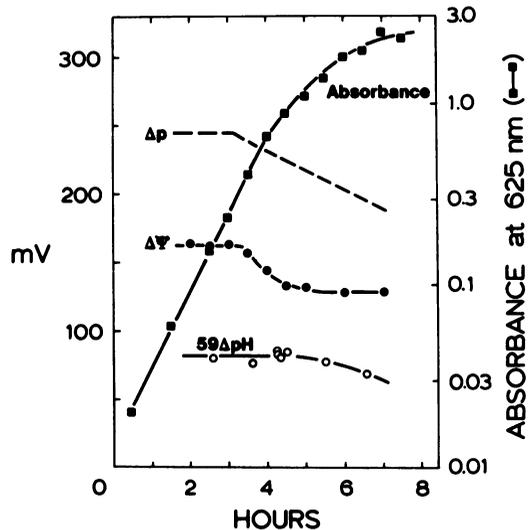


FIG. 4. PMF of *K. pneumoniae* during batch culture under aerobic conditions. The cells were grown at pH 6.5, as described in the text. Each  $\Delta\psi$  value (●) is the mean of triplicate determinations (standard deviation, 0.8 to 5.4 mV). Each 59 $\Delta$ pH value (○) is the mean of three to four replicates (standard deviation 0.6 to 2.6 mV). The doubling time was 43 min.

## DISCUSSION

The advantage of using several species of bacteria for the examination of the PMF of growing cells that the cells serve as each others' controls. For example, with *S. lactis* cells, which are aerotolerant anaerobes, I was able to show that the

TABLE 3. Uptake of [ $^3\text{H}$ ]TPP $^+$  and  $\text{S}^{14}\text{CN}^-$  by exponential phase *K. pneumoniae* cells $^a$ 

Cell treatment $^b$	Uptake (cell-associated cpm) $^c$	Significance ( $P$ ) $^d$	$\Delta\psi$ $^e$
$[^3\text{H}]\text{TPP}^+$ added under growth condition of:			
Anaerobic + $\text{NH}_4^+$ , -BT	2,572 $\pm$ 523	0.57	0
Anaerobic + $\text{NH}_4^+$ , +BT	3,038 $\pm$ 384		
Anaerobic + $\text{N}_2$ , -BT	2,642 $\pm$ 489	0.66	0
Anaerobic + $\text{N}_2$ , +BT	2,889 $\pm$ 280		
Aerobic, -BT	40,593 $\pm$ 943	<0.001	164
Aerobic, +BT	3,331 $\pm$ 208		
$\text{S}^{14}\text{CN}^-$ added under growth condition of:			
Anaerobic + $\text{NH}_4^+$ , -BT	80 $\pm$ 11	0	0
Anaerobic + $\text{NH}_4^+$ , +BT	135 $\pm$ 60		

$^a$  Cell samples were derived from 1.0 ml of culture, normalized to an absorbance at 625 nm of 1.0, containing 100,000 cpm of  $^3\text{H}$  or  $^{14}\text{C}$ . The cells were grown and tested as described in the text. Aerobically growing cells were sampled at an absorbance at 625 nm of <0.235 and a pH of 6.5; anaerobic cultures were sampled at an absorbance at 625 nm of 0.6 to 1.0 and a pH of 6.2 to 6.5.

$^b$  -BT, Not treated with butanol; +BT, butanol treated.

$^c$  Data are expressed in counts per minute per cell sample as the mean  $\pm$  standard deviation of one to six experiments.

$^d$   $P$ , Significances of differences as determined by Student's  $t$  test. Anaerobically growing cells with or without  $\text{NH}_4^+$  were not significantly different ( $P = 0.84$ ).

$^e$  Millivolts.

filtration and centrifugation methods gave the same values for the  $\Delta\psi$  and the  $\Delta\text{pH}$  (13). Therefore, when the degree of aerobiosis was not a factor, the two techniques were equally valid. Interference from the outer membrane of the gram-negative cells per se can be discounted in these experiments, since the PMF measured under aerobic conditions of growth was higher than 200 mV in both *S. aureus* and in the two gram-negative facultative organisms. Indeed, treatment of growing gram-negative cells with EDTA seemed to be unnecessary, in contrast to resting cells. Third, the fact that *K. pneumoniae* cells were able to grow with  $\text{N}_2$  as the sole nitrogen source suggested that the anaerobic technique used was sufficiently anaerobic to permit this oxygen-sensitive process to occur (11, 16). The difference in the PMF seen in a *S. aureus* heme-

deficient mutant with or without hemin added (13) suggests that trace amounts of oxygen may have been available to the cells; nevertheless, the gram-negative cells had no  $\Delta\psi$  under these incubation conditions.

From the studies presented here and previously (13), it is clear that the degree of aerobiosis affects the PMF of growing bacteria profoundly, whereas other growth conditions have little effect. In all cells tested, the PMF was constant during exponential phase of batch growth in both aerobic and anaerobic conditions. The PMF was not affected by the growth rate of the cells, nor by the composition of the medium. On the other hand, the medium pH affected the contribution of  $\Delta\text{pH}$  to the PMF in all cells tested. It is evident that the cells, whether growing aerobically or anaerobically, maintain a high and relatively constant internal pH. The mechanisms for that homeostasis (reviewed in references 29 and F. M. Harold, *Curr. Top. Membr. and Transp.*, in press) probably include  $\text{K}^+$

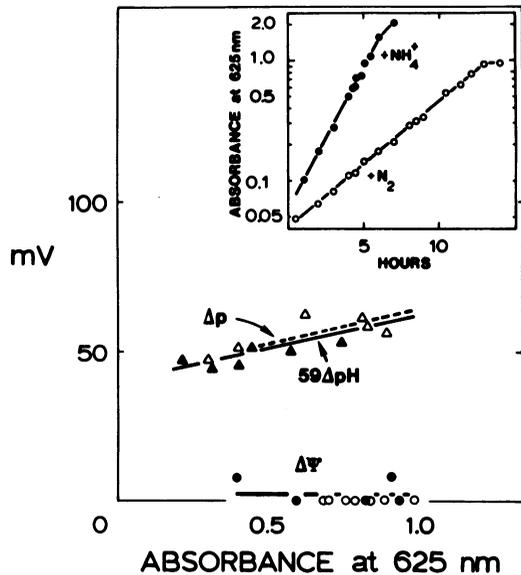


FIG. 5. PMF of *K. pneumoniae* growing anaerobically with  $\text{NH}_4^+$  or  $\text{N}_2$  as the nitrogen source. The cells were grown and the  $\Delta\psi$  and  $\Delta\text{pH}$  measured as described in the text. The graph is the composite of six experiments. With  $\text{NH}_4^+$  added (closed symbols), the cells doubled every 78 min, whereas with  $\text{N}_2$  (open symbols), the culture doubling time was 165 min. Each value is the mean of triplicate determinations (standard deviation, <2 mV). The  $\Delta\psi$  (○) was 0 (see also Table 3). The PMF ( $\Delta\text{p}$  line) is the regression line through the 59 $\Delta\text{pH}$  points (▲). The pH of the medium decreased from 6.8 at an absorbance at 625 nm of 0.2 to a pH of 6.2 at an absorbance at 625 nm of 1.0.

TABLE 4. Effect of alternate nitrogen sources on the PMF of *K. pneumoniae* growing anaerobically at pH 6.2<sup>a</sup>

Nitrogen source added	Cell-associated [ <sup>3</sup> ]TTPP <sup>+</sup> (cpm) <sup>b</sup>		$\Delta\psi$ (mV)	59 $\Delta$ pH (mV) <sup>c</sup>
	Untreated	Butanol-treated		
Arginine	4,719 $\pm$ 641 (5)	4,646 $\pm$ 280 (2)	0	67
Aspartic acid	3,462 $\pm$ 444 (5)	3,961 $\pm$ 64 (2)	0	64
Glutamine	4,799 $\pm$ 845 (15)	4,735 $\pm$ 452 (6)	0	72
Serine	4,415 $\pm$ 1,005 (5)	5,145 $\pm$ 501 (2)	0	75

<sup>a</sup> The experiment was performed as described in the text and in footnote *a* to Table 3. Amino acid (25  $\mu$ g/ml) was added to the medium instead of ammonium sulfate, as indicated. Samples were taken when the cultures were at an absorbance value at 625 nm of 0.5.

<sup>b</sup> Data expressed in normalized counts per minute as the mean  $\pm$  standard deviation (number of replicates of one to three experiments).

<sup>c</sup> A similar number of determinations as explained in footnote *b* were done for the 59 $\Delta$ pH values, which had standard deviations of 1 to 4 mV.

transport systems such as the one described for *Streptococcus faecalis* (2) and the *E. coli* K<sup>+</sup>/H<sup>+</sup> antiporter (3).

During aerobic exponential growth, the gram-negative organisms tested, including the obligate aerobe *Paracoccus denitrificans* (unpublished observations), had a PMF of 200 mV or more. Comparing with published values at the same external pH, the magnitude of the PMF of aerobically growing *E. coli* cells was greater (by 35 to 60 mV) than that of respiring, non-growing, EDTA-treated cells (33) or of giant *E. coli* cells (8), but similar to that of valinomycin-treated *E. coli* spheroplasts (7).

The PMF of two energy-coupling mutants of *E. coli* was found to be the same as that of their parental types during exponential growth. Cells of AN120 (*uncA*) have a faulty BF<sub>0</sub>F<sub>1</sub>, but a normal respiratory chain (4). As expected, these cells were able to maintain a normal proton gradient during exponential phase of aerobic growth. Interestingly, their PMF was not greater than that of their wild type, AN180, even though the BF<sub>0</sub>F<sub>1</sub> of the mutants presumably did not translocate H<sup>+</sup> ions and thus did not dissipate the PMF. The other energy coupling mutant tested was MN-2M (24; Newman et al., in press). These highly pleiotropic mutants have a phenotype similar to energy-uncoupled mutants described by Plate (25) and by Thorbjarnardotter and co-workers (31), in that they grow poorly on oxidizable substrates such as succinate, are deficient in active transport of chemiosmotically coupled substrates, yet have a normal respiratory chain and a normal BF<sub>0</sub>F<sub>1</sub>. The defect maps at about 86 min on the *E. coli* chromosome map (24, 31). I found that these cells, during aerobic growth, had a normal PMF. Thus, when the cells are able to grow, their defect presumably can be compensated for since it is not demonstrable in the steady-state transmembrane gradient of H<sup>+</sup> ions.

The PMF values measured in *E. coli* and *K. pneumoniae* cells during anaerobic growth were 70 mV or less and consisted of only the  $\Delta$ pH component. Thus, the gram-negative cells differed considerably from the gram-positive cells during anaerobic growth (13). The gram-negative cells tested were grown in minimal salts media with a sugar added, whereas the gram-positive cells were grown in richer media containing amino acids and other nutrients. Since *E. coli* cells growing in a rich medium had no  $\Delta\psi$  under anaerobic conditions, the differences in  $\Delta\psi$  between anaerobic gram-positive and -negative cells are not likely to be due to the composition of the medium.

Although the internal pH was relatively constant, the  $\Delta\psi$  and the PMF varied greatly in these growing cells. The gram-negative cells, in particular, grew anaerobically with a very low proton motive force. This was an unexpected finding, and it is not obvious to me why the  $\Delta\psi$  value falls to 0 under these conditions. A suggested explanation would be that the reaction carried out by the BF<sub>0</sub>F<sub>1</sub> is away from its equilibrium position, and hydrolysis of ATP can occur at any PMF value below about 200 mV. The poise of the PMF is then a function of the rate of H<sup>+</sup> reentry by the various secondary porters (reviewed in reference 27). The fluxes of ions and nonelectrolytes must be sufficiently rapid and occur in the right direction, since the cells are able to grow. Therefore, there must be an electrogenic flux of at least one other ion to dissipate the  $\Delta\psi$ . In the gram-positive bacteria, which probably have a different complement of membrane carriers, this would not be the case.

The process of N<sub>2</sub> fixation, which is expensive in terms of ATP requirements (28), was expected to be reflected in decreased levels of the H<sup>+</sup> gradient compared to the PMF of cells not carrying out this reaction. However, under anaerobic conditions there was no  $\Delta\psi$ . Therefore, it was

not possible to see a decrease in the  $H^+$  gradient. It should be noted that these experiments measure the steady-state proton gradients; thus, it is not possible to detect whether unidirectional fluxes of  $H^+$  or other ions have been affected by a process such as  $N_2$  fixation. However, the absence of  $\Delta\psi$  in growing, nitrogen-fixing *Klebsiella* should be contrasted to resting, respiring preparations of *Rhizobium* bacteroids. In these obligate aerobes, the  $\Delta\psi$  apparently regulates the generation of reducing equivalents for nitrogenase (17). In *Klebsiella*, this would not seem to be the case. In addition, any oxygen available to the cells under these anaerobic growth conditions is not likely to contribute to ATP synthesis by the mechanism of oxidative phosphorylation, as suggested by Hill (11, 12) for *Klebsiella* growing on  $N_2$  in oxygen-limited chemostat cultures.

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