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# A Protonmotive Force as the Source of Energy for Galactoside Transport in Energy Depleted *Escherichia Coli*

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*Summary.* An artificially produced electrochemical potential difference for protons (protonmotive force) provided the energy for the transport of galactosides in *Escherichia coli* cells which were depleted of their endogenous energy reserves. The driving force for the entry of protons was provided by either a transmembrane pH gradient or a membrane potential. The pH gradient across the membrane was created by acidifying the external medium. The membrane potential (inside negative) was established by the outward diffusion of potassium (in the presence of valinomycin) or by the inward diffusion of the permeant thiocyanate ion. The magnitude of the electrochemical potential difference for protons agreed well with magnitude of the chemical potential difference of the lactose analog, thiomethylgalactoside. The observations are consistent with the view that the carrier-mediated entry of each galactoside molecule is accompanied by the entry of one proton.

In 1963 Mitchell proposed that lactose enters *Escherichia coli* via a membrane carrier by cotransport with protons. According to this view energy stored as an electrochemical potential difference of hydrogen ions is utilized to drive lactose transport via an obligatory coupling between proton and lactose entry. He also proposed that the ability of uncouplers to inhibit active transport is due to their ability to transfer protons across the cell membrane, thus collapsing the driving force for accumulation. In 1968 Pavlasova and Harold demonstrated that uncouplers blocked transport of $\beta$-galactosides in anaerobic cells of *E. coli* without reducing the ATP levels. The authors proposed that the uncouplers reduced the electrochemical potential of protons in the manner proposed by Mitchell. Further support for the cotransport hypothesis came from the work of West (1970) and West and Mitchell (1972, 1973) who showed that when lactose was added to energy-depleted cells protons entered the cell against an electro-

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chemical gradient. Furthermore, they demonstrated a stoichiometry of one proton to one lactose molecule (West & Mitchell, 1973).

Another experimental approach to this problem has been to test the ability of an inwardly directed movement of protons to induce sugar uptake against a concentration gradient. An electrical driving force derived from a potassium diffusion potential (in the presence of valinomycin) can provide the energy for proton entry and sugar accumulation in *Streptococcus lactis* (Kashket & Wilson, 1972; 1973) and in membrane vesicles of *E. coli* (Hirata, Altendorf, & Harold, 1973; 1974). A pH gradient may also provide a driving force for proton entry and thiomethylgalactoside (TMG) accumulation in *S. lactis* (Kashket & Wilson, 1973). The subject has recently been reviewed by Simoni and Postma (1975).

In this paper experiments are described in which both electrical and chemical driving forces have been used to induce galactoside accumulation in energy depleted cells of *E. coli*. In addition, quantitative experiments suggest that the chemical potential difference of TMG is in equilibrium with the electrochemical potential difference for protons.

## Materials and Methods

### *Bacteria*

Several strains of *E. coli* were studied. ML-308 which is constitutive for both lactose transport and β-galactosidase was isolated by Monod and his collaborators at the Pasteur Institute. DL-54 (Simoni & Shallenberger, 1972) is an adenosine triphosphatase negative mutant derived from ML 308-225 (Winkler & Wilson, 1966) which is constitutive for the lactose transport system but lacks β-galactosidase. After completion of each experiment using DL-54 the cell suspension which had been used was tested for the presence of revertants by streaking onto succinate minimal plates containing the necessary supplements. In the experiments reported here no significant reversion to ATPase-positive cells had occurred. Strain 1100, a derivative of Strain 1000 was isolated by Fox and Wilson (1968). SASX76-2 was a lactose positive derivative of the *hem A* mutant SASX76 which was a generous gift from Dr. A. Sasarman (Sasarman, Surdeanu & Horodniceanu, 1968). SASX76-2 was obtained by mating SASX76 ($lac^-$, $F^-$, $Sm^R$) with a streptomycin-sensitive, lactose positive cell which contained the genes of the lactose operon on a F factor (Δ lac/W3747). The latter cell was obtained from Dr. M. Malamy. Lactose positive cells (SASX76-2) were isolated as red clones on McConkey plates containing streptomycin.

1100 was routinely grown in minimal Medium 63 (Cohen & Rickenberg, 1956) supplemented with 1% tryptone (Difco), 1 mM isopropylthiogalactoside (IPTG) and $B_1$ (0.5 μg/ml). Growth was followed turbidimetrically with a Klett-Summerson colorimeter (filter no. 42). Cells were harvested by centrifugation at 4 °C and washed once in tris buffer (120 mM, pH 8). When required, the cells were incubated with 10 mM EDTA (Kaback, 1971) for 30 min (125

mg cells/ml tris). The cells were then centrifuged at 4 °C and resuspended in phosphate buffer at pH 6.8.

SASX76-2 was grown on Penassay broth. If added, γ-aminolevulenic acid was present at a final concentration of 50 μg/ml. In cases where γ-aminolevulenic acid was omitted the cells grew very slowly and cultures were allowed to grow for approximately 12 hr. Otherwise, the cultures were grown for approximately 6 hr. Depending on the experiment to be carried out the cells were harvested by centrifugation and washed in either phosphate buffer (pH 7) or Tris buffer at pH 8. When required, the Tris-washed cells were incubated in the presence of EDTA as described above.

### *Preparation of Energy-depleted Cells*

Cells were grown to late stationary phase in minimal medium 63 (Cohen & Rickenberg, 1956) supplemented with 0.2% glycerol and $B_1$ (0.5 μg/ml). Cells were then diluted into fresh growth medium and allowed to double once according to the method of Wood (1975). Growth was monitored turbidimetrically with a Klett-Summerson colorimeter (filter no. 42). The cells were harvested by centrifugation at $10{,}000 \times g$ for 5 min and washed two times in Medium 63 at room temperature. The washed cells were depleted of their energy reserves in 2,4-dinitrophenol (DNP) according to the method of Berger (1973). The cells (1.2 to $1.8 \times 10^9$ cells/ml) were incubated in Medium 63 containing 5 mM DNP on a shaker (80 rpm) for 10 to 12 hr at 37 °C. The energy-depleted cells were washed 3 times in 0.2 M potassium phosphate buffer containing 2 mM cyanide and resuspended in the same solution. This energy-depletion procedure reduced the capacity of cells to accumulate galactosides. Normal cells exposed to 25 μm TMG accumulate the sugar analog to a concentration 100 to 200 times that in the external medium, while energy-depleted cells do not accumulate. When glucose or D-lactate were added to such depleted cells an accumulation of 20- to 50-fold was observed. When O-nitrophenyl-β-D-galacto pyranoside (ONPG) entry was examined, these cells possessed 10 to 15% normal carrier activity.

When the ATPase negative mutant DL-54 was used, the incubation time in DNP was decreased to one hr. After the energy depletion procedure the cells were washed three times in potassium phosphate buffer. All washes contained 2 mM cyanide and 2 mM iodoacetate. Cyanide was added to block respiration and iodoacetate to inhibit glycolysis, the latter process might produce a protonmotive force in these mutants via slight residual activity of the ATPase. The ATPase negative mutant was treated with 0.1 mM dicyclohexylcarbodiimide (DCCD) before the third wash. In the earlier experiments DCCD was added at a final concentration of 1 mM, however later it was found that one could produce the desired results using much lower concentrations. A peculiar feature of the ATPase negative mutant used in this study (DL-54) was its abnormally high permeability to protons and its inability to transport amino acids (Simoni & Shallenberger, 1972; Altendorf, Harold & Simoni, 1974) to normal levels. The ATPase inhibitor DCCD has been shown to block the high proton permeability and restore the normal transport of amino acids (Altendorf *et al.*, 1974). The use of DCCD in the present studies was to reduce the proton permeability so that large protonmotive potential differences could be maintained.

### *Determination of Cell* $H_2O$

Energy-depleted cells were incubated at a final concentration of $1 \times 10^9 - 1 \times 10^{10}$ cells/ml in 3 ml of 0.2 M potassium phosphate buffer, pH 7 containing [$^3$H] water. [$^{14}$C] inulin (4.2 μg/ml; 0.5 μC/ml) was then added as an extracellular marker. The tubes were

covered tightly to prevent evaporation and after 10 to 15 min three 1-ml aliquots were pipeted into 1.5 ml microfuge tubes containing 0.5 ml of a Dow Corning silicone oil mixture (75% 550 fluid; 25% 510 fluid). The samples were centrifuged for one min at 10,000 × *g* at 25 °C in a Beckman Microfuge. The cells pass through the silicone and pellet at the bottom with only a small amount of adhering extracellular fluid. After the removal of the water and oil layers the tip of the centrifuge tube was removed with a razor blade and placed in a vial containing 1 ml of 1.2 N KOH (Dolais-Kitabgi & Perlman, *personal communication*). The pellet was partially digested in the alkali for 30 min and then neutralized with 1 N HCl (0.5 ml). After neutralization, 10 ml of a counting solution (3.6 g omnifluor, 330 ml Triton X 100 plus 900 ml toluene) were added to the vials. The samples were analyzed for [$^{14}$C] and [$^{3}$H]. One ml of energy-depleted DL-54 cells at an optical density of 100 ($8 \times 10^8$ cells/ml) contains 0.33 μl of cell water (average of three experiments).

### *Transport Assays*

*(a) Valinomycin stimulated transport.* Washed energy-depleted cells were resuspended to a very concentrated suspension (about $3 \times 10^{11}$ cells/ml) in 0.2 M potassium phosphate buffer, pH 6 or 7 containing 2 mM cyanide unless specified otherwise in the legends. Valinomycin was added to a final concentration of 10 to 20 μM. After 1 hr incubation at 25 °C the cells were diluted 500-fold (about $7 \times 10^8$ cells/ml) into either sodium or potassium phosphate plus 2 mM cyanide, pH 6 or 7 containing [$^{14}$C] TMG (either 25 or 50 μM). At various intervals 0.5-ml aliquots of the suspension were withdrawn and filtered through Millipore filters (pore size 0.65 μm). The filters were then washed with 5 ml of buffer and placed in vials containing 15 ml of Bray's solution for counting. In all assays where ATPase negative cells were used 2 mM cyanide and 2 mM iodoacetate were present.

*(b) Acid stimulated transport.* Washed energy depleted cells, pH 8 or 6 were resuspended to a density of $1.6 \times 10^8$ cells/ml in 0.2 M potassium phosphate buffer at the appropriate pH. [$^{14}$C] TMG was added and at various intervals 2 to 3 aliquots (0.2 ml) were removed, and the cells were filtered, and washed. At a specified time enough 2 N acid was added to the cells at pH 8 to decrease the pH to 6. After the acid or buffer addition, further aliquots were removed, the cells were filtered, washed and counted. In all assays using ATPase negative cells both cyanide and iodoacetate were present in the assay buffer at a final concentration of 2 mM.

### *Quantitative Experiments*

In some experiments (Tables 3 and 4) both TMG uptake and total protonmotive force were determined. The protonmotive force was estimated from the sum of the membrane potential and pH gradient. The membrane potential was calculated using the Nernst equation from the potassium distribution ratio in the presence of valinomycin. The $\Delta$ pH was calculated from the distribution of the weak acid dimethyloxazolidinedione. In these experiments oxidizing cells (strain 1100) previously treated with EDTA were resuspended in two parallel tubes, one for the measurement of $K^+$ ratios and pH gradient and the second for estimation of the sugar accumulation. In each tube cells ($1.6 \times 10^9$ cells/ml) were suspended in a mixture of 75 mM sodium phosphate and 25 mM potassium phosphate at pH 6.8. To one tube [$^{14}$C] dimethyloxazolidinedione (2.2 μg/ml, 0.1 μCi/ml), [$^{3}$H] inulin (4.2 μg/ml; 0.5 μCi/ml), and nonradioactive TMG (25 μM) were added. [$^{14}$C] TMG (25 μM; 0.1 μCi/ml) was added to the second tube. Valinomycin was added to both tubes at a final concentration of 10 μM. After 20 to 25 min, six 1-ml aliquots were removed from the first tube (containing nonradioactive

TMG) and filtered immediately. Intracellular potassium was extracted from three of the filters using a method previously described (Maloney, Kashket & Wilson, 1975) and was measured by flame photometry. Values for internal potassium ranged from 250 to 300 mM for the wild type cell examined. The three remaining filters were analyzed for both [$^{14}$C] and [$^{3}$H]. The internal pH was calculated from the distribution of the weak acid, [$^{14}$C] DMO (pK 6.3) using inulin as a marker for extracellular water (Kashket & Wong, 1969; Harold, Pavlasova & Baarda, 1970). Under these conditions the internal pH ranged from 7.6 to 8.0 in the different experiments.

[$^{14}$C] TMG uptake was measured in the second tube. After 20 to 25 min in the presence of valinomycin, two 1-ml aliquots of the suspension were removed and the cells were filtered and washed. The filters were analyzed for [$^{14}$C]. Under the conditions of the experiment, the intracellular concentration of TMG ranged from 3 to 3.4 mM. The intracellular space of the EDTA-treated cells was determined from the distribution of [$^{3}$H] water and [$^{14}$C] inulin using the microfuge technique previously described. In the presence of 100 mM phosphate buffer pH 6.8 one ml of EDTA-treated cells at an optical density of 100 contains 0.3 µl of intracellular water. This value represents the average of 2 experiments.

For other quantitative experiments spheroplasts were prepared from 1100 according to the method of Kaback (1971). These experiments were performed in a buffer containing 0.28 M sucrose, 0.05 M KCl, 0.09 M sodium phosphate and 0.01 M potassium phosphate at pH 6.7. The protocol for these experiments was the same as that previously described for the quantitative experiments with the intact cells of 1100. The intracellular space of the spheroplasts was determined from the distribution of [$^{3}$H] water and [$^{14}$C] inulin using the microfuge technique previously described. One milliliter of spheroplasts in the sucrose containing buffer at a density of $8 \times 10^{8}$ cells/ml contained 0.2 µl of intracellular water. This value represents the average of three experiments.

### *Chemicals*

Valinomycin and N,N-dicyclohexylcarbodiimide (DCCD) were purchased from Calbiochem. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (CCFP) was a gift of Dr. E.P. Kennedy (Harvard Medical School). Valinomycin, DCCD, CCFP and 3,3-dipropylthiodicarbocyanine were added to cell suspensions as small volumes of stock solution in 95% ethanol. Final ethanol concentrations did not exceed 0.2%. The fluorescent dye, 3,3-dipropylthiodicarbocyanine was a gift from Dr. A. Waggoner, Amherst College. [$^{14}$C] 5,5-Dimethyloxazolidine-2-4-dione (DMO), [$^{14}$C] thiomethylgalactoside, [$^{3}$H] inulin and [$^{14}$C] inulin were obtained from New England Nuclear Corp. The inulin was purified by paper chromatography prior to use. Nonradioactive isopropylthiogalactoside was obtained from Sigma Chemical Co.

### *Calculation of the Electrochemical Potential for Protons*

When expressed in millivolts the electrochemical potential difference for protons $\bar{\mu}_{H^+}$ is given by:

$$\bar{\mu}_{H^+} = \Delta\Psi - Z\Delta\text{pH}$$

where $\Delta\Psi$ is the membrane potential (in millivolts) and $\Delta$pH is the transmembrane pH difference (the internal pH minus the external pH). At 25 °C the coefficient $Z$ (2.3 $RT/F$) has a value of 59. $R$, $T$ and $F$ represent the gas constant, absolute temperature and the Faraday, respectively. The pH gradient was estimated from the distribution of the weak acid dimethyl-

oxazolidinedione (DMO) (*see* Maloney *et al.*, 1975). In the presence of valinomycin the membrane potential could be determined from the measured ratio of internal to external potassium. Assuming that the valinomycin-treated cells were far more permeable to the potassium ion than to any other ion, the chemical potential difference of the potassium ions on the two sides of the membrane would be in equilibrium with the electrical potential as described by the Nernst equation:

$$\Delta\Psi = -\frac{RT}{F} \ln \frac{[K]_{in}}{[K]_{out}}$$

where $[K]_{in}$ and $[K]_{out}$ refer to the internal and external concentrations of potassium, respectively. It is assumed for simplicity that the activity coefficient for $[K]_{in}$ is the same as that for $[K]_{out}$.

## Results

Two different methods were utilized to induce an inwardly directed electrochemical potential for protons. The first was to produce a chemical gradient for protons by acidifying the external medium. The second was to generate an electrical potential difference (inside negative) by inducing an outward diffusion potential for $K^+$ (in the presence of valinomycin) or an inward diffusion potential for $CNS^-$.

### *TMG Accumulation in Response to a pH Gradient*

In preliminary experiments energy-depleted cells were suspended in 0.2 M potassium phosphate buffer at pH 8 and incubated in the presence of valinomycin for one hr. This permitted entry of valinomycin which was very slow to penetrate the membrane of normal *E. coli*. The purpose of the valinomycin was to allow movement of $K^+$ across the membrane and prevent the development of a membrane potential during entry of $H^+$. When the lactose analog TMG was added to the starved cells at pH 8 the sugar entered the cell attaining a steady state concentration within the cell only slightly higher than that in the medium (Fig. 1). When sufficient HCl was added to reduce the external pH from 8 to 5.9, the intracellular TMG concentration rose to a level 14 times that in the medium. Cells which were washed at pH 6 (to equilibrate the pH across the membrane) and then exposed to TMG at pH 6 showed no accumulation (Flagg & Wilson, 1976).

Since an inwardly directed protonmotive force may result in ATP synthesis in *E. coli* (Maloney, Kashket & Wilson, 1974; Maloney & Wilson, 1975; Grinius, Slusnyte & Griniuviene, 1975; Wilson, Alderete,

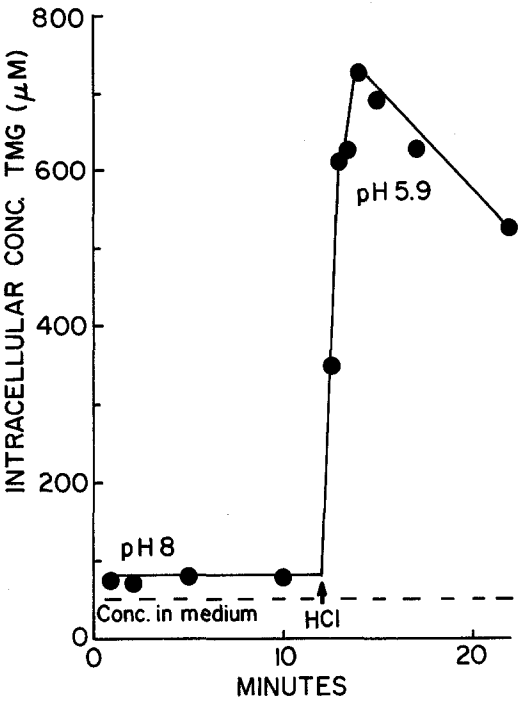


Fig. 1. TMG accumulation in response to a pH gradient. Starved ML-308 cells were washed and resuspended in 0.2 M potassium phosphate at pH 8. Cells ($1.6 \times 10^9$ cells/ml) were exposed to 10 μM valinomycin for 1 hr, then TMG was added to a final concentration of 50 μM (0.2 μCi/ml). Aliquots were removed initially for the determination of control levels of TMG uptake by the energy-depleted cells at pH 8. Next, sufficient 2 N HCl was added to decrease the pH from 8 to 5.9 and additional aliquots were removed for further TMG uptake determinations

Table 1. Sugar accumulation and ATP synthesis in response to a pH gradient[a]

| Δ pH (pH units) | TMG Accumulation in/out | ATP Synthesis (mM) |
|---|---|---|
| 2.0 | 6.0 | 0.04[b] |
| 4.8 | 1.3 | 1.00 |
| 5.3 | 0.9 | 1.10 |

[a] Aliquots for ATP and TMG determinations were removed from the same tube. The values given represent accumulation (2 min) and ATP levels (45 sec) after the addition of the acid.
[b] No significant increase in ATP levels. The initial ATP level was 0.04 mM.

Maloney & Wilson, 1976; Tsuchiya & Rosen, 1976), it was important to determine whether the observed sugar transport was secondary to ATP synthesis. Energy-depleted cells were examined for their ability to synthesize ATP under conditions in which maximal TMG up-

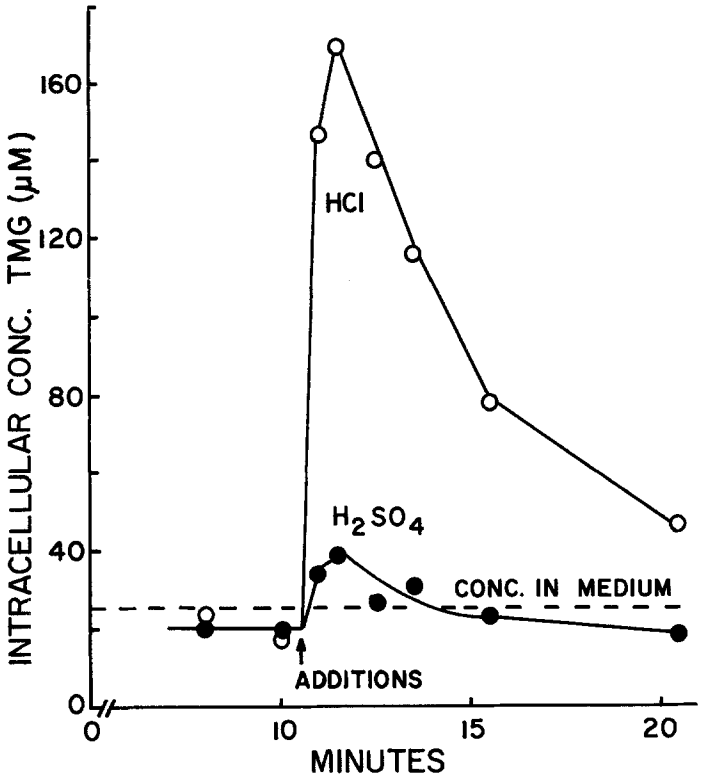


Fig. 2A

Fig. 2. Comparison of hydrochloric acid- and sulfuric acid-induced thiomethylgalactoside transport. Energy-depleted DL-54 cells were washed and resuspended in 0.2 M potassium phosphate, pH 8 containing 2 mM cyanide plus 2 mM iodoacetate ($2 \times 10^9$ cells/ml). The experiment was initiated by adding 25 μM TMG (0.1 μCi/ml). At the indicated intervals, aliquots of the cell suspensions were filtered, washed and counted for the determination of control levels of TMG uptake at pH 8. For (*A*), 2 N HCl (o——o) or 2N $H_2SO_4$ (●——●) was added at the arrow and aliquots of the suspensions were removed for further TMG uptake determinations. For (*B*), the cells were exposed to 10 μM valinomycin for 1 hr prior to the initiation of the experiment. Both hydrochloric acid and sulfuric acid were added as before. For (*C*), the cells in one of three tubes were exposed to 0.1 M KCl (▲——▲) for several min prior to the initiation of the experiment. At the arrow either $H_2SO_4$ (▲——▲; ●——●) or $H_2SO_4$ plus KCl (o——o) was added to the cell suspensions. The final concentration of potassium chloride in the latter tube was approximately 0.1 M. In all assays the final pH of the external medium was approximately 5.7

take was observed. No significant ATP synthesis was observed when depleted cells were exposed to a 2 pH unit reduction in the external medium (Table 1). The same cells accumulated TMG to an intracellular concentration 6 times that in the external medium. When the protonmotive force was increased by lowering the external pH from 8 to 3.2 or 2.7, there was a

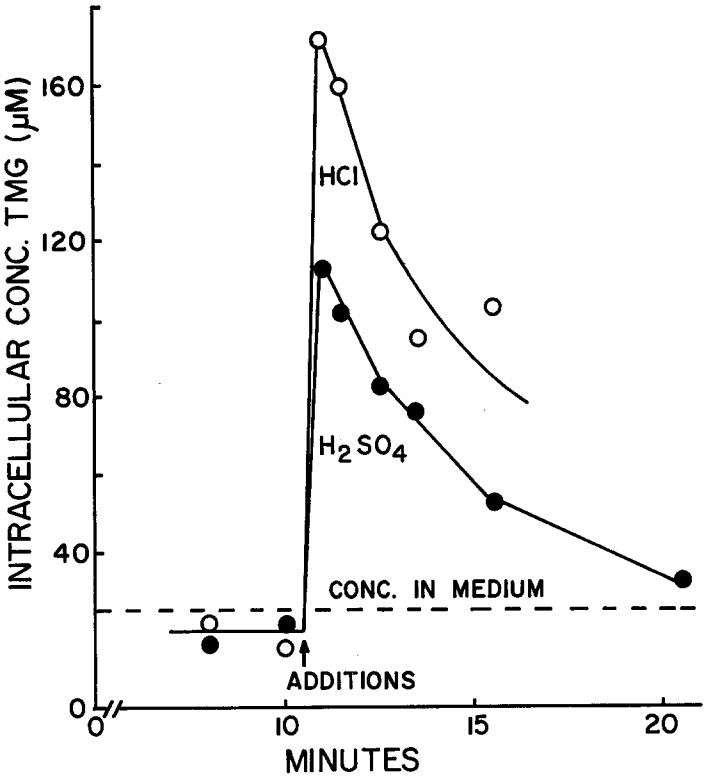
INTRACELLULAR CONC. TMG (μM)
160
120
80
40
0
HCl
$H_2SO_4$
CONC. IN MEDIUM
ADDITIONS
0
10
15
20
MINUTES

Fig. 2B

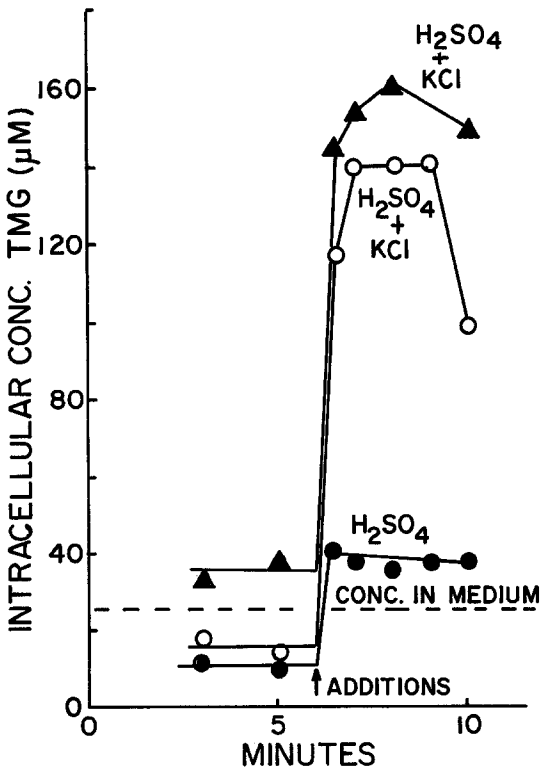
INTRACELLULAR CONC. TMG (μM)
160
120
80
40
0
$H_2SO_4$ + KCl
$H_2SO_4$ + KCl
$H_2SO_4$
CONC. IN MEDIUM
ADDITIONS
0
5
10
MINUTES

Fig. 2C

burst of ATP synthesis but no significant TMG accumulation. The cells failed to accumulate TMG under the latter conditions because the TMG transport system shows no activity at a pH lower than about 4.5. A second type of experiment which excludes ATP as the source of energy for transport under these conditions involves the use of ATPase negative mutants. Such mutants do not carry out oxidative phosphorylation (Butlin, Cox & Gibson, 1971) nor do they synthesize ATP as a result of an artificially induced protonmotive force (Maloney *et al.*, 1974; Wilson *et al.*, 1976). However, sugar accumulation can be induced in such mutants by an inwardly directed protonmotive force (Fig. 2, Fig. 5 and Table 2).

Although valinomycin had been added to all preliminary experiments with HCl, it was subsequently found that valinomycin was not essential for the HCl induced sugar accumulation. However, in the absence of valinomycin $H_2SO_4$ gave only a slight response (Fig. 2*A*). To account for these observations it was postulated that the membrane of energy-depleted cells was permeable to $Cl^-$ but not $SO_4^{--}$. The entry of $H^+$ via the sugar transport system and other electrogenic pathways would tend to carry positive charges into the cell and reduce the driving force for further entry of protons. Entry of $Cl^-$ would alleviate the adverse electrical potential and permit continued inward movement of $H^+$.

To test this hypothesis, valinomycin was added to allow $K^+$ exit to balance $H^+$ entry. Under these conditions $H_2SO_4$ induced a significant sugar accumulation (Fig. 2*B*). Likewise, the addition of $Cl^-$ ion before or during the $H_2SO_4$ addition resulted in a marked improvement in TMG uptake (Fig. 2*C*). A small but significant stimulation of sugar uptake was observed on the addition of 0.05 M NaCl or KCl, but not $K_2SO_4$ (not shown). Since the degree of stimulation of TMG accumulation by $Cl^-$ was much less than that by HCl it was clear that the pH gradient was responsible for the major effect of HCl. Nevertheless these findings are consistent with the view that the membrane is sufficiently permeable to $Cl^-$ to give rise to a $Cl^-$ diffusion potential (inside negative) under these conditions.

Additional experiments were carried out to confirm the view that $Cl^-$ entered the cell much faster than $SO_4^{--}$. When energy-depleted cells were exposed to either $^{36}Cl$ or $^{35}SO_4$ (Fig. 3) the cells appeared somewhat permeable to $Cl^-$ but not to $SO_4^{--}$. In subsequent experiments cells at pH 8 were exposed to radioactive HCl (decreasing the external pH from 8 to 6) and $Cl^-$ uptake was observed. As a control, $Cl^-$ entry was examined in cells which were washed at pH 6 and exposed to radioactive chloride at pH 6. In both cases the final concentration of $Cl^-$ was about

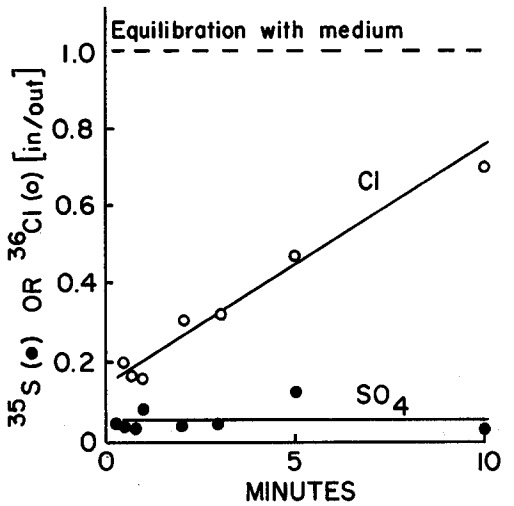


Fig. 3. Uptake of chloride and sulfate by energy-depleted cells. Starved DL-54 cells were washed and resuspended in 0.2 M potassium phosphate, pH 7 containing 2 mM cyanide and 2 mM iodoacetate ($2.8 \times 10^9$ cells/ml). The experiment was initiated by adding either radioactive KCl [$^{36}Cl$] (1 mM 0.2 μCi/ml) or $K_2SO_4$ [$^{35}S$] (2 mM 0.2 mCi/ml). At the indicated intervals aliquots (0.2 ml) were removed and the cells were filtered, washed and counted

the same (0.1 M) however, the presence of the added $H^+$ appeared to enhance $Cl^-$ entry by a factor of 2 (data not shown).

### *Accumulation in Response to a Membrane Potential*

In the next series of experiments an inwardly directed driving force for protons was created with an artificially produced electrical potential across the membrane (inside negative). The first method was that of adding valinomycin to $K^+$-containing cells suspended in low $K^+$ incubation medium. The magnitude of the $K^+$ diffusion potential can be controlled by the addition of various concentrations of $K^+$ to the external medium.

Energy-depleted cells were suspended potassium phosphate and exposed to valinomycin for 1 hr. This procedure rendered the cells permeable to potassium and at the same time prevented losses in the intracellular concentration of this ion. The cells were then diluted 500-fold into a $K^+$-free medium containing radioactive TMG. The cells accumulated the sugar to a concentration 20 times that in the external medium (Fig. 4). As a control, valinomycin-treated cells were diluted into 0.2 M potassium

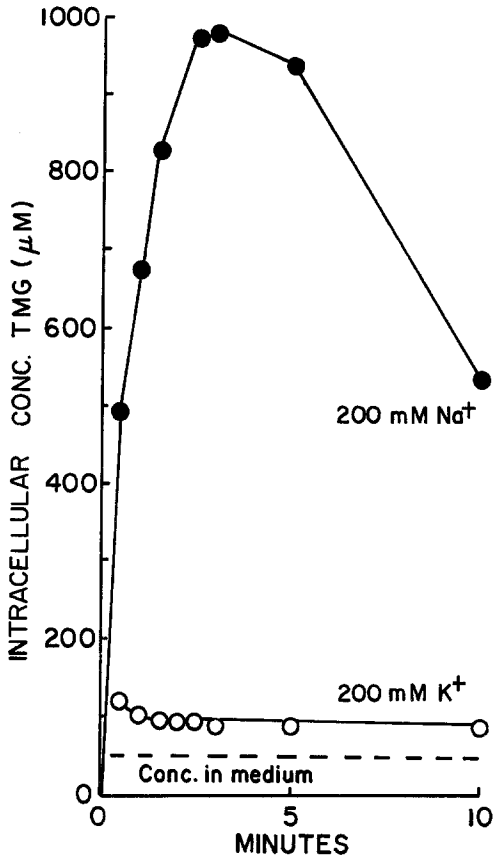


Fig. 4. TMG accumulation in response to a membrane potential. Starved ML-308 cells were washed and resuspended in 0.2 M potassium phosphate, pH 6. Cells were exposed to valinomycin for 1 hr, then diluted 500-fold into either 0.2 M sodium or potassium phosphate buffer (pH 6) containing radioactive TMG (50 μM; 0.2 μCi/ml). Aliquots were removed, and the cells were filtered, washed and counted

Table 2. Effect of CCFP on TMG accumulation in response to a protonmotive force in DL-54

| Energy source | CCFP (10 μM) | TMG Accumulation[b] in/out |
|---|---|---|
| None | – | 3 |
| Membrane potential[a] | + | 2 |
| Membrane potential | – | 28 |
| None | – | 1 |
| pH gradient | + | 0.9 |
| pH gradient | – | 7 |

[a] Membrane potential was induced with valinomycin.

[b] The values given represent the peak level of TMG accumulation occurring 1 min after acid addition or 3 min after the dilution of valinomycin treated cells into the assay buffer.

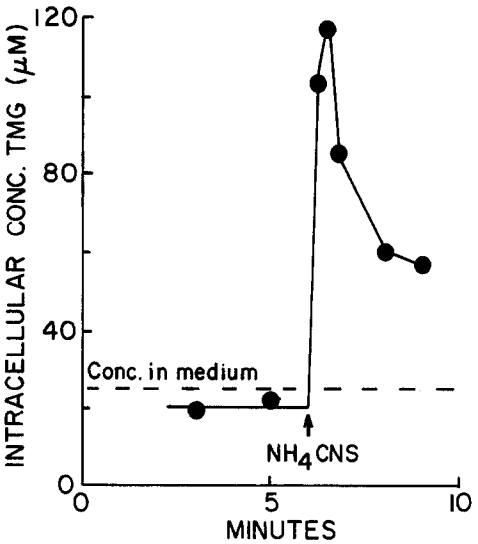


Fig. 5. Thiocyanate-induced thiomethylgalactoside uptake. Energy-depleted DL-54 cells were washed and resuspended ($3.1 \times 10^9$ cells/ml) in 0.2 M potassium phosphate (pH 7) plus 2 mM cyanide and 2 mM iodoacetate. Radioactive TMG (25 μM 0.1 μCi/ml) was added and aliquots were initially removed to determine control levels of TMG uptake. At the arrow $NH_4CNS$ was added to a final concentration of 0.05 M and additional aliquots (0.2 ml) were removed for further TMG uptake determinations

phosphate containing radioactive TMG. Under these conditions the $K^+$ concentrations inside and outside the cell were approximately the same, giving a membrane potential which was very low. Such cells showed very little sugar accumulation. When such valinomycin-treated cells were exposed to intermediate concentrations of $K^+$ intermediate levels of TMG accumulation were observed (not shown). The addition of the proton conductor CCFP completely blocked the accumulation induced either by a membrane potential or a pH gradient (Table 2). Dinitrophenol also blocked accumulation in a manner similar to that shown for CCFP.

An alternative method of producing a membrane potential was to suddenly expose the cell to an anion to which the membrane is very permeable. Under these conditions, an anion diffusion potential is created (inside negative). The addition of a sudden pulse of $NH_4CNS$ to starved cells produced a transient accumulation of TMG (Fig. 5). Two additional experiments gave similar results. Either NaCNS or KCNS produced a similar result in several experiments. As indicated in a previous section the addition of $Cl^-$ ion to energy-depleted cells results in a small but measurable TMG accumulation, suggesting that the membrane is permeable to $Cl^-$ as well as $CNS^-$.

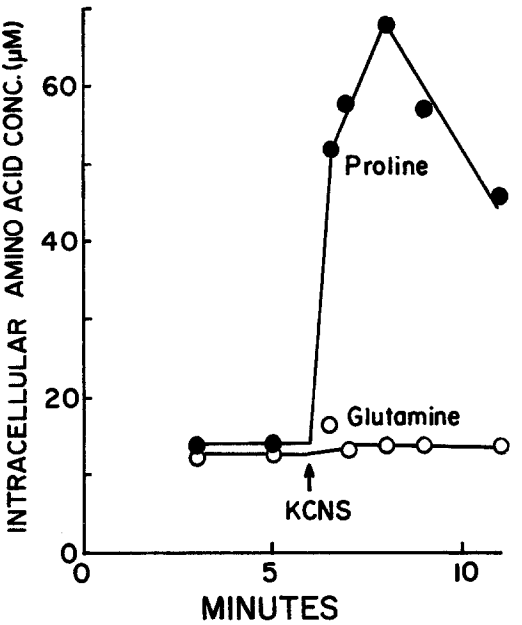


Fig. 6. Thiocyanate-induced amino acid uptake. Energy-depleted DL-54 cells were washed and resuspended in 0.2 M potassium phosphate, pH 7 ($2.6 \times 10^9$ cells/ml) containing 2 mM cyanide and 2 mM iodoacetate. The experiment was initiated by adding either proline or glutamine to a final concentration of 25 μM (0.2 μCi/ml). Aliquots of the cell suspensions were then filtered, washed and counted for the determination of control levels of amino acid uptake by the energy-depleted cells. 0.05 M KCNS was added at the arrow and additional aliquots were removed for further amino acid uptake determinations

In subsequent experiments it was found that proline but not glutamine uptake could be stimulated by the addition of KCNS (Fig. 6). These results were consistent with the idea that glutamine uptake was energized directly by phosphate bond energy while proline transport is coupled to an electrochemical potential for protons (Berger, 1973; Berger & Heppel, 1974).

### *Galactoside Accumulation in E. Coli Cells Which Lack Endogenous Energy Reserves*

The results presented in the preceding section were obtained in cells which were depleted of their energy reserves by exposure to the uncoupler, 2–4-dinitrophenol. While this procedure effectively reduces the ability of cells to energize transport, it also severely reduces carrier activity, especially when the cells are exposed to the uncoupler for several hours.

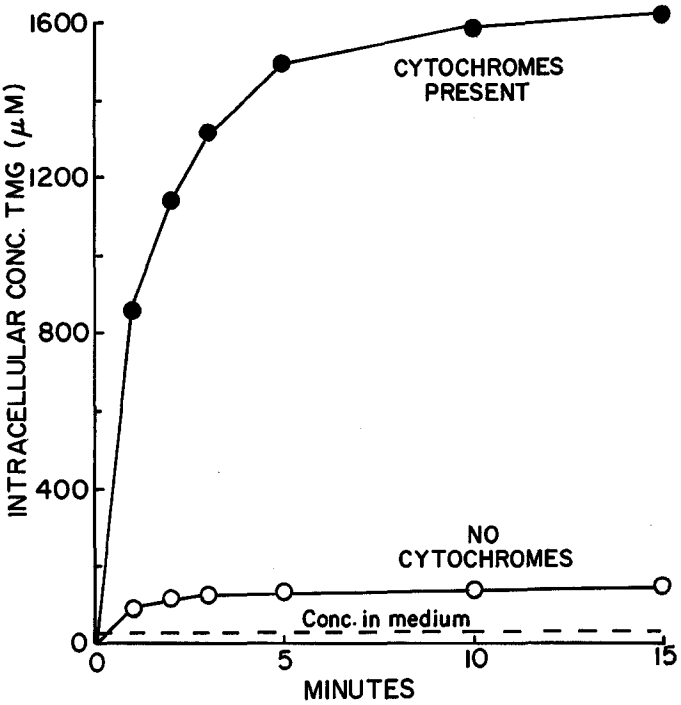


Fig. 7. TMG accumulation by SASX76-2 cells. Washed cells were resuspended in 0.1 M sodium phosphate (pH 7) containing TMG (25 μM; 0.1 μCi/ml). At the indicated intervals, samples (0.2 ml) were withdrawn and the cells were filtered, washed and counted. The optical density of the assay suspensions was $1.8 \times 10^9$ and $2.3 \times 10^9$ cells/ml for the cytochrome-containing (●——●) and the cytochrome-deficient (○——○) cells, respectively. The ONPG entry rate by the latter cells was about 30% of that found in the cytochrome-containing cells

An alternative approach was to study a cell which did not store significant energy reserves and therefore did not require energy depletion. A cell of this type was found in the heme deficient mutant SASX76 of Sasarman, Sundeanu and Horodniceanu (1968). Washed cells of SASX76-2 (lacking cytochromes) were unable to transport TMG normally while the cytochrome-containing SASX76-2 (grown in the presence of γ-aminolevulinic acid) transported TMG normally (Fig. 7). This is similar to the observation of Singh & Bragg (1974) that phenylalanine transport was reduced in cytochrome-negative cells of SASX76 but normal in cytochrome containing SASX76 grown in the presence of γ-aminolevulinic acid.

The exposure of the washed cytochrome-deficient cells to valinomycin in $K^+$-free medium did not result in galactoside accumulation. A fluorescent probe, 3,3-dipropylthiodicarbocyanine was used to examine the possibility that valinomycin had failed to penetrate the cell wall and thus did

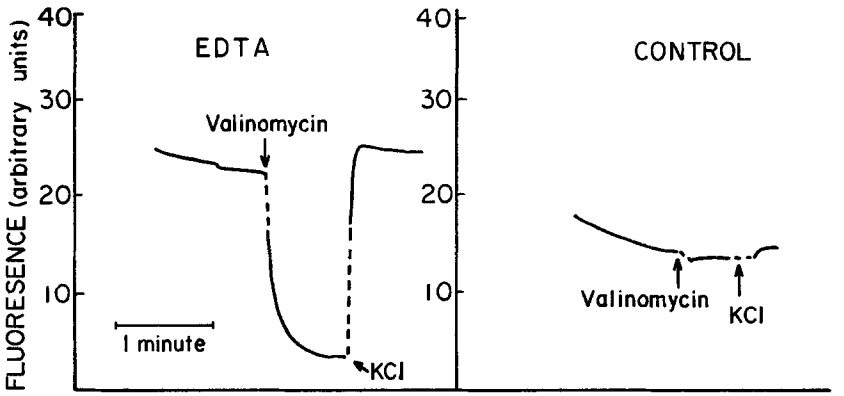


Fig. 8. Valinomycin effected fluorescence changes in cytochrome-deficient SASX76-2 cells. Washed cells (control) and EDTA-treated cells (each at a concentration of $4 \times 10^{10}$ cells/ml) were diluted 600-fold into cuvettes containing 0.1 M sodium phosphate (pH 7) and the fluorescent dye 3,3-dipropylthiodicarbocyanine ($1.3 \times 10^{-6}$ M). At the first arrow, valinomycin was added to a final concentration of 3 μM. At the second arrow, KCl was added to give a final concentration of 27 mM

not produce a membrane potential in these cells. Studies in several laboratories indicated that these probes could effectively monitor the membrane potential (Davila, Salzberg, Cohen & Waggoner, 1973; Hoffman & Laris, 1974; Laris & Pershadsingh, 1974; Kashket & Wilson, 1974). A hyperpolarization of the membrane (inside negative) results in a decrease in the fluorescent intensity of the dyes while depolarization (inside more positive) leads to an increase. When valinomycin was added to washed cells suspended in a dilute dye suspension, there was essentially no change in fluorescence indicating the absence of a valinomycin-induced potential difference across the membrane (Fig. 8). The implication of this observation was that the SASX76-2 cells were probably impermeable to valinomycin. When the washed cells were exposed to EDTA before the experiment was initiated, the addition of valinomycin resulted in a marked decrease in the fluorescent intensity of the dye (Fig. 8) indicating the presence of a membrane potential (interior negative). When potassium was added to this cuvette, the fluorescent intensity of the dye increased, indicating that a reduction in membrane potential was associated with the increase in external potassium. These findings indicated that valinomycin was only effective in inducing a membrane potential in washed cells which had been exposed to EDTA. When EDTA-treated cells were exposed to valinomycin in the presence of radioactive TMG, significant galactoside transport was observed (Fig. 9). Untreated cells showed no accumulation of TMG when exposed to valinomycin.

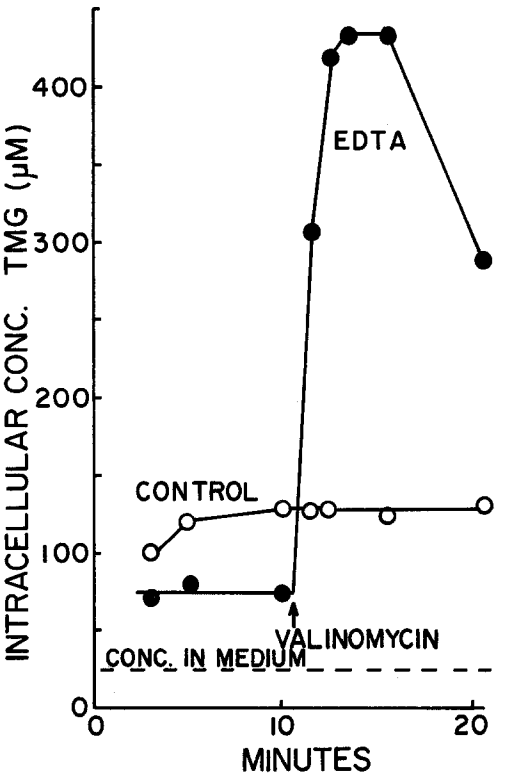


Fig. 9. Valinomycin-induced TMG accumulation in cytochrome-deficient cells. EDTA-treated SASX76-2 cells ($2.0 \times 10^9$ cells/ml) were exposed to 25 μM TMG (0.1 μCi/ml) in sodium phosphate, pH 7. Aliquots were initially removed to determine control levels of TMG uptake. Next the cells were exposed to valinomycin (10 μM) and additional samples were removed for further TMG uptake determinations. The same procedure was followed with the cells which had not been exposed to EDTA (control experiment). All manipulations were carried out at 25 °C

### *Correlation of TMG Accumulation and the Protonmotive Force in Oxidizing Cells*

It was important to describe the quantitative relationship between the protonmotive force and TMG accumulation under conditions where the source of the protonmotive force was primarily cellular oxidation. To do this, TMG accumulation was monitored in Strain 1100 under conditions where the total protonmotive force could be evaluated from direct measurements of the membrane potential and the pH gradient. The results of these experiments are given in Table 3. For each of the 12 experiments shown, independent cultures of oxidizing cells were studied. The

Table 3. Correlation of the energy of the TMG concentration gradient ($\mu_{TMG}$) with the calculated protonmotive force ($\bar{\mu}_{H^+}$)[a]

| pH ($pH_{in} - pH_{out}$) | K (in/out) | TMG (in/out) | $\mu_{TMG}$[b] (mV) | Protonmotive force | | |
|---|---|---|---|---|---|---|
| | | | | $-59\,\Delta pH$ (mV) | $+\Delta\Psi$[c] (mV) | = Total (mV) |
| 1.41 | 13.6 | 184 | −134 | −83 | −67 | −150 |
| 1.15 | 14.4 | 185 | −134 | −68 | −68 | −136 |
| 1.05 | 11.3 | 92 | −116 | −62 | −62 | −124 |
| 1.11 | 11.3 | 82 | −113 | −65 | −62 | −127 |
| 1.12 | 12.9 | 138 | −126 | −66 | −66 | −132 |
| 0.91 | 10.8 | 128 | −124 | −54 | −61 | −115 |
| 1.07 | 12.7 | 148 | −128 | −63 | −65 | −128 |
| 1.07 | 14.6 | 150 | −128 | −63 | −69 | −132 |
| 1.13 | 13.3 | 150 | −128 | −67 | −66 | −133 |
| 0.81 | 16.0 | 152 | −129 | −48 | −71 | −119 |
| 1.16 | 10.9 | 162 | −130 | −68 | −61 | −129 |
| 1.13 | 12.3 | 162 | −130 | −67 | −64 | −131 |
| 1.1 ± 0.1 | 12.8 ± 0.5 | 144 ± 9 | −127 ± 2 | −65 ± 3 | −65 ± 1 | −130 ± 2 |

[a] EDTA-treated cells (Strain 1100) were exposed to valinomycin either in the presence of radioactive or nonradioactive TMG plus DMO and inulin (marker for the extracellular space). Intracellular pH was calculated from the DMO ratios (in/out). TMG accumulation was determined from the TMG ratios (in/out), intracellular and extracellular potassium was determined by flame photometry. *See Materials and Methods; Quantitative Experiments* for details.

[b] Chemical potential difference for $TMG = -59 \log \frac{TMG_{in}}{TMG_{out}}$.

[c] Membrane potential $(\Delta\Psi) = -59 \log \frac{K_{in}}{K_{out}}$.

value of the membrane potential was estimated from the Nernst equation using measured values for intracellular and extracellular potassium in the presence of valinomycin. The distribution of DMO was used to estimate the internal pH. The external pH was measured with a glass electrode. Under the conditions of the experiments, the pH gradient across the membrane ranged from 0.8 to 1.4 pH units (47 to 83 mv) and the potassium ratios ranged from 10 to 14 (59 to 68 mV). When the total protonmotive force was calculated from the measured chemical and electrical potentials for protons there was a good correlation between the magnitude of this potential difference and the chemical potential difference for TMG ($-59 \log [TMG]_{in}/[TMG]_{out}$). There was approximately a 1 to 1 correlation (within experimental error).

Spheroplasts which were made from strain 1100 also exhibited a moderately good correlation between the calculated protonmotive force

Table 4. The protonmotive force and TMG accumulation in *E. coli* spheroplasts[a]

| $\mu_{TMG}$ (mv) | $\bar{\mu}_{H^+}$ (mv) |
|---|---|
| $-102 \pm 3$ | $-120 \pm 3$ |

[a] Spheroplasts were made from Strain 1100 according to the method of Kaback (1971). The data are the mean values of 4 experiments $\pm$ the SEM.

and TMG accumulation (Table 4). In these preparations, however, the chemical potential difference for TMG was consistently somewhat lower than the calculated electrochemical potential difference for protons. Perhaps this discrepancy may be due to a "leak" of accumulated sugar. Spheroplasts were found to accumulate only 50% of that found with cells exposed to EDTA only. As with intact cells, however, spheroplasts maintain their impermeability to protons. Under similar conditions the pH gradient across the membrane of spheroplasts was about the same as that found in the intact cells.

## Discussion

Evidence was presented that strengthens the view that an inwardly directed protonmotive force drives $\beta$-galactoside accumulation in *E. coli*. Acidification of the external medium provided a chemical driving force for the inward migration of protons, resulting in a marked stimulation of TMG uptake. A similar observation has been made in *Streptococcus lactis* (Kashket & Wilson, 1974). An interesting observation made in the course of the present studies was that the addition of HCl but not $H_2SO_4$, induced sugar accumulation. Evidence was presented which was consistent with the view that proton entry resulted in the generation of a considerable membrane potential (inside positive) which restricted further entry of protons. When proton entry was accompanied by the permeant $Cl^-$ ion, electrical neutrality could be maintained and considerable proton entry sustained. The sulfate ion, on the other hand, could not enter the energy-depleted cell and proton entry was apparently restricted by the adverse electrical potential. These studies are consistent with previous work indicating the electrogenic movement of $H^+$ with galactosides (West, 1970; West & Mitchell, 1972; Hirata *et al.*, 1973; Hirata *et al.*, 1974; Grinuviene *et al.*, 1975; Schuldiner & Kaback, 1975).

An electrical potential difference was also able to provide the driving force for proton entry and sugar accumulation, as previously demonstrated

in *Streptococcus lactis* (Kashket & Wilson, 1972; Kashket & Wilson, 1973) and membrane vesicles of *E. coli* (Hirata *et al.*, 1973; Hirata *et al.*, 1974; Schuldiner & Kaback, 1975). In the present study a membrane potential (inside negative) was induced either by outward directed diffusion potential of $K^+$ (in the presence of valinomycin) or an inwardly-directed diffusion potential of $CNS^-$. In each case a transient TMG accumulation resulted from the imposed membrane potential. Thus, sugar accumulation was observed when an inwardly directed protonmotive potential difference was produced either by a chemical or an electrical driving force.

The accumulation of TMG was independent of any direct involvement of ATP since the magnitude of the artificially induced protonmotive force was insufficient to energize ATP synthesis (Table 1). Furthermore, acid-induced and potential-induced transport was observed in ATPase negative cells which lacked the capacity to synthesize ATP in response to a protonmotive force (Figs. 2 and 5; Table 2).

In an attempt to correlate the protonmotive force with the sugar accumulation both the membrane potential and the pH gradient were estimated experimentally. The membrane potential was estimated indirectly from the distribution of potassium in the presence of valinomycin. This technique makes three assumptions: (1) that cells are permeable to valinomycin, (2) that in the presence of the antibiotic, the final distribution of potassium is in equilibrium with the electrical potential difference across the membrane according to the Nernst equation, and (3) that intracellular $K^+$ is free (and not bound). Evidence was presented that EDTA-treated cells of *E. coli* are permeable to valinomycin in confirmation of Pavlasova & Harold (1968) and West & Mitchell (1973). Cells exposed to this ionophore in this manner showed very rapid changes in membrane potential (measured with a fluorescent dye) when potassium was added in increments to the external medium. Thus, it is reasonable to assume that under these conditions potassium ions on the two sides of the membrane are close to their electrochemical equilibrium. Furthermore, evidence from several laboratories (*see* the review by Harold & Altendorf, 1974) suggest that most of the intracellular $K^+$ in bacteria is free and osmotically active. Thus, the use of the Nernst equation with the measured potassium concentration ratio in the presence of valinomycin is believed to provide a satisfactory estimate of the membrane potential under the conditions of these experiments.

The pH gradient across the membrane was estimated from the transmembrane distribution of the metabolically inert weak acid, DMO. It was assumed that the undissociated form of the acid crossed the membrane

by simple passive diffusion, and that its concentrations on the two sides rapidly equilibrated. The ionized form of the acid, on the other hand, does not cross the membrane or does so extremely slowly. Evidence that DMO readily passes the bacterial membrane and is not transported comes from the work of Harold *et al.* (1970). These authors estimated the permeability coefficient of the charged ($P_A$) and undissociated ($P_{HA}$) forms of the acid and found that $P_{HA}/P_A$ was approximately 200. According to Roos (1965) the maximal error in the estimation of the internal pH would be 0.1 pH units when the membrane potential was 100 mV and $P_{HA}/P_A$ was 200. It is therefore likely that this method provides a reasonable estimate of pH differences across the cell membrane.

If there is an obligatory coupling between proton movement and sugar movement (with a stoichiometry of 1:1), then the electrochemical potential difference of protons should be in equilibrium with the chemical potential difference of TMG. Such a relationship was observed for TMG transport in *S. lactis* (Kashket & Wilson, 1974). A correlation between lactose concentration ratio and membrane potential in membrane vesicles of *E. coli* was observed by Schuldiner & Kaback (1975). Experiments with intact cells of *E. coli* presented in Tables 3 and 4 indicate a good quantitative agreement between the protonmotive force ($\Delta\Psi - 59\,\Delta$pH) and the chemical potential difference for TMG ($-59 \log \mathrm{TMG_{in}}/\mathrm{TMG_{out}}$). These experiments therefore provide quantitative support for the proton-lactose cotransport hypothesis in *E. coli.*

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