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Stoichiometry of Proton Movements Coupled to ATP Synthesis Driven by a pH Gradient in *Streptococcus lactis*

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Summary. An electrochemical potential difference for H^+ was established across the plasma membrane of the anaerobe *Streptococcus lactis* by addition of sulfuric acid to cells suspended in potassium phosphate at pH 8 along with valinomycin or permeant anions. Subsequent acidification of the cell was measured by the distribution of salicyclic acid. A comparison between cells treated or untreated with the inhibitor N,N′-dicyclohexylcarbodiimide was used to reveal that portion of net proton entry attributable to a direct coupling between H^+ inflow and synthesis of ATP catalyzed by the reversible proton-translocating ATPase of this microorganism. When the imposed electrochemical proton gradient was below 180–190 mV, proton entry was at the rate expected of passive flux, for both control cells and cells treated with the ATPase inhibitor. However, at higher driving force acidification of control cells was markedly accelerated, coincident with ATP synthesis, while acidification of cells treated with the inhibitor continued at the rate characteristic of passive inflow. This observed threshold (180–190 mV) was identified as the reversal potential for this H^+ "pump". Parallel measurements showed that the free energy of hydrolysis for ATP in these washed cells was 8.4 kcal/mole (370 mV). The comparison between the reversal (threshold) potential and the free energy of hydrolysis for ATP indicates a stoichiometry of 2 H^+/ATP for the coupling of proton movements to ATP formation in bacteria.

Key words chemiosmotic theory · stoichiometry · ATP synthesis · proton-translocating ATPase · membrane potential · pH gradient

Introduction

The membranes of bacteria contain a proton-translocating ATPase (BF_0F_1) that operates in one of two modes under physiological conditions – to either establish and maintain an electrochemical proton gradient at the expense of ATP hydrolysis, or to catalyze ATP formation subsequent to the generation of an electrochemical proton gradient by other reactions (for recent reviews, *consult* Harold, 1977; Rosen & Kashket, 1978; Mitchell, 1979; Maloney, 1982). In the latter case, this enzyme carries out the terminal step in oxidative or photosynthetic phosphorylations, as do its counterparts in mitochondria and chloroplasts. It is presumed that the mechanism of coupling between proton movements and ATP synthesis (hydrolysis) is the same in the prokaryotic and eukaryotic systems, for these enzymes share three important characteristics: (*i*) there is a similar, rather complex physical structure, incorporating at least 8 different polypeptide subunits; (*ii*) the coupling between ion movement and ATP synthesis (hydrolysis) requires participation of both integral (F_0) and periferal (F_1) membrane proteins; and (*iii*) there is no covalent interaction between reactants and protein during the catalytic cycle. These features distinguish this class of ion "pumps" from all others so far studied, in which only a relatively few, integral membrane proteins are required, at least one of which is likely to be phosphorylated during the overall reaction (*see* Guidotti, 1979).

Several bacterial systems have proven useful to the study of this proton-translocating ATPase. While genetic and biochemical studies have been especially fruitful using *Escherichia coli* (Downie, Gibson & Cox, 1979; Futai & Kanazawa, 1980; Fillingame, 1981) or PS3, a thermophilic bacterium (Kagawa, 1978), questions concerning the operation of this enzyme within its native membrane have been more conveniently addressed using the Streptococci. Thus, work with *Streptococcus faecalis* (Harold, Pavlasova & Baarda, 1970; Harold & Papineau, 1972) was the first to identify this enzyme as the link between ATP hydrolysis and generation of both the membrane potential and pH gradient of metabolizing cells (*see also* Kashket, 1981), and studies of *S. lactis* have provided convincing evidence that this ATPase activity can be reversed to yield net ATP synthesis when the appropriate electrical or chemical (pH) gradients are imposed (Maloney, Kashket & Wilson, 1974; Maloney & Wilson, 1975).

Work using *S. lactis* has also been important in documenting the obligatory nature of the coupling between proton movements and ATP formation (Ma-

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loney, 1977). But in that earlier work, it was possible to study this coupling only under conditions where an imposed membrane potential (inside negative) dominated the electrochemical proton gradient. The experiments reported here provide the necessary companion study, in which a pH gradient drives the coupled reaction. As well as confirming conclusions reached in the earlier work, the results presented here provide strong support for two additional proposals: (*a*) that the stoichiometry of the reaction is $2H^+$/ATP; and (*b*) that the rate of the reaction (measured as proton entry) is indifferent to the relative proportion of membrane potential and pH gradient (*see also* Maloney & Schattschneider, 1980). These findings are consistent with predictions made by a model that supposes a direct interaction between all reactants (H^+, phosphate and ADP) during the synthesis of ATP (Mitchell, 1974).

Materials and Methods

Bacterial Strain and Growth Conditions

Streptococcus lactis (ATCC 7962) was grown as described earlier (Maloney & Wilson, 1975; Maloney, 1977). In most experiments cells were twice washed and finally resuspended using 0.1 M potassium phosphate, pH 8. The cell density of this concentrated stock was about 8000 Klett Units (No. 42 filter), corresponding to 25 μl of intracellular water/ml suspension (*see below*) or 16.5 mg dry weight/ml (Kashket & Wilson, 1972). All experiments were done at 21–22 °C.

Measurement of Intracellular Water

Intracellular water was determined from the distribution of tritiated water (2 μCi/ml) after a 1-min centrifugation through silicone oil (specific gravity of about 1.05) using a Beckman Microfuge B. [^{14}C]-Inulin (0.8 μCi/ml; 0.4 mg/ml) was used to monitor the extracellular space. The silicone oil was a 3:1 (vol/vol) mixture of Dow Corning Silicone Fluids Nos. 550 and 510, respectively. Provided that a minimum cell mass was used (equivalent to 0.6 μl cell water), internal and external water spaces were directly proportional to total cell mass and independent of the volume used for centrifugation. Calculated internal water space was 0.31 ± 0.01 (SEM, 6 experiments) for 1 ml of a suspension at 100 Klett Units; extracellular water represented $42 \pm 1\%$ of total pellet water. These internal and external water spaces did not change after addition of valinomycin to 10 μM.

Determination of Intracellular Potassium

Two techniques gave the same results. In one case cells suspended in 0.1 M potassium phosphate, pH 8, were filtered (0.65 μ pore size), and then washed once with an equal volume of 0.1 M sodium phosphate (pH 8) to remove residual external potassium. Alternatively, cells suspended in potassium phosphate were centrifuged through silicone oil, as described above, and the known external water space was used to account for contaminating extracellular potassium in the pellets. In either case, total potassium on filters or in pellets was measured by flame photometry. The results of seven experiments indicated internal potassium of 380 ± 30 mM (SEM), in agreement with other work (Maloney, 1977).

Assays of Intracellular Phosphate

For these measurements cells were washed and resuspended using 0.1 M potassium sulfate maintained at pH 8 with 20 mM potassium-morpholinopropane sulfonate. In routine experiments aliquots containing about 2.5 μl cell water were extracted on ice with 0.5 N perchloric acid for 20–30 min before neutralization with potassium hydroxide. After the extracts were clarified by centrifugation, inorganic phosphate was determined using a method described by Ames (1966). In several experiments this method was compared with a procedure (Martin, Berberich, Ames, Davis, Goldberger & Yourno, 1971) designed to eliminate contributions from acid labile compounds such as pyrophosphate. The two assays gave identical results. Measurements from eight separate experiments indicated an inorganic phosphate pool of 53 ± 3 mM (SEM). This represents about one-third of the total organic and inorganic phosphate measurable (Ames, 1966) after acid extraction. Control experiments showed that all (>95%) inorganic phosphate measured in this way was contained within cells, and that there was no significant (<20%) increase of internal phosphate after a 1-hr incubation of cells in the phosphate-based medium used for other work. No (<5%) release of phosphate into the medium was found after cells were subjected to a pH jump (pH 8 to pH 3.5) in the presence of valinomycin.

Determination of Thiocyanate Distribution

In some experiments the presence of a membrane potential (inside positive) was inferred from the distribution of the thiocyanate anion. In such cases, cells were suspended at a density of about 1100 Klett Units in 0.1 M potassium phosphate, pH 8, along with potassium [^{35}S]-thiocyanate (0.3 μCi/ml; 15 μM final concentration). No valinomycin was present. After 10 min, duplicate 0.2 ml samples (0.68 μl cell water) were centrifuged through silicone oil. Sulfuric acid was then added to lower external pH to 3.5, and further samples removed for centrifugation 1 and 10 min later. Calculations of the distribution of thiocyanate assumed the external and internal water spaces measured in other experiments (*see above*). The membrane potential in equilibrium with this distribution was determined using the Nernst relationship, assuming no nonspecific binding of the anion to cells. The concentration of thiocyanate used was without effect on the ATP synthesis that occurred when valinomycin was also present. However, higher levels (100 μM or above) were inhibitory (tested in cases where cell density was reduced 15-fold). It is not clear whether this inhibition reflects a direct toxic effect of the anion, or an indirect effect due to association of the anion with the potassium-valinomycin complex (Blok, Gier & Deenan, 1974).

Measurement of Intracellular pH

Two procedures were used. To estimate internal pH of valinomycin-treated cells suspended in 0.1 M potassium phosphate at pH 8, aliquots containing about 2.5 μl cell water incubated with either tritiated water (2 μCi/ml) and [^{14}C]-methylamine (0.4 μCi/ml; 15 μM final concentration) or tritiated water (2 μCi/ml) and [^{14}C]-inulin (0.8 μCi/ml; 0.4 mg/ml). The pellets obtained after centrifugation through silicone oil had the same total water spaces, and a comparison of the inulin and methylamine contents allowed calculations of the distribution of the weak base. In three separate experiments, the calculated ratio of internal to external methylamine was 2.3 ± 0.1 (SEM), indicating a mean value for internal pH of 7.6 (Maloney, Kashket & Wilson, 1975).

Other experiments required assays of the kinetics of change of internal pH after external pH had been lowered by addition of an acid to the medium. In these cases internal pH was estimated (Maloney et al., 1975) from the distribution of salicylic acid (pK =

3.0). Unless otherwise stated, the following general procedure was used. Cells were suspended at a density of 75–150 Klett Units in 0.1 M potassium phosphate, pH 8, along with [^{14}C]-salicylic acid (0.1 μCi/ml; 1.5 μM final concentration) and (when required) valinomycin (10 μM final concentration). During the 5- to 10-min preincubation period, duplicate samples were removed to estimate nonspecific binding of salicylate and to measure zero time levels of ATP (*see below*). A small volume of 1 N acid (usually sulfuric) was then added to lower external pH to the desired value. Subsequently, 0.2-ml samples (0.05–0.10 μl cell water) were filtered on prewetted, prechilled (4 °C) Millipore filters (0.65 μ pore size). This was followed by a brief wash with 4 ml of iced 0.1 M potassium phosphate that had been similarly acidified (to within ±0.2 pH units). Time from filtration to completion of the wash was about 5 sec. Duplicate samples removed before addition of acid gave an estimate of nonspecific binding to both filters and cells; these blank values (100–150 cpm) were subtacted from sample values (typically 300–3,000 cpm) before calculation of internal pH. In such experiments, the weak acid was accumulated maximally to an internal level of about 5 mM when large pH gradients were imposed. Measurements of internal buffering power (Maloney, 1979) lead one to expect that of itself such accumulation would not lower internal pH by more than 0.2 pH units (from 7.6 to 7.4).

In a control experiment, cells were subjected to a pH jump (pH 8 to pH 3.5, using sulfuric acid) that resulted in a several thousandfold accumulation of radioactivity. The material accumulated within cells was extractable (98%) with ethanol and was identified as unaltered salicylic acid by cochromatography with the unlabeled compound on silica gel, using hexane/acetic acid/chloroform (85:15:10; vol/vol). In one other experiment, after external pH had been lowered to pH 3.5, samples were removed at 45-sec intervals and cells separated from the medium, alternately, using either filtration and washing or a centrifugation through silicone oil. The two methods gave identical results for the decay of internal pH during the first 3–4 min after the pH jump, although at later times the centrifugation method indicated a more alkaline internal pH (by as much as 0.5 pH unit after 10 min). Several additional findings also suggest that the filtration method provided adequate estimates of the initial changes of internal pH: (*i*) When large pH gradients were imposed (3.5–4.5 pH units) a several thousandfold accumulation of the anion occurred within 20 sec, indicating that the later and less rapid changes of internal pH could be accurately tracked by the probe. (*ii*) Although external pH was routinely lowered by 2–4.5 pH units, extrapolated zero time internal pH was usually pH 6.8–7.2, reasonably close to the expected range (pH 7.4–7.6, depending on the size of the pH jump; note that since internal buffering power increases as pH falls (Maloney, 1979), extrapolations to zero time may underestimate initial internal pH). (*iii*) The rates of acidification observed for cells treated with both valinomycin and N,N′-dicyclohexylcarbodiimide (DCCD) were used to calculate net membrane conductance to H^+ (*see* Discussion). This calculation agreed well with values obtained by entirely different methods (*see* Maloney, 1982). The major deficiency of this assay is a quantitative uncertainty when internal pH is only 1–1.5 pH units above outside pH, since sample counts are only 2–3 times higher than the assay blank. This was of particular concern when outside pH was near the pK of the probe. Thus, when outside pH was 3–3.5, no quantitative arguments were based on values for internal pH of 5 or below.

Assays of ATP and ADP

For routine experiments, 0.4-ml aliquots (containing 0.09–0.18 μl cell water) were mixed with 0.1 ml iced 1 N perchloric acid, and maintained in an ice bath for at least 20 min before neutralization with 0.3 ml 3 N potassium hydroxide. The supernatant was assayed for ATP using the firefly assay, essentially as described earlier (Maloney & Wilson, 1975), except that photon counting was performed with a Nuclear Chicago Mark II Liquid Scintillation Counter in which both coincidence and dead time circuitries were disconnected. The mean of at least three trials was used to estimate the ATP content of a sample. In cases where both ATP and ADP were measured, cell density was increased fivefold to ensure accurate assay of the low basal level for ATP. ADP was measured after its conversion to ATP in the presence of pyruvate kinase and excess phospho(enol)pyruvate (Maloney, 1977).

Chemicals

Sigma Chemical Co. was the source of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), phospho(enol)pyruvate, pyruvate kinase (Type II), valinomycin, and the luciferin-luciferase preparation (FLE-50) used in the assay of ATP. N,N′-dicyclohexylcarbodiimide (DCCD) was purchased from Schwarz/Mann Co. and sodium tetraphenylborate from Fisher Chemical Co. Dr. P.L. Pedersen (The Johns Hopkins Univerisity School of Medicine) generously provided carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. The radioactive materials, all used without further purification, were from the New England Nuclear Corp.: carboxyl [^{14}C]-salicylic acid; [^{14}C]-methylamine; [^{14}C]-inulin; potassium [^{35}S]-thiocyanate; and tritiated water.

Valinomycin was routinely used at 10 μM final concentration, DCCD at 1 mM. The valinomycin, DCCD, and labeled salicylic acid or methylamine were added to cells from concentrated stocks of ethanolic solutions. Final ethanol concentrations did not exceed 0.3%.

Calculation of Thermodynamic Quantities

The difference in electrochemical potential for H^+ ($\Delta\tilde{\mu}_{H^+}/F$) was expressed in electrical units (mV) and was calculated according to the expression:

$$\Delta\tilde{\mu}_{H^+}/F = \Delta\psi - (2.3\ RT/F)\Delta\text{pH} \quad (1)$$

where R, T and F have their usual meanings, and where $\Delta\psi$ indicates the value of the membrane potential and ΔpH gives the difference between internal and external pH. The membrane potential was calculated by assuming an equilibrium distribution for potassium in the presence of valinomycin. In cases where calculation of $\Delta\tilde{\mu}_{H^+}/F$ was appropriate, cells were initially suspended in 0.1 M potassium phosphate, pH 8, along with valinomycin. Under these conditions measurements of internal potassium and internal pH (*see above*) indicated that H^+ was distributed at or near equilibrium ($\Delta\tilde{\mu}_{H^+}/F = +6$ mV). This small apparent disequilibrium was neglected in calculation of imposed driving force, since the value of $\Delta\tilde{\mu}_{H^+}/F$ after additions of sulfuric acid was large (*see for example*, Fig. 5). Moreover, with this assumption, the value of $\Delta\tilde{\mu}_{H^+}/F$ established by addition of acid at zero time could be calculated directly from the measured deflections in outside pH, along with known changes (if any) of external potassium. External pH was measured in each sample using Radiometer pH meter (PHM64) equipped with a combined electrode (GK2321C), after aliquots for assay of internal pH and intracellular ATP had been removed. Cell density was sufficiently low so that internal buffering power would not significantly alter outside pH as a result of K^+/H^+ exchange.

In other experiments, the free energy for hydrolysis of ATP ($\Delta G'_{ATP}$) was calculated for cells suspended in 0.1 M potassium phosphate, pH 8, in the presence of valinomycin. Calculations were made from the following relationship:

$$\Delta G'_{ATP} = \Delta G^0_{ATP} + RT \ln [ATP]/[ADP][Pi], \quad (2)$$

where ΔG^0_{ATP} indicates the standard free energy for hydrolysis,

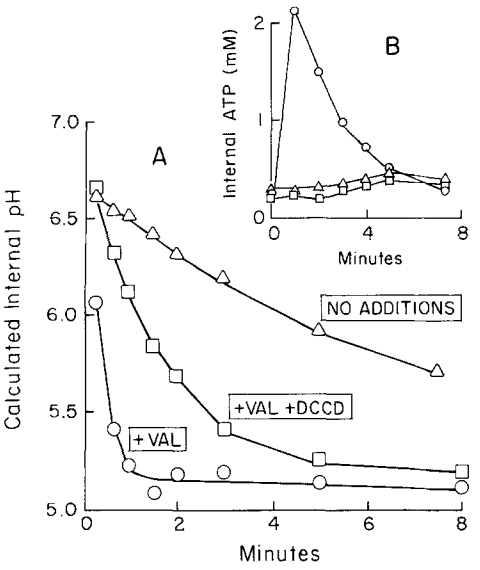

Fig. 1. ATP synthesis and proton entry after addition of sulfuric acid. Stock cells were diluted to a final density of 100 Klett Units in 0.1 M potassium phosphate, pH 8, along with labeled salicylic acid and either 10 μM valinomycin (○) or the equivalent volume of ethanol (△). For pretreatment with N,N′-dicyclohexylcarbodiimide (DCCD), stock cells were incubated with the inhibitor (1 mM) for 60 min at 21 °C before dilution into phosphate buffer and addition of the ionophore and salicyclic acid (□). At zero time 1 N sulfuric acid (about one-tenth volume) was added to lower external pH to 3.5. Samples were then removed for assay of either internal pH (*A*) or intracellular ATP (*B*)

and where terms in brackets give the molar concentrations of nucleotides (ATP, ADP) and phosphate (Pi). Division by Faraday's constant was used to express this calculated "phosphate potential" in equivalent electrical units.

Results

Factors Affecting ATP Synthesis and Proton Entry After Imposition of a pH Gradient

The basic experimental design is illustrated in Fig. 1. In that experiment washed cells were first suspended at pH 8 in 0.1 M potassium phosphate, conditions that yield an internal pH of 7.6 (*see* Materials and Methods). Sulfuric acid was then added to lower outside pH to 3.5, imposing an initial pH gradient of 4.1 pH units across the cell membrane. At later times aliquots were removed to estimate changes of both internal pH (Fig. 1 *A*) and intracellular ATP (Fig. 1 *B*) for each of three samples: cells to which acid alone was added (triangles); cells also made permeable to potassium by use of valinomycin, the potassium ionophore (circles); and potassium permeable cells having an inactivated BF_0F_1 because of prior treatment with N,N′-dicyclohexylcarbodiimide (DCCD) (squares) (Fillingame, 1981). As expected from earlier work (Maloney & Wilson, 1975), ATP synthesis occurred by reversal of BF_0F_1 when acid was added to cells also exposed to valinomycin. There was in addition a prompt net acidification of these cells, at an initial rate of about 3 pH units/min. In this case a portion of the observed proton entry might be attributed to a coupling with ATP formation, since there was partial reduction (to 0.7 pH units/min) of this rapid initial acidification when ATP synthesis was blocked by the carbodiimide inhibitor. But other factors must also significantly affect both ATP formation and net proton entry, for ATP synthesis did not occur when acid alone was added, despite the persistence of a pH gradient sufficient to reverse BF_0F_1 in valinomycin-treated cells. Moreover, net acidification of the cell was substantially slowed, to only 0.15 pH units/min.

It seemed likely that the differing responses of cells untreated and treated with valinomycin reflected an effect of the ionophore on the membrane potential. Thus, when sulfuric acid is added to otherwise untreated cells, the electric gradient may be significantly influenced by the chemical gradient for H^+ (OH^-), so that the membrane potential would move towards the H^+ equilibrium potential (inside positive). If this does occur, one might expect results of the sort illustrated by Fig. 1. For example, earlier work (Maloney & Wilson, 1975; Maloney, 1977) showed that reversal of BF_0F_1 in *S. lactis* required an imposed electrochemical proton gradient of at least 175–215 mV. For the experiment shown in Fig. 1, inward driving force contributed by the pH gradient corresponded to about 240 mV, so that a membrane potential, inside *positive*, of only 25–65 mV would reduce net inward driving force on H^+ to below the required threshold value. However, if valinomycin were also present, increased membrane permeability to K^+ should ensure that the membrane potential rests near the potassium equilibrium potential (−17 mV at zero time), and the imposed electrochemical proton gradient would exceed threshold. In a qualitative sense, such reasoning would also predict the valinomycin dependence of net proton entry that was observed (Fig. 1, compare circles and triangles). Without the ionophore net acidification may be limited by the (relatively) slow entry of external anions (phosphate, sulfate) or efflux of internal cations (largely potassium). Consequently, one expects an acceleration of net proton entry when the membrane is made permeable to potassium with valinomycin. Several experiments were performed to test the arguments given above, for if they are correct, the use of valinomycin would greatly simplify an analysis of the relationship between ATP formation and proton movements under these conditions.

In one series of experiments the distribution of thiocyanate was used to monitor the appearance of

Table 1. Distribution of thiocyanate before and after addition of sulfuric acid[a]

Time (min)	Observed ratio In/out	Derived membrane potential (mV)
0	1.9±0.2	+16
1	23.9±1.3	+81
10	31.5±2.8	+88

[a] Accumulation of thiocyanate was determined as described in Materials and Methods. Addition of sulfuric acid lowered external pH from 8 to 3.5 in each of 3 separate experiments. Mean values along with standard errors are shown.

a membrane potential (inside positive) after addition of sulfuric acid (Table 1). Those studies showed that before addition of acid there was an apparent 1.9-fold accumulation of the anion, corresponding to a small positive potential (+16 mV). However, after acid was added, the striking increase of intracellular thiocyanate indicated a large positive potential [+81 to +88 mV), of size sufficient to lower the electrochemical proton gradient below the threshold needed for ATP synthesis.

A second series of experiments examined the effects of permeant anions on ATP formation after addition of sulfuric acid (Fig. 2). In these cases a parallel inflow of the permeant anion was expected to limit the size of the positive potential that would form as H^+ moves inward, down the chemical gradient. Thus, a variety of permeant anions (as valinomycin) should be permissive to the observation of ATP synthesis driven by an imposed pH gradient (*see also* Maloney, 1978). Figure 2*A* shows the example in which cells were treated with acid in the presence of 0.1 M halide anion. In that experiment the negative control (sulfate alone added) showed little ATP formation (an initial rate of 0.04 mmol ATP/min liter cell water), whereas significantly increased rates of synthesis were observed for cells also exposed to chloride, bromide, iodide or valinomycin (0.16, 0.25, 0.98 and 6 mmol ATP/min liter cell water, respectively). Other trials employed either 3–100 mM nitrate (Fig. 2*B*) or 1–10 μM tetraphenylborate (Fig. 2*B*) as permeant anions; in each instance, increasing levels of the anion were associated with increasing initial rates of ATP appearance (with the exception of tetraphenylborate at >10 μM; *see* legend to Fig. 2*C*). Other work (*data not given*) showed that ATP synthesis rescued by these permeant anions was sensitive to a proton conductor (carbonylcyanide-*p*-trifluoromethroxyphenylhydrazone, 1 μM) and was absent when cells were pretreated with DCCD, the inhibitor of BF_0F_1. Thus, under these conditions a productive reversal of the proton-translocating ATPase is recovered by external anions with an effectiveness that parallels their lipid solubility: tetraphenylborate > nitrate > iodide > bromide ≳ chloride.

Two additional studies compared proton entry

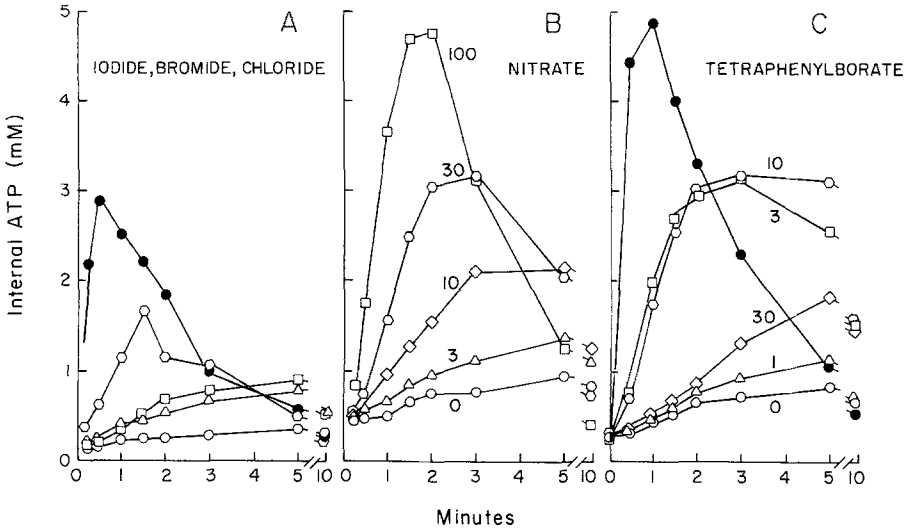

Fig. 2. Effect of permeant anions on ATP synthesis driven by a pH gradient. Results of three separate experiments are shown: (*A*) Stock cells at 7400 Klett Units in 0.1 M potassium phosphate, pH 8, were diluted 50-fold into this same buffer acidified to pH 3.3 with sulfuric acid. The dilution buffer also contained either 0.1 M potassium iodide (○), bromide (□) or chloride (△), or 0.05 M potassium sulfate (○, ●); for the positive control, cells were treated with 10 μM valinomycin before dilution (●). (*B*) The experiment was performed as in part *A*, except that cells were diluted into buffer acidified (pH 3.3) with varying amounts of nitric and sulfuric acids, along with potassium sulfate to maintain 0.1 M sulfate in all samples. Final nitrate concentrations (mM) are shown on the graph. (*C*) Cells were placed at 75 Klett Units in 0.1 M potassium phosphate, pH 8, along with the indicated levels (μM) of sodium tetraphenylborate, before addition of sulfuric acid brought external pH to 3.1; when valinomycin was present (●), no tetraphenylborate was added. During the preincubation at pH 8, a potassium-tetraphenylborate precipitation occurred when the anion was at 30 μM; the free anion level in this sample may have been considerably less than the stated value

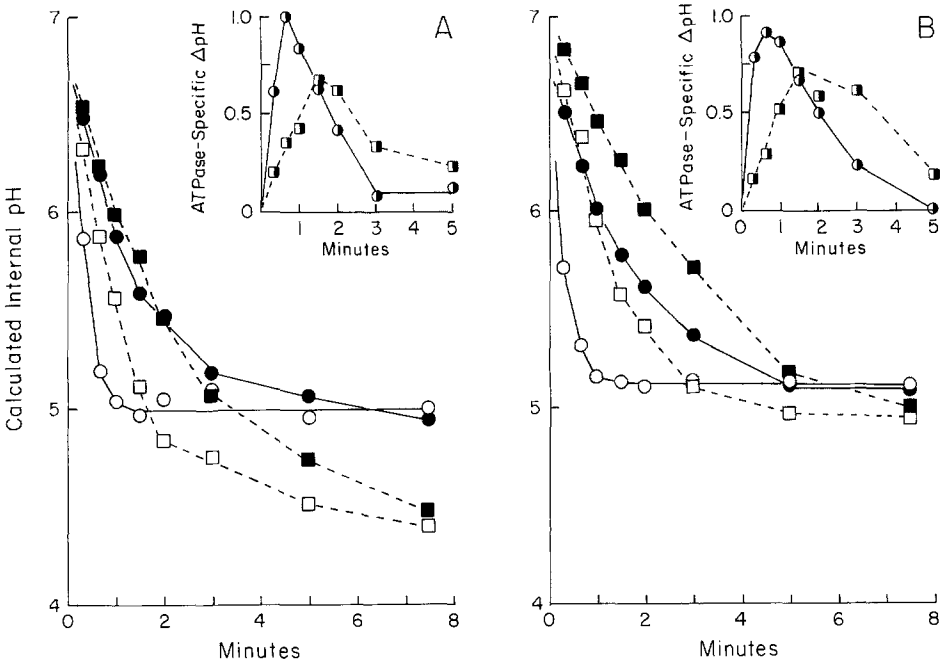

Fig. 3. Proton entry after addition of acid in the presence of valinomycin or a permeant anion. Results of two separate experiments are shown: (*A*) Experimental details are as described in the legend to Fig. 1, except that addition of either sulfuric (○, ●) or nitric (□, ■) acids lowered outside pH to 3.4–3.5. Other data (*not given*) showed ATP synthesis in the control cells (○, □) but not in the DCCD-treated samples (●, ■). Initial rates of proton entry for DCCD-treated cells were calculated as the slope of a line connecting the 20-, 40- and 60-sec time points: 0.83 and 0.78 pH units/min, respectively, for cells exposed to valinomycin or nitrate. For control cells, initial rates of proton entry were estimated using the zero time value for internal pH calculated from the paired sample treated with DCCD (6.75 for both DCCD-treated samples in this experiment) and the subsequent time point(s) where internal pH was 5.5 or higher: initial rates were 2.6 or 1.3 pH units/min, respectively, for control cells exposed to valinomycin or nitrate. *Inset:* "ATPase-specific ΔpH" is given as the difference between internal pH of control and DCCD-treated cells, exposed to either valinomycin (◑) or nitrate (◨). (*B*) Experimental details are as described in the legends to Figs. 1 and 2*C*. Tetraphenylborate was used at 3.5 μM final concentration, and sulfuric acid lowered outside pH to 3.0–3.2 in all samples. Initial rates of proton entry, calculated as described in part *A*, were 0.75 or 0.55 pH units/min for DCCD-treated cells exposed to valinomycin (●) or the anion (■), respectively. In control cells, proton entry was estimated as 3.0 or 0.95 pH units/min for samples exposed to the ionophore (○) or anion (□). *Inset:* As described in part *A*

into cells treated with valinomycin or exposed to a permeant anion, either 0.1 M nitrate or 3.5 μM tetraphenylborate. In the same work, the effect of DCCD was also examined, to provide the basis for determining whether ATP synthesis rescued by the anions was accompanied by an accelerated net proton entry, as noted earlier (e.g., Fig. 1). Data in Fig. 3*A* show results for cells treated with nitrate or valinomycin. In that experiment proton entry was relatively slow when only sulfuric acid was added, proceeding from an apparent zero time internal pH of 6.9 at a rate of 0.19 pH units/min (*data not shown* in the Figure). But when either nitrate or valinomycin were present, acidification of the cell was clearly accelerated, by four- to 10-fold, depending on whether prior treatment with DCCD had occurred (legend to Fig. 3*A*). Acceleration of proton entry was also found when tetraphenylborate replaced nitrate (Fig. 3*B*). Moreover, when either anion was used, ATP synthesis in the control cells was paralleled by an enhanced initial rate of acidification compared to the DCCD-treated cells in which reversal of BF_0F_1 had been prevented. Thus, valinomycin and permeant anions have qualitatively similar effects on proton entry: both accelerate acidification in the absence of ATP synthesis (DCCD-treated cells), and each may be used to reveal net proton movements that are presumed to reflect an influx by way of the ATPase during its reversal (Fig. 3, insets).

Taken together, work summarized by Table 1 and Figs. 2 and 3 strongly supports the idea that an inside positive membrane potential is established as H^+ moves towards equilibrium after addition of sulfuric acid to the outside phase. The net effect of this "back potential" is to lower the inward driving force on H^+, with understable consequences for both ATP synthesis and proton movements. Therefore, as argued before (Fig. 1 and accompanying text) the effect of valinomycin on each of these is most simply interpreted by assuming that the ionophore allows an experimental control over the size and polarity of the membrane potential. It has been important

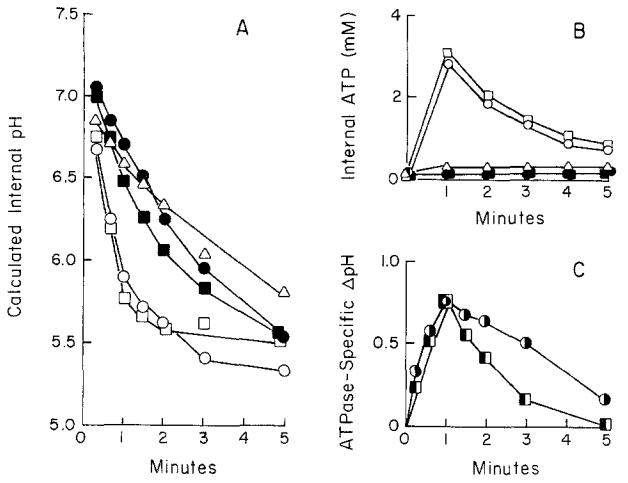

Fig. 4. Proton entry coupled to ATP synthesis. Unless otherwise specified, experimental details for measurement of internal pH (panel *A*) and intracellular ATP (panel *B*) are as given in the legends to Figs. 1 and 3. Solid symbols indicate a pretreatment with 1 mM DCCD, as described in Fig. 1. In three cases, cells were suspended in 0.1 M potassium phosphate, pH 8, with valinomycin before addition of sulfuric acid brought outside pH to 4.9 (△), 4.0 (□) or 3.9 (■). In two instances (●, ○), stock cells in 0.1 M potassium phosphate, pH 8, were diluted into 0.09 M choline *plus* 0.01 M potassium phosphate (pH 8) before addition of the acid lowered pH to 5.0; valinomycin was added 15 sec before the acid. Panel *C* shows "ATPase-specific ΔpH" (legend to Fig. 3) for the paired samples in which increased driving force above threshold for ATP formation was due to an increased pH gradient (◨) or membrane potential (◑). Rates of proton entry during the first 60 sec, calculated as described for data in Fig. 3, were (in pH units/min): 0.43 (△), 0.46 (●), 0.75 (■), 1.5 (○) and 1.7 (□)

to support this conclusion using a variety of approaches, since the experiments summarized below have taken advantage of this ionophore to analyze the coupling between proton entry and ATP synthesis.

Proton Entry Coupled to Synthesis of ATP

In work described by the preceding section, an examination of control and DCCD-treated cells revealed an elevated rate of proton entry that correlated with a reversal of the proton-translocating ATPase. The experiment illustrated in Fig. 4 was performed to provide further tests of the idea that this enhanced acidification was mediated by BF_0F_1 itself. The intent was to examine ATP formation and proton entry under two conditions: (*a*) when the imposed electrochemical proton gradient was raised from below to above threshold for ATP formation; and (*b*) when a variable proportion of membrane potential and pH gradient contributed to a total driving force exceeding threshold. The sample used as the basis for these comparisons is shown by the triangles (Fig. 4). In that case, as in the remaining trials, a pH gradient was generated by adding sulfuric acid to valinomycin-treated cells. For these cells, imposed driving force (about 180 mV) did not elicit ATP synthesis, and the initial rate of proton entry was relatively slow (about 0.4 pH units/min). These responses were in contrast to those of the sample (open squares) in which driving force (about 230 mV) was raised above threshold by an increased pH gradient; this supported both ATP synthesis and a fourfold acceleration of proton entry. The increment in proton entry was clearly greater than predicted by the fractional change of driving force, and was greater than that observed in the paired sample (closed squares) where driving force was similarly elevated for cells having nonfunctional BF_0F_1. This experiment also included samples where elevations of driving force above threshold were achieved by altering the membrane potential, rather than the pH gradient. For those samples (open and closed circles), outside pH was maintained near pH 5 while cells were diluted into media with choline in partial replacement of potassium, increasing the potassium diffusion potential by about 60 mV. Thus, it was possible to compare cases in which net driving force was the same (230–240 mV), but represented by different proportions of the electrical and chemical gradients. In both instances, proton entry that paralleled ATP synthesis was relatively rapid (1.5–1.7 pH units/min), and was increased over that found in paired samples treated with DCCD (0.5–0.75 pH units/min). The results of this experiment, along with data presented in the preceding section (Fig. 3), support the conclusion that under such conditions it is possible to monitor a H^+ influx that arises from the coupling of proton movements to the synthesis of ATP. A qualitative index of this coupling is given by Fig. 4*C* as the difference between internal pH of control and DCCD-treated cells ("ATPase-specific ΔpH").

To provide a quantitative basis for the comparison of control and DCCD-treated cells, initial rates of proton entry were measured in a number of experiments of the kind illustrated in Figs. 1 and 4. These data are summarized by Fig. 5, which shows initial rates of proton entry as a function of the imposed electochemical proton gradient. In such work, the imposed gradient was determined largely by the size of the pH gradient, although in three separate experiments (identified by the squares) driving force included a 60-mV increment in the membrane potential due to an increase in the potassium diffusion potential (as in Fig. 4). These results clearly distinguish the responses of control and DCCD-treated cells. Thus, for cells without functional ATPase, proton entry was directly related to driving force in the region 150–

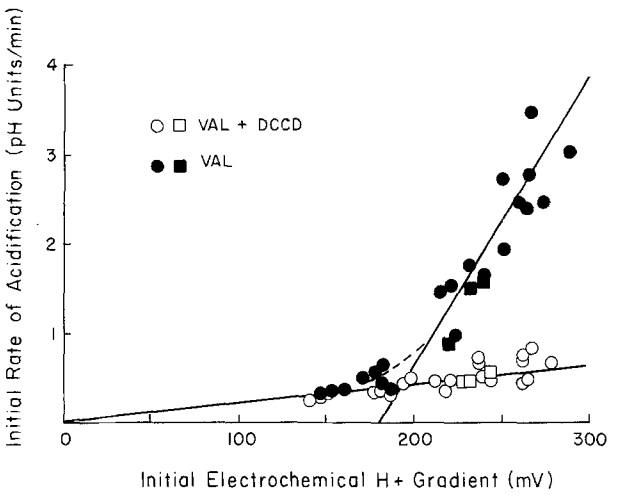

Fig. 5. Relationship between initial rates of proton entry and the initial value of the electrochemical gradient for H^+. The graph shows results obtained in nine separate experiments, and includes data from Figs. 1, 3 and 4, as well as other results from those experiments not given in the earlier Figures. Calculations of the initial value of the imposed electrochemical gradient were performed as described in Materials and Methods; initial rates of proton entry were determined from the observed changes of internal pH during the first 60 sec, as outlined in the legend to Fig. 3. In 20 of the paired comparisons of control (●) and DCCD-treated (○) cells, the imposed electrochemical proton gradient was varied by addition of varying amounts of sulfuric acid. Three separate experiments also contained one pair in which control (■) and DCCD-treated (□) cells were examined when driving force included a 60-mV increment in membrane potential, as described in the legend to Fig. 4. For all paired comparisons, the mean difference of final external pH was 0.04 pH units (range: 0–0.3 pH units). The regression line drawn for control cells considers only cases where driving force was 200 mV or greater, and shows a slope of 0.0276 pH units/min × mV with an abscissa intercept of 174 mV; the standard deviation of the vertical scatter corresponds to ±10 mV on the horizontal scale. For DCCD-treated cells, the slope of the line drawn is 0.0021 pH units/min × mV. This line assumes an ordinate intercept of 0 mV, and at 200 mV passes through a point representing the mean (least-squares) value for data where driving force was less than 260 mV (*see* Discussion). The lines given in the Figure reflect measured *rates* of acidification. This method of relating proton entry to driving force was preferred to the alternative in which a single (20-sec) time point was used to calculate net proton entry. Nevertheless, the two methods were in good agreement. For example, in the latter method proton entry was derived using known internal buffering power (Maloney, 1979) and the difference between initial internal pH (pH 7.6) and internal pH 20 sec after addition of acid: for control cells (> 200 mV driving force) the least-squares regression had a slope of 3.6 μmol H^+/min × mV × g dry weight, and an ordinate intercept of 183 mV; for DCCD-treated cells (< 260 mV), the least squares line had a slope of 0.45 μmol H^+/min × mV × g dry weight, and an ordinate intercept of 10 mV. The intersection of the two lines occurred at 208 mV

280 mV, and the analysis given later (*see* Discussion) indicates that this proton entry for DCCD-treated cells reflects a purely passive net movement of H^+, down the electrochemical gradient. The behavior of control cells, however, was more complex. With driving force centered about 180 mV it was not possible to differentiate proton entry into control cells from that found in DCCD-treated cells. But as driving force rose above 200 mV, acidification of control cells became more rapid than expected for passive flow. The arguments given earlier indicate that such elevated proton entry must include a component reflecting inflow of H^+ by way of the ATPase itself, during ATP synthesis. Accordingly, the intersection of the lines drawn (Fig. 5) corresponds to an observed threshold of 188 ± 10 mV for proton movements associated with the reversal of the ATPase reaction. As noted in Materials and Methods, calculations of imposed driving forces (Fig. 5) assumed an equilibrium distribution for H^+ before additions of sulfuric acid. But at pH 8 in 0.1 M potassium phosphate there was a small apparent gradient of 6 mV (directed outward). If this is taken at face value, the observed threshold for ATPase-mediated proton entry would correspond to 182 mV.

Stoichiometry of Coupling Between H^+ Entry and ATP Synthesis

Net proton entry coupled to ATP formation was observed only when the imposed electrochemical proton gradient exceeded a threshold value of 180–190 mV. Similar threshold values (175–215 mV) have been reported for the measurements of ATP synthesis in washed cells of *S. lactis* (Maloney & Wilson, 1975; Maloney, 1977) or a related organism (Drift, Janssen & Wezenbeek, 1978), The simplest interpretation of these observations is that these required thresholds identify the reversal potential for the proton-translocating ATPase under these conditions. With this assumption, one may derive the stoichiometry of coupling between proton entry and ATP formation, provided that measurements of the phosphate potential are also available.

The data in Table 2 show levels of ATP, ADP and inorganic phosphate (Pi) measured for washed cells of *S. lactis* suspended in 0.1 M potassium phosphate at pH 8. Assuming a value of 7.6 kcal/mole (Guynn & Veech, 1973) for the standard free energy of ATP hydrolysis (ΔG^0_{ATP}), these data have been used to calculate the phosphate potential, $\Delta G'_{ATP}$.

As shown by Table 2, the phosphate potential of these resting cells is 8.4 kcal/mole, or about 370 mV when expressed in the equivalent electrical units. For two reasons the phosphate potential of these cells is significantly less than that observed during active glycolysis (11–11.5 kcal/mole, or 480–500 mV; Maloney, *unpublished*). In the resting state the ratio ATP/ADP is about 0.2 (Table 2), whereas during glycolysis this ratio rises to between 5 and 10 (Mason, Carbone,

Table 2. The phosphate potential of resting cells[a]

[ATP]	[ADP]	[Pi]	ΔG^0_{ATP}	$RT\ln$ [ATP]/[ADP][Pi]	$\Delta G'_{ATP}$
(moles/liter cell water $\times 10^3$)			(kcal/mole)		
1.18 ± 0.02	0.78 ± 0.01	53 ± 3	7.60	0.84	8.44

[a] Values given at the left show the means and standards errors of 4 (ATP, ADP) and 8 (Pi) separate experiments. The nucleotide and phosphate assays are described in Materials and Methods. Calculation of $\Delta G'_{ATP}$ was performed as described in Materials and Methods.

Cushman & Waggoner, 1981; Maloney, *unpublished*). In addition, the inorganic phosphate pool is considerably higher in the resting condition (about 50 mM) than in the active state (about 5 mM) (Mason et al. 1981; Maloney, *unpublished*).

The value of the phosphate potential determines the minimum free energy which must be made available to form ATP under these conditions. Thus, if entry of a single proton were coupled to ATP synthesis, imposed driving force could be no less than 370 mV. However, for a stoichiometry of 2 H^+/ATP the reversal potential of the coupled reaction would fall to 185 mV. Similarly, for coupling ratios of 3 H^+/ATP or higher, one expects both proton entry and ATP synthesis when driving force is 125 mV or below. Since the ratio of the phosphate potential to the observed reversal potential is 2.0, the data given in Fig. 5 and Table 2 indicate a stoichiometry of 2 H^+/ATP for the proton-translocating ATPase of *S. lactis*.

Discussion

By exploiting several characteristic features of the anaerobe *S. lactis* it has been possible to document the coupling between proton entry and ATP synthesis catalyzed by the proton-translocating ATPase of bacteria. Of particular importance to these experiments has been the sensitivity of this cell to valinomycin, which provides an experimental control over the membrane potential, and the high internal potassium and high intracellular buffering capacity, which allow one to impose electrical and chemical gradients that subsist over a time appropriate for manual sampling. With this strategy in mind, earlier work had studied proton entry when a membrane potential (a potassium diffusion potential) dominated an externally applied driving force, and in that case it proved convenient to infer proton entry from changes of external pH (Maloney, 1977). The work described here presents the parallel study, in which a pH gradient was used to drive the coupled reaction, and where the measurement of internal pH served to verify proton entry.

The overall objective of such studies has been to classify the routes by which proton entry might occur when driving force was artificially imposed. For this reason, it was important to arrange circumstances so that net ion movements were restricted to either the exchange of H^+ with some internal cation (e.g., K^+) or the simultaneous inflow of H^+ with an external anion. Under such conditions, observed pH changes reflect the electrophoretic movement of H^+, rather than merely a transmembrane diffusion of acid or base. This requirement was satisfied earlier by showing a 1:1 exchange of H^+ and K^+ in the presence of valinomycin, but for technical reasons, that criterion was not set in the present study. Instead, it was thought sufficient to demonstrate that observed proton entry depended strongly on the presence of a permeant internal cation (K^+, when valinomycin was present) or any one of several permeant external anions (Figs. 2 & 3, Table 1). However, it is clear that some net proton entry occurs in the absence of an added permeant species (Fig. 1, triangles), and it is not known whether this represents an electrophoretic H^+ inflow, or the diffusion of a neutral species. For example, entry of H_3PO_4 might have taken place when high driving forces were imposed (260 mV or more; external pH of 3.5 or less), as outside pH approached the pK or the acid. Especially at high imposed driving force when low absolute rates were found (DCCD-treated cells), measured acidification may overestimate electrophoretic inflow of H^+. This bias is not expected to be significant in other instances.

The results of such studies suggest that protons pass across the membrane by only two routes of quantitative significance under these conditions. In the present work, a numerical argument is used to identify one pathway as a passive or "leak" component. The data given in Fig. 5 show that for DCCD-treated cells proton entry had a mean value of 0.43 pH units/min when driving force was 200 mV. This corresponds to about 30 μmol H^+/min × g dry weight, since the bulk of those measurements were made as internal pH changed between pH 6 and 7, where buffering power is about 70 μmol H^+/pH × g dry weight (Maloney, 1979). This may be compared to the rate predicted by the basal membrane conductance to H^+, which has been measured in "acid pulse" experiments (Mitchell & Moyle, 1967; Scholes & Mitchell, 1970) that used low driving force (0.05–0.10 pH units 3–6 mV) to assess the passive properties of this bacterial membrane (Maloney, 1979). These latter assays lead one to expect a passive proton entry of about 40 μmol H^+/min × g dry weight if driving force were raised to 200 mV. It is concluded, therefore, that proton

entry into DCCD-treated cells results from a passive flow, down the electrochemical gradient. It is also of interest to note that a similar absolute rate (about 24 μmol H^+/min × g dry weight) was observed for DCCD-treated cells when the inward driving force (200 mV, at internal and external pH of about 6) was dominated by the membrane potential (Maloney, 1977). Clearly, these comparisons indicate that net passive flow of H^+ is linearly related to driving force over a wide range of both the electrical and chemical gradient, but it is relatively insensitive to the absolute concentration of H^+ itself (*see also* Maloney, 1979). One must note, however, that these rate estimates are based on measurements of pH and so must reflect the balance between H^+ and OH^- fluxes as these ions move down their respective gradients. The work of Nichols and Deamer (1980) and Nichols, Hill, Bangham and Deamer (1980) reports similar observations using an artificial system (liposomes) rather than a biological membrane, and their analysis accounts for the unusual behavior of net H^+ fluxes by suggesting that H^+ and OH^- do not move across a membrane as discrete ionic species. Instead, these authors suggest that H^+ and OH^- move by way of a common intermediate (H_2O), during a reassortment of hydrogen bonds within the temporary water-filled pores that appear in the lipid phase (*see* Finkelstein, 1976).

Other observations suggest that a second major route for proton entry is the proton-translocating ATPase itself, but only during the synthesis of ATP. Evidence strongly supporting this conclusion comes from the comparison of control and DCCD-treated cells (Fig. 5), which shows that this additional pathway is available only to cells containing functional ATPase. But work that avoids use of the inhibitor is equally convincing. Thus, proton entry in excess of that expected for passive flow shows a threshold value for driving force (180–190 mV) that is the same as that observed for ATP synthesis (175–215 mV) (Maloney & Wilson, 1975; Maloney, 1977; Drift et al. 1978), and this threshold may be exceeded by either an increased electrical or chemical gradient (Fig. 4). Moreover, with reasonable accuracy initial rates of proton entry (in excess of the passive component) account for the initial rates of ATP synthesis observed in an independent study using identical conditions (Maloney & Schattschneider, 1980). In those experiments rates of 1.5–3 μmol ATP/min × g dry weight were obtained at a driving force near threshold (200 mV). This would require proton entry of at least 6–12 μmol H^+/min × g dry weight, assuming an excess of intracellular adenylate kinase (Maloney, 1977) and a stoichiometry of 2 H^+/ATP for the vectorial reaction. For this same driving force the data in Fig. 5 indicate about 20 μmol H^+/min × g dry weight for proton entry by way of the ATPase. A similar agreement is found for driving force well above threshold: at 280 mV, rates of ATP synthesis would require about 180 μmol H^+/min × g dry weight, while observed proton entry was 170 μmol H^+/min × g dry weight (assuming the inner buffering power used in the earlier calculation). These estimates use initial rates to illustrate the adequacy of the measured pump current to account for ATP synthesis under these conditions, but it is not appropriate to derive a stoichiometry for the vectorial reaction from such comparisons, for the contributions of cytoplasmic ATPase activity (including adenylate kinase) has not been examined directly. For example, since the sum of ATP, ADP and AMP is only about 4 μmol nucleotide/g dry weight (Maloney, 1977), it is apparent that the absolute deflections of internal pH, expecially at high driving force, require turnover of the adenine nucleotide pool (*see* Maloney, 1977, 1978). Nevertheless, the reasonable match between initial rates of proton entry and ATP synthesis suggests that the coupling between the two events is an obligatory one: significant proton entry by way of the ATPase does not occur unless ATP formation takes place at the same time. A thermodynamic argument leads to the same conclusion, since the behavior of DCCD-treated cells (Fig. 5) demonstrates that imposition of an electrochemical proton gradient above threshold does not disrupt the integrity of the cell membrane. Thus, proton entry in excess of passive flow requires that H^+ movements be coupled to some energy-consuming reaction (ATP synthesis) when the membrane is crossed.

As noted earlier, conditions for these assays ensured that measured proton entry represents the electrophoretic movement of H^+. Since neither ATP synthesis nor its associated proton entry were found in the absence of permeant ions (Fig. 1), these data demonstrate directly that the ionic species involved in the coupling to ATP synthesis is H^+ (or its equivalents in aqueous phases, OH^- or O^{2-}). Furthermore, these data show that the coupled reaction, measured as proton entry, has the same rate for driving forces of equal thermodynamic weight, without regard to the precise value of the membrane potential or pH gradient (Fig. 4; *see also* Fig. 5, solid circles and squares). The finding is consistent with the results obtained when ATP synthesis is studied (Maloney & Schattschneider, 1980), but an examination of proton movements had not yet been reported. Taken together, such observations exclude the possibility that the membrane potential and pH gradient have different mechanistic targets during the coupled reaction. In turn, these results are in accord with either of two proposals (Mitchell, 1969; 1974; Morowitz, 1978), each of which predicts equal rate for equal

driving force, although for different reasons. Other models for coupling are not yet formulated in the detail required to assess the relative effects of electrical and chemical gradients (Boyer, 1975; Kozlov & Skulachev, 1977).

Data collected here show that the threshold for ion flow mediated by the proton-translocating ATPase is the same as that found for synthesis of ATP (*see above*; and Maloney, 1982). A similar threshold (175 mV) for proton entry was observed in the earlier study (Maloney, 1977) where an imposed membrane potential was used to drive the reaction. Because the same threshold describes appearance of both products of the reaction (ATP and internal H^+), it seems reasonable to identify it with the reversal potential of the coupled process. With this assumption, a stoichiometry of 2 H^+/ATP was calculated after comparing the reversal (threshold) potential with the phosphate potential (Table 2 and text). Currently, there is some disagreement regarding the stoichiometry of this reaction in mitochondrial systems, where values of either 2 H^+/ATP or 3 H^+/ATP have been assigned (*see* Mitchell, 1979; Lehninger, Reynafarje, Alexandre & Villalobo, 1980). Because of this uncertainty, it is worth noting three general criticisms that might be made of the interpretation given to the results summarized here. First, the calculations of thermodynamic quantities have neglected the effect of high internal ionic strength on the activities of intracellular ions. This appears to have been a justifiable simplification. The initial near equilibrium distribution of H^+ was derived from measurements of membrane potential (potassium ratios, in the presence of valinomycin) and pH gradient (methylamine ratios) using probes of similar ionic radii (Kielland, 1937), so that errors associated with use of chemical, rather than activity, ratios do not affect the final calculation. As a result, estimates of the imposed electrochemical proton gradient depended only on changes of external pH measured with a glass electrode. In addition, while intracellular activities of ATP, ADP or phosphate may be different from their concentrations, the activity (molar) ratio [ATP]/[ADP][Pi] is not much altered by ionic strength. A second general criticism focuses on the role of intracellular enzymes that hydrolyze ATP. Thus, if the true stoichiometry of coupling were 3 H^+/ATP, then an imposed driving force of 200 mV should move the resting phosphate potential towards a new equilibrium value of 600 mV, with an increase in the ratio [ATP]/[ADP][Pi] of nearly 10^4. Instead, under these conditions increases of ATP (Maloney & Wilson, 1975; Maloney, 1977) suggest that this ratio rises by only about six- to 10-fold, as expected if stoichiometry were 2 H^+/ATP (the presence of adenylate kinase does not affect this argument, since the equilibrium constant for that reaction is close to 1). But measurement of ATP gives only the balance between synthetic and hydrolytic events, so that a rapid and irreversible hydrolysis of newly made ATP by other ATPases might have suppressed the expected rise in the phosphate potential, leading to an overestimate of the reversal potential for ATP formation. However, if this were true, such intracellular hydrolysis of ATP would be evident as an equally rapid net proton entry. This alternative is now ruled out by the work reported here, for no significant proton entry over the passive leak is found at driving forces below the reversal (threshold) potential for ATP synthesis. A final criticism is warranted because of the design of these experiments. It is known that proton-translocating ATPases of the F_0F_1 category can be subject to a kinetic control. For example, regulation of the mitochondrial enzyme is realized by a separate protein (Gomez-Fernandez & Harris, 1978; Pedersen, Schwerzmann & Cintron, 1981). Although the analogous "inhibitor protein" has not been found in bacterial systems (Kagawa, 1978), modulation of ATP hydrolysis by the isolated BF_1 sector of *E. coli* is associated with reversible binding of the ε subunit (Smith & Sternweis, 1977). Despite the more recent findings showing that the ε does not regulate the reconstituted BF_0F_1 complex (Sternweis & Smith, 1980), the observation using isolated BF_1 illustrates a general case. One might suppose that any observed threshold for proton entry and ATP synthesis includes two separate elements – a thermodynamic barrier represented by the phosphate potential, as well as a kinetic barrier associated with the functional dissociation of some (poorly described) regulatory process. In this context, two comments are appropriate. (*i*) The threshold observed here correlates with total driving force, while the analysis in chloroplasts (Harris & Crofts, 1978) suggests that relief from kinetic control is determined only by the membrane potential. (*ii*) On the time scale of these experiments, no unusual kinetic behavior was apparent when initial rates of ATP synthesis were examined (Maloney & Schattschneider, 1980). Thus, it is concluded that the stoichiometry of coupling between proton movements and ATP synthesis is 2 H^+/ATP in *S. lactis*. This conclusion supports the earlier suggestion (Maloney & Wilson, 1975) that was based only on measurements of ATP synthesis, and agrees with other work using that same approach (Drift et al. 1978). An extension of this line of reasoning leads to the same conclusion for the ATPase of *E. coli* – in the experiments of Wilson, Alderete, Maloney and Wilson (1976) the ratio of the phosphate potential to the reversal (threshold) potential for ATP formation was likely to have been about 2. Since entirely different techniques give 2 H^+/ATP for the

ATPase of bacterial chromatophores (Petty & Jackson, 1979), it is probable that all bacterial examples of this proton-translocating ATPase employ a stoichiometry of 2 H^+/ATP.

Of several models that describe the coupling between ATP synthesis and proton movements (Mitchell, 1969; Boyer, 1975; Kozlov & Skulachev, 1977; Morowitz, 1978), the proposal of Mitchell is the most strongly supported by data shown here. From that model one may derive two predictions: (*i*) that stoichiometry of the reaction reflects entry of 2 H^+ per ATP made; and (*ii*) that rate-limiting steps in ATP synthesis and net proton entry are located *after* the transmembrane electrical field has been trasversed by incoming H^+, so that an increased membrane potential should have the same effect on the rate of the reaction as an increased pH gradient of equivalent thermodynamic weight (internal pH remaining constant). The work reported here and elsewhere (Maloney & Schattschneider, 1980) verifies each of these predictions for the proton-translocating ATPase of bacteria.

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