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ATP Synthesis Driven by a Protonmotive Force in *Streptococcus lactis*

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Summary. An electrochemical potential difference for hydrogen ions (a protonmotive force) was artificially imposed across the membrane of the anaerobic bacterium *Streptococcus lactis*. When cells were exposed to the ionophore, valinomycin, the electrical gradient was established by a potassium diffusion potential. A chemical gradient of protons was established by manipulating the transmembrane pH gradient. When the protonmotive force attained a value of 215 mV or greater, net ATP synthesis was catalyzed by the membrane-bound Ca^{++}, Mg^{++}-stimulated ATPase. This was true whether the protonmotive force was dominated by the membrane potential (negative inside) or the pH gradient (alkaline inside). Under these conditions, ATP synthesis could be blocked by the ATPase inhibitor, dicyclohexylcarbodiimide, or by ionophores which rendered the membrane specifically permeable to protons. These observations provide strong evidence in support of the chemiosmotic hypothesis, which states that the membrane-bound ATPase couples the inward movement of protons to the synthesis of ATP.

In recent years, studies of the membrane-bound Ca^{++}, Mg^{++}-stimulated ATPase (EC 3.6.1.3) in microorganisms have been stimulated by the realization that this enzyme plays a central role in a number of the energy transductions catalyzed by bacterial membranes (for a review *see* West, 1974). Important information has come from the examination of mutants of *Escherichia coli* which lack this ATPase (for a review, *see* Cox & Gibson, 1974). Although the oxidation of substrates proceeds normally in ATPase-negative strains (Butlin, Cox & Gibson, 1971; Schairer & Haddock, 1972; Simoni & Shallenberger, 1972; Nieuwenhuis, Kanner, Gutnick, Postma & Van Dam, 1973; Rosen, 1973; Cox, Gibson & McCann, 1974) respiration does not support the phosphorylation of ADP (Butlin *et al.*, 1971; Gutnick, Kanner & Postma, 1972; Cox *et al.*, 1974; Kobayashi, Kin & Anraku, 1974; Mevel-Ninio & Yamamoto, 1974). Thus, this ATPase is required for the synthesis of ATP during oxidative phosphorylation. It is also known that under anaerobic conditions this ATPase participates in reactions which allow the cell to couple

the hydrolysis of ATP to essential membrane events which require the expenditure of metabolic energy. In the absence of respiration, ATPase-negative mutants are unable to use ATP from substrate level phosphorylations to drive flagellar movements (Larsen, Adler, Gargus & Hogg, 1974; Thipayathasana & Valentine, 1974), the ATP-linked transhydrogenase (Cox, Newton, Butlin & Gibson, 1971; Kanner & Gutnick, 1972; Bragg & Hou, 1973; Cox *et al.*, 1974; Kobayashi *et al.*, 1974), or the active transport of certain amino acids and sugars (Schairer & Haddock, 1972; Berger, 1973; Or, Kanner & Gutnick, 1973; Van Thienen & Postma, 1973; Yamamoto, Mevel-Ninio & Valentine, 1973; Berger & Heppel, 1974; Kobayashi *et al.*, 1974).

The results of inhibitor studies confirm the view that this ATPase plays an important role in both aerobic and anaerobic energy transformations. N,N′-Dicyclohexylcarbodiimide (DCCD), an inhibitor of the ATPase (Harold, Baarda, Baron & Abrams, 1969; Evans, 1970), blocks oxidative phosphorylation in *E. coli,* and prevents both oxidative and photophosphorylation in *Halobacterium halobium* (Danon & Stoeckenius, 1974). In *E. coli* the transhydrogenase reaction is inhibited by DCCD when ATP is used as the energy source, but not when energy is provided by respiration (Gutnick *et al.*, 1972; Kanner & Gutnick, 1972; Bragg & Hou, 1973). Similarly, DCCD blocks the active transport of proline by *E. coli,* but only under anaerobic conditions (Klein & Boyer, 1972). Moreover, in the *Streptococci,* which lack oxidative metabolism, DCCD inhibits the active transport of a number of metabolites (Harold *et al.*, 1969; Abrams, Smith & Baron, 1972; Kashket & Wilson, 1972*a*; Asghar, Levin & Harold, 1973).

Thus, it seems clear that in microorganisms the membrane-bound ATPase can function in at least two ways which are of importance to the cell. On the one hand, under aerobic conditions the ATPase catalyzes the synthesis of ATP during oxidative phosphorylation. On the other hand, under anaerobic conditions, or in bacteria lacking a respiratory chain, the ATPase can link the hydrolysis of ATP to several energy-dependent membrane reactions. These different aspects of ATPase function are readily understood within the framework of the chemiosmotic hypothesis of Mitchell (Mitchell, 1961, 1966; for reviews, *see* Greville, 1969; and Harold, 1972). This model views the ATPase as a reversible ion translocating pump, one which catalyzes the movement of hydrogen ions (H^+) across the cell membrane as a consequence of the hydrolysis or synthesis of ATP. The operation of this hydrogen ion pump under anaerobic conditions is illustrated in Fig. 1*A*. In this case, the ATPase

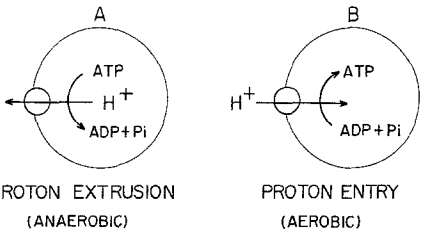

Fig. 1. The proton translocating ATPase of bacteria. (*A*) Hydrolysis of ATP is coupled to the extrusion of protons in anaerobes (e.g. *S. lactis*), or in facultative aerobes (e.g. *E. coli*) under anaerobic conditions. (*B*) Under aerobic conditions, the ATPase couples the inward movement of protons to the synthesis of ATP during oxidative phosphorylation

couples the hydrolysis of ATP to the extrusion of protons from the cell. Since hydrogen ions pass from the inside to the outside of the cell, the activity of the ATPase generates a transmembrane pH gradient, alkaline inside. In addition, because positively charged particles move from the inside to the outside of the cell, the activity of such an ATPase establishes a membrane potential, negative inside. Thus, metabolic energy which is dissipated by the hydrolysis of ATP is conserved as a "protonmotive force," a difference in the electrochemical potential for hydrogen ions across the cell membrane. This protonmotive force would then be used to drive a variety of energy-dependent reactions. Under aerobic conditions (Fig. 1 *B*) the protonmotive force arises from the activity of the respiratory chain, which couples the oxidation of its substrates to the extrusion of hydrogen ions. In this case, synthesis of ATP during oxidative phosphorylation would occur when protons, moving down their electrochemical gradient, re-enter the cell via the ATPase.

There are several lines of evidence which argue in favor of a chemiosmotic view of energy transductions in microorganisms. It is now well documented that oxidative reactions in bacteria are associated with the extrusion of protons from the intact cell (Scholes & Mitchell, 1970; West & Mitchell, 1972; Lawford & Haddock, 1973; Meyer & Jones, 1973; Drozd, 1974). The same observation has been made using membrane vesicles whose orientation is the same as that of the intact cell (Reeves, 1971; Altendorf, Harold & Simoni, 1974), whereas if vesicles are inverted with respect to the intact cell, oxidation removes protons from the medium (Hertzberg & Hinkle, 1974). One would expect such proton translocations to generate a transmembrane pH gradient and a membrane potential. Although measurements have not yet been made of the pH gradient which may be associated with respiration, it is known that an electrical potential (negative inside) is developed by metabolizing

cells (Griniuviene, Chmieliauskaite & Grinius, 1974) or as a result of the oxidation of substrates by membrane vesicles (Hirata, Altendorf & Harold, 1973; Altendorf *et al.*, 1974; Altendorf, Hirata & Harold, 1975).

There is also strong evidence that supports the idea that the bacterial ATPase can operate to extrude protons from the cell (Fig. 1*A*). Harold and his collaborators have shown that during glycolysis, cells of the anaerobe *S. faecalis* establish both a pH gradient (alkaline inside) and a membrane potential (negative inside); DCCD prevents the formation of each of these components of the protonmotive force (Harold, Pavlasova & Baarda, 1970; Harold & Papineau, 1972*a*, *b*; Laris & Pershadsingh, 1974). More recently, West and Mitchell (1974*a*) and Hertzberg and Hinkle (1974) have examined this ATPase in membrane vesicles from *E. coli*. These experiments have shown that hydrolysis of ATP is associated with the net movement of protons across the vesicle membrane. However, without the direct demonstration of ATP synthesis driven by a protonmotive force (Fig. 1*B*), the evidence supporting a chemiosmotic interpretation of energy transductions in bacteria remains incomplete.

Recently we reported experiments which showed that in both *S. lactis* and *E. coli* the membrane-bound ATPase catalyzes the net synthesis of ATP in response to an inwardly directed protonmotive force (Maloney, Kashket & Wilson, 1974). These initial observations are extended by the experiments reported here, which examine ATP synthesis in *S. lactis*. This organism is particularly suited to such investigations, because one can study the ATPase apart from possible interactions with a respiratory chain. These results show that ATP synthesis catalyzed by the ATPase can be driven by potential energy stored as either a membrane potential, a pH gradient, or the appropriate combination of these two components of the protonmotive force.

Materials and Methods

Preparation of Cell Suspensions

Streptococcus lactis (ATCC 7962) was used in the experiments reported here. Cells were grown using a complex medium (Citti, Sandine & Elliker, 1965) which contained: Bacto-Yeast Extract (Difco), 10 g; Bacto-Tryptone (Difco), 10 g; Bacto-Gelatin (Difco), 2.5 g; potassium chloride, 4 g; sodium acetate, 1.5 g; ascorbic acid, 0.5 g; and distilled water to a final volume of 1 liter. The pH was adjusted to pH 7 using sodium hydroxide. D-Galactose was added to a final concentration of 1%. For routine experiments, 200 ml of medium was inoculated with 10 ml of an overnight culture (stationary phase) and incu-

bated in a 250 ml flask at 37 °C without shaking. After 10–12 hr of growth (early stationary phase) the cells were harvested by centrifugation at 4 °C. Unless otherwise indicated, cells were then washed twice with 100 mM sodium phosphate, pH 6, and finally resuspended as a concentrated stock in about 5 ml of this buffer. Washed cells were maintained at 25 °C, and all subsequent operations were performed at this temperature.

Cell density was measured turbidimetrically using a Klett-Summerson colorimeter (No. 42 filter). For *S. lactis,* 1 ml of a cell suspension of 100 Klett units is equivalent to 165 μg dry weight or 0.24 μl of intracellular water (Kashket & Wilson, 1972*b*).

Assays

(a) *Measurement of ATP.* The procedure of Cole, Wimpenny and Hughes (1967) was followed, with minor modifications. Intracellular ATP was extracted by placing 0.4 ml of a cell suspension (100–200 Klett units) onto 0.1 ml of iced 3 N perchloric acid. After about 30 min, the extract was neutralized with 0.3 ml of 1 N potassium hydroxide and kept on ice for an additional 30 min. Firefly lantern extract (FLE-50, Sigma Chemical Co.) was prepared according to the manufacturer's directions, and then clarified by centrifugation at $12{,}000 \times g$ for 10 min at 4 °C. For the assay of ATP, 0.025 ml of cell extract was mixed with 0.9 ml of 45 mM glycylglycine buffer, pH 7.4, in a 1 dram glass vial (No. 7475, Rochester Scientific Co.). Firefly lantern extract (0.025 ml) was carefully pipetted into the center of the plastic cap, and at zero time the sample and firefly extract were mixed by inversion. The vial was then placed in the well of a Nuclear Chicago Mark I liquid scintillation counter which had been set for maximum sensitivity, with the coincidence circuit off. Fifteen seconds after mixing, the sample was counted for 6 sec. With no added ATP, background counts were about 2000; when 25 pmoles ATP were added, about 60,000 counts were obtained. Unknowns contained 0–25 pmoles ATP, and over this range there was a linear relationship between counts and ATP. The intracellular concentration of ATP in the original cell suspension was calculated from the amount of ATP found in cell extracts, using the known relationship between cell density and intracellular water. In different experiments, the composition of the original cell suspension varied with respect to pH, salt concentrations or the presence of various inhibitors; in no case was there any significant effect on the assay of ATP in cell extracts.

(b) *Measurement of ADP.* ADP was measured after its conversion to ATP in the presence of pyruvate kinase and excess phosphoenolypyruvate. Perchloric acid extracts of cell suspensions were prepared as described for the assay of ATP. After neutralization with potassium hydroxide, 0.2 ml of the extract was mixed with 0.2 ml of 100 mM glycylglycine buffer, pH 7.4, which contained 150 mM potassium chloride, 15 mM magnesium sulfate, 0.5 mM phosphoenolpyruvate and 2 μl/ml of pyruvate kinase (Sigma Chemical Co., type I). After incubation for 10 min at 25 °C the reaction was stopped by the addition of 0.1 ml of 3 N perchloric acid. Samples were then placed in an ice bath and 0.3 ml 1 N potassium hydroxide was added after 20 min. ATP was then assayed as described in the preceding section. The amount of ADP present in the original perchloric acid extract was calculated from the difference between the levels of ATP measured before and after the extract had been incubated with pyruvate kinase and phosphoenolpyruvate.

(c) *Measurement of Intracellular Potassium.* One ml of a cell suspension (about 400 Klett units) was passed through a dry Millipore filter (1.2 μ pore size), without washing. The filter was then placed in a glass tube along with 2 drops of *n*-butanol and incubated in a boiling water bath for 10 min. After cooling, an appropriate volume of lithium chloride was added, and potassium measured by flame photometry. Intracellular potassium was always determined under conditions where the level of extracellular potassium was low (less than 0.5 mM), so that extracellular fluid trapped on the filter made no significant

contribution (less than 5%) to the total potassium recovered. The internal concentration of potassium was calculated using the known relationship between cell density and intracellular water volume. Extracellular potassium was also determined by flame photometry. The cell suspension was centrifuged, a sample of the supernatant removed, and lithium chloride then added as an internal standard.

(d) *Measurement of Intracellular pH.* When the transmembrane pH gradient was alkaline inside, the internal pH was determined from the distribution of the weak acid, 5,5-dimethyloxazolidine-(2,4-dione) (DMO). The rationale behind this method (Kashket & Wong, 1969; Harold *et al.*, 1970) is that bacterial cell membranes are much more permeable to the unionized (acid) form of DMO than to the ionized (anionic) species. Thus, DMO will accumulate within the cell if the internal space is more alkaline than the suspension medium. Cells were placed in 100 mM sodium phosphate, pH 6, which contained 100 mM sodium chloride, (^{14}C)-DMO (0.02 mM, 0.20 μC/ml), and ^{3}H-D-sorbitol (0.06 mM, 0.15 μC/ml) as a marker for the extracellular space. Final cell density was about 400 Klett units. After measuring the external pH, 1 ml aliquots were filtered through dry Millipore filters (1.2 μ pore size) without washing. Radioactivity retained on the filters was determined with a liquid scintillation counter set for efficient discrimination between ^{3}H and ^{14}C. For each sample, the volume of extracellular fluid trapped on the filter could be determined from the ^{3}H counts, allowing a calculation of the internal concentration of DMO. The value for the pK of DMO at 25 °C was taken as 6.32 (Addanki, Cahill & Sotos, 1968). The intracellular pH was then calculated from the Hendersen-Hasselbalch equation, using measured values for the external pH, the external concentration of DMO and the internal concentration of DMO.

When the transmembrane pH gradient was acid inside, the internal pH was measured from the distribution of the weak base, methylamine (Rottenberg, Grunwald & Avron, 1972; Kashket & Wilson, 1973). This method also assumes that the cell membrane is more permeable to the neutral (unprotonated) species than to the ionized (cationic) form of the probe. The procedure employed was essentially the same as that described in the preceding paragraph, except that (^{14}C)-methylamine (0.02 mM, 0.17 μC/ml) replaced (^{14}C)-DMO, and cells were suspended in potassium phosphate buffer at pH 8 (*see text*). Since the pK of methylamine is high (about 10.6) compared to the pH values encountered, it was assumed that the ratio of methylamine in/out was equal to the ratio of hydrogen ions out/in.

Chemicals

Valinomycin and N,N′-dicylohexylcarbodiimide (DCCD) were purchased from Calbiochem. Pentachlorophenol was obtained from Eastman Kodak, and 2,4-dinitrophenol was from Mann Research Laboratories. Tetrachlorasalicylanilide was a gift of Dr. F.M. Harold (National Jewish Hospital and Research Center, Denver, Colorado) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (CCFP) was a gift of Dr. E.P. Kennedy (Harvard Medical School, Boston, Massachusetts). Valinomycin, DCCD, CCFP, tetrachlorosalicylanilide and pentachlorophenol were added to cell suspensions as small volumes of stock solutions in 95% ethanol. Final ethanol concentrations were never more than 0.2%.

5,5-Dimethyloxazolidine-(2,4-dione-2-^{14}C), (^{14}C)-methylamine and D-(1-^{3}H)-sorbitol were obtained from New England Nuclear Corp.

Calculation of the Protonmotive Force

The term "protonmotive force" is used to represent the difference in electrochemical potential for hydrogen ions across a membrane (Mitchell, 1966). When expressed in milli-

volts, the protonmotive force, Δp, is given by

$$\Delta p = \Delta\Psi - Z\Delta \text{pH} \tag{1}$$

where $\Delta\Psi$ is the membrane potential (in millivolts) and Δ pH is the transmembrane pH gradient (the internal pH minus the external pH). At 25 °C the coefficient Z (2.3 RT/F) has a value of 59. In some of the experiments reported here it was possible to evaluate the protonmotive force from measurements of the membrane potential and the pH gradient. The pH gradient was estimated from the distribution of a weak acid or base as described above. In the presence of valinomycin the membrane potential could be determined from the measured ratio of internal to external potassium. Assuming that valinomycin-treated cells are far more permeable to the potassium ion than to any other ion, the electrical gradient across the membrane arises from the outward diffusion of the potassium ion as it travels down its concentration gradient. Such movement of the potassium ion carries positive charge out of the cell and thus generates a membrane potential (negative inside) which opposes subsequent loss of potassium. At equilibrium the magnitude of this potassium diffusion potential is given by the Nernst equation:

$$\Delta\Psi = -\frac{RT}{F} \ln \frac{[\text{K}]_{\text{in}}}{[\text{K}]_{\text{out}}} \tag{2}$$

where R, T and F have their usual meanings, and where $[\text{K}]_{\text{in}}$ and $[\text{K}]_{\text{out}}$ refer to the internal and external concentrations of potassium, respectively. It is assumed that the activity of the potassium ion is given by its concentration.

Results

ATP Synthesis Driven by a Membrane Potential

To impose an electrical gradient across the cell membrane, intact cells were suspended in a potassium-free medium and then treated with the potassium ionophore, valinomycin. The action of valinomycin, on both artificial and natural membranes, is to greatly increase permeability to the potassium ion (Pressman, 1968). Thus, when *S. lactis* is exposed to this ionophore, efflux of the potassium ion removes positive charge from the cell and generates a membrane potential, negative inside.

The experiment described in Fig. 2 serves to introduce the basic observation. Cells were suspended in 100 mM sodium phosphate buffer of varying pH, and valinomycin was added after samples had been taken to determine basal levels of ATP (about 0.2 mM ATP). For cells suspended at pH 8, there was no change in the intracellular levels of ATP following valinomycin. At pH 7, ATP levels increased to a peak value which was only twice that found at zero time. However, when cells were suspended at pH 6 or below, there was a rapid rise in the internal concentration of ATP after cells were exposed to the ionophore. Under these conditions, peak levels of ATP were attained within 1 min, and

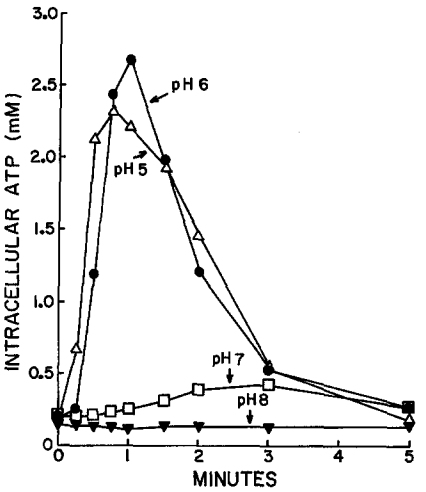

Fig. 2. Valinomycin-induced synthesis of ATP. Cells were washed and resuspended in 0.1 M sodium phosphate at pH 5, 6, 7 or 8. For treatment with valinomycin, 0.1 ml cells (10,000 Klett units) was mixed with 5.3 ml of 0.1 M sodium phosphate of the appropriate pH. After removing samples for measurement of basal levels of ATP, 5 μl of 10 mM valinomycin was added (10 μM final concentration) and additional samples taken at the indicated times. In parallel tubes, 0.1 ml of 1.1 M glucose was added (22 mM final concentration) instead of valinomycin. After 25 min with glucose, steady-state levels of ATP were 2.3–2.6 mM

represented at least a 10-fold increase over the basal value. This increase was transient, and after 5 min ATP levels had fallen to approximately their original value. For clarity, data for cells suspended at pH 4 have been omitted in Fig. 2; the behavior of these cells was similar to that found for cells suspended at pH 5. Additional data from this experiment showed that between pH 4 and pH 6, the peak levels of ATP seen after valinomycin were comparable to the steady-state levels of ATP maintained by glycolytic reactions (*see* legend to Fig. 2).

Because intracellular levels of ATP reflect the balance between synthetic and degradative reactions, increases in ATP after the addition of valinomycin could result from either an inhibition of ATP breakdown or a stimulation of ATP synthesis. To distinguish between these alternatives, the experiment described in Fig. 3 was performed. High intracellular levels of ATP were obtained by allowing cells to metabolize glucose. Glycolysis was then stopped by the addition of iodoacetate, and samples taken to determine the rate of breakdown of ATP. To eliminate hydrolysis of ATP catalyzed by the ATPase itself, cells were pretreated with the inhibitor, DCCD. As shown by Fig. 3*A*, in DCCD-treated cells the rate of loss of ATP was about 1 μmole ATP/min/ml of intracellular water;

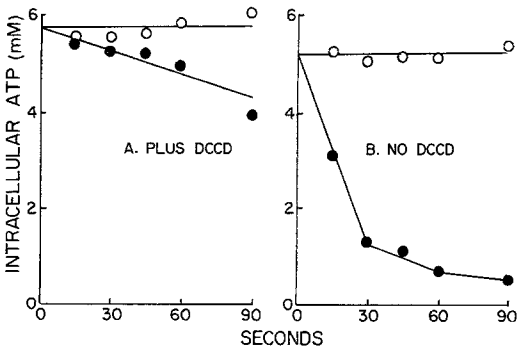

Fig. 3. ATP breakdown in the presence and absence of DCCD. Cells were washed and resuspended in 0.1 M sodium phosphate, pH 8. To 1 ml of cells (8700 Klett units) either 1 μl of 95% ethanol or 1 μl of 1 M DCCD in 95% ethanol was added (the final concentration of DCCD was 1 mM). After 30 min, cells were centrifuged and resuspended in 1 ml of 0.1 M sodium phosphate, pH 6. Fifteen minutes later a portion of each suspension was diluted 50-fold using this buffer containing 30 mM glucose. After an additional 15 min, one part of the diluted cell suspensions received 1 mM iodoacetate (●); the other part served as a control (○). The time of addition of iodoacetate was designated zero time. In parallel tubes, cells exposed to ethanol or DCCD were treated in the same way except that the final cell suspensions contained no glucose. To these cells valinomycin (10 μM final concentration) was added 5 min after iodoacetate. One minute later samples were removed for the measurement of ATP. After valinomycin, ATP in cells not treated with DCCD had risen to 4.5 mM, from a basal level of 0.2 mM; iodoacetate had no effect on ATP levels observed after valinomycin. In DCCD-treated cells there was no increase in ATP after valinomycin

for cells with a functional ATPase (Fig. 3*B*) ATP breakdown, as estimated from its initial rate, was about 8 times more rapid. In parallel samples, valinomycin was added to washed cells which had not been given either glucose or DCCD (*see* legend to Fig. 3). One minute after valinomycin, ATP had risen from a basal level of 0.2 mM to 4.5 mM, indicating a minimum rate of increase of 4.3 μmoles ATP/min/ml cell water. This cannot be accounted for by a generalized inhibition of ATP breakdown, since even if all degradative reactions (excluding hydrolysis by the ATPase itself) were blocked by valinomycin, the data of Fig. 3*A* predict a maximal rate of increase of only 1 μmole ATP/min/ml cell water. It is concluded, therefore, that the rise in ATP levels seen after the addition of valinomycin represents an increased synthesis of ATP rather than an inhibition of ATP breakdown. Since no net ATP formation was observed in DCCD-treated cells (Maloney *et al.*, 1974; *see also* legend to Fig. 3), it may also be concluded that this ATP synthesis is catalyzed by the membrane-bound ATPase.

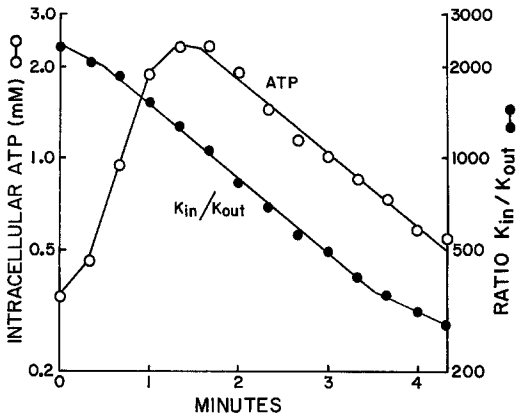

Fig. 4. Relationship between ATP levels and the ratio of internal to external potassium in valinomycin-treated cells. Three samples were used which contained washed cells (435 Klett units) in 0.1 M sodium phosphate, pH 6, with 0.1 mM potassium phosphate to ensure that there would be measurable extracellular potassium. After removing samples for zero time measurements, valinomycin (3.3 μM final concentration) was added to two samples. Later samples were withdrawn at the indicated times for measurement of internal ATP (first suspension) and internal potassium (second suspension). To measure the initial level of extracellular potassium, the third cell suspension was centrifuged and the potassium concentration in the supernatant was determined. In calculating the ratio of potassium in/out, a correction was made for the increase in external potassium due to loss of potassium from the cell. See the text for further details

The addition of valinomycin to washed cells was followed by a temporary increase in ATP levels (Fig. 2). This transient response would be expected if there were net loss of internal potassium since this would lower the ratio of internal to external potassium and thus reduce the membrane potential. As shown by the data in Fig. 4, one can demonstrate a correlation between the decay of the potassium ratio in/out and the loss of ATP in valinomycin-treated cells. In this experiment intracellular potassium was 460 mM at zero time while extracellular potassium was about 0.2 mM, giving a potassium ratio in/out of 2300. After the addition of valinomycin, the potassium ratio began to fall, reaching a value of about 300 within 4 min. It is apparent that after reaching their peak value, levels of ATP fell in parallel with the potassium ratio. Under these conditions ATP levels, as well as the potassium ratio in/out, showed exponential decay with a $t_{1/2}$ of about 1.2 min.

According to the chemiosmotic hypothesis, synthesis of ATP occurs in response to a difference in the electrochemical potential for hydrogen ions across the membrane. This difference in electrochemical potential would be reduced in the presence of agents which render the membrane

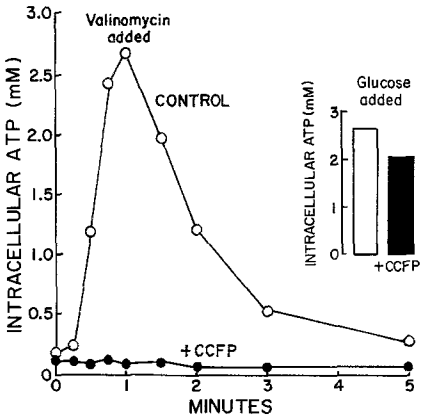

Fig. 5. Effect of a proton conductor on ATP synthesis in valinomycin-treated cells. Washed cells (150 Klett units) were suspended in 0.1 M sodium phosphate, pH 6. Valinomycin (10 μM final concentration) or glucose (25 mM final concentration) was added after cells had been exposed for 20 min to either CCFP (0.5 μM final concentration) or ethanol. ATP levels in glucose-treated cells were measured after 25 min

permeable to the hydrogen ion. Thus, such "proton conductors" should inhibit the synthesis of ATP which is observed after the addition of valinomycin. The results shown in Fig. 5 confirm this prediction. When cells were exposed to the proton conductor, CCFP, before the addition of valinomycin, there was complete inhibition of the ATP synthesis observed in the control, although CCFP did not interfere with the intracellular reactions which generate ATP from substrate level phosphorylations (inset to Fig. 5). Other proton conductors, such as tetrachlorosalicylanilide (1 μM), pentachlorophenol (1 μM) or 2,4-dinitrophenol (1 mM) were also effective inhibitors of the ATP synthesis found in valinomycin-treated cells (experimental conditions as in Fig. 5).

The relationship between ADP and ATP levels in valinomycin-treated cells was next investigated (Fig. 6). As ATP levels increased, ADP levels fell, so that the peak level of ATP coincided with the lowest level of ADP. The subsequent fall in ATP was then accompanied by a rise in ADP. However, increases and decreases in ATP levels were not reflected by stoichiometric changes in ADP, presumably because of the presence of AMP and adenylate kinase in these cells. The observed ratios of ATP/ADP are given by the inset to Fig. 6. Before the addition of valinomycin this ratio was about 0.15. One minute after valinomycin this ratio had reached a peak value of 3.4; within 5 min the ATP/ADP ratio had returned to near its basal value. Because ATP showed greater fluctuations than ADP, changes in the ratio of ATP/ADP were due primarily to changes in the levels of ATP.

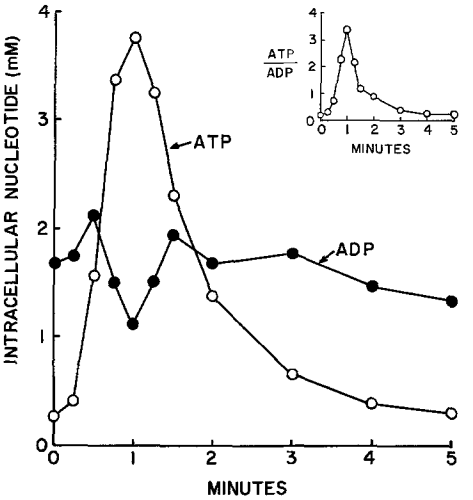

Fig. 6. Levels of ATP and ADP in valinomycin-treated cells. Washed cells (150 Klett units) were suspended in 0.1 M sodium phosphate, pH 6. After removing a sample for measurement of basal levels of nucleotides, valinomycin (6.7 μM final concentration) was added. The inset gives ratio of ATP to ADP

ATP Synthesis Driven by a Membrane Potential and a pH Gradient

The results presented in the preceding section dealt with cases in which ATP synthesis responded to variations in the electrical gradient across the membrane. The experiments given in this section were performed to determine whether ATP synthesis responded to manipulations of both the membrane potential and the pH gradient.

The first of these experiments is shown in Fig. 7. As in the preceding experiments, a membrane potential was generated by treating cells with valinomycin. In this case, however, when ATP levels had reached their peak, the pH of the external medium was suddenly changed by adding either acid or base. When acid is added, the protonmotive force established by the electrical gradient would be supplemented by the higher concentration of hydrogen ions in the external medium. Consequently, ATP synthesis should be potentiated. On the other hand, when base is added, the new pH gradient would act in opposition to the membrane potential, reducing the tendency for protons to flow into the cell, and shifting the ATPase reaction in the direction of hydrolysis rather than synthesis. Changes in ATP levels which accompany the acid transition are shown in Fig. 7*A*. In response to an acid transition (pH 6 to pH 4) ATP levels reached higher peak values, and the time course of ATP

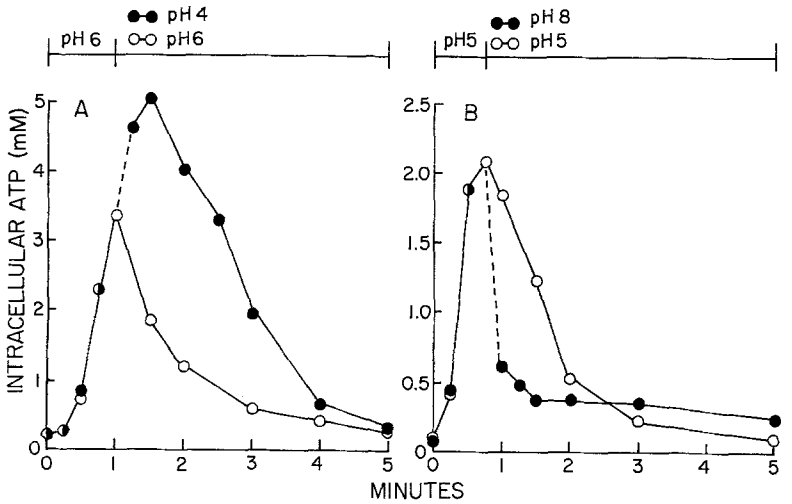

Fig. 7. Effect of acid or base transition during ATP synthesis in valinomycin-treated cells. In the first experiment (*A*) parallel tubes contained washed cells (158 Klett units) suspended in 0.1 M sodium phosphate, pH 6. After samples were removed for zero time measurements, valinomycin (6.7 μM final concentration) was added to each. One (○) received no further additions; the second (●) received hydrochloric acid 1 min after valinomycin to lower the external pH to 4. In the second experiment (*B*), parallel tubes contained cells (190 Klett units) washed and resuspended in 0.1 M sodium phosphate, pH 5. One suspension was given 10 μM valinomycin but no further additions (○); to the other (●), sodium hydroxide was added 45 sec after valinomycin to raise the external pH to 8

synthesis was extended. Fig. 7*B* shows the results of the reciprocal experiment in which base, rather than acid, was added. Immediately after the alkaline shift (pH 5 to pH 8) there was a rapid decrease in ATP levels. Thus, the results of these experiments provide qualitative evidence to support the idea that ATP synthesis catalyzed by the membrane-bound ATPase responds to the combined effects of both a membrane potential and a pH gradient, as predicted by the chemiosmotic hypothesis.

It was important to describe the quantitative relationship between the protonmotive force and ATP synthesis in these experiments. To do this, ATP synthesis was monitored under conditions where the initial value of the total protonmotive force could be evaluated from direct measurements of the membrane potential and the pH gradient. These results are given in Fig. 8. For each of the six samples shown, cells were originally suspended as a concentrated stock in 100 mM sodium phosphate, pH 6, containing 100 mM sodium chloride. Sodium chloride was present so that in the next steps potassium chloride could be used

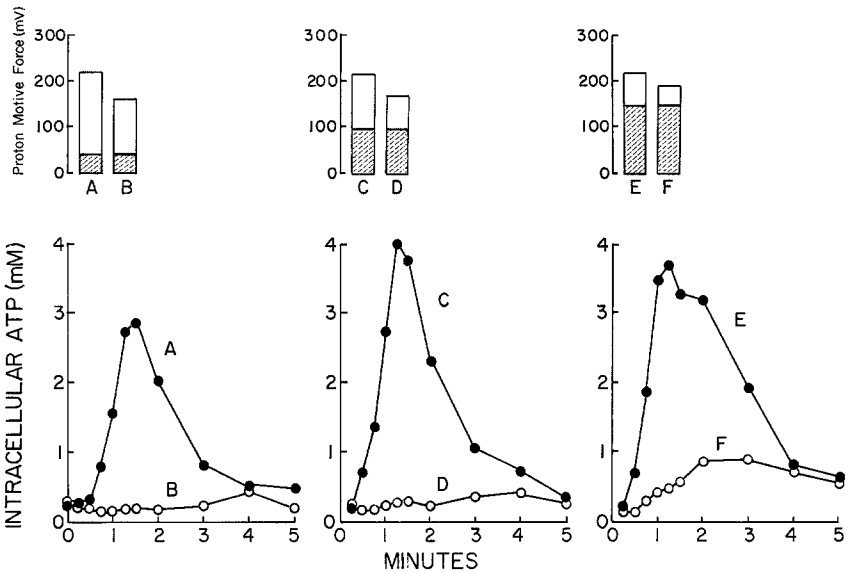

Fig. 8. Sequential inhibition and restoration of ATP synthesis in valinomycin-treated cells. Washed cells (8200 Klett units) were suspended in 0.1 M sodium phosphate, pH 6, which contained 0.1 M sodium chloride. For measurement of ATP synthesis, cells were diluted 50-fold with 0.1 M sodium phosphate, pH 6 (*A* and *B*), pH 5 (*C* and *D*) or pH 4 (*E* and *F*) containing varying levels of sodium and potassium chloride (total equal to 100 mM). Final concentrations of potassium were 0.5 mM (*A*), 5 mM (*B* and *C*), 30 mM (*D* and *E*) or 100 mM (*F*). For *A* and *B*, valinomycin (6.7 μM final concentration) was added after aliquots had been removed to determine basal levels of ATP. The remaining samples received valinomycin 10 sec after dilution. For each sample, extracellular pH was measured 6–7 min after valinomycin. Intracellular pH was measured in a parallel tube containing cells (410 Klett units) in 0.1 M sodium phosphate, pH 6, 0.1 M sodium chloride, ^{14}C-DMO and ^{3}H-D-sorbitol. After 5 min in this medium, samples were withdrawn for filtration (*see* Materials and Methods). An identical procedure was used for measurement of intracellular potassium (*see* Materials and Methods) except that no radioactive materials were present in the final suspension. For each of the six samples examined, the initial value of the total protonmotive force was calculated (bar graphs) as described in Materials and Methods. For clarity in presentation, the negative sign of the protonmotive force has been omitted in the bar graphs

to replace sodium chloride without a large change in the osmotic strength of the medium. For the first two samples (*A* and *B*, left panel) cells were diluted into buffer at pH 6 which contained either 0.5 mM potassium (*A*) or 5 mM potassium (*B*). After the addition of valinomycin, net synthesis of ATP was observed in sample *A*, but not in sample *B* where the membrane potential was reduced by the presence of 5 mM extracellular potassium. A pH jump was introduced for the next two samples (*C* and *D*, middle panel). As in sample *B*, for *C* the final cell suspension contained

5 mM potassium, to reduce the membrane potential. However, the pH of the external medium was pH 5.1, rather than pH 6, so that a new pH gradient (alkaline inside) was established. This increase in the total protonmotive force was sufficient to restore net synthesis of ATP. Sample *D* was treated in the same way except that the final cell suspension contained 30 mM potassium, instead of 5 mM potassium, to further lower the membrane potential. This decrease in the protonmotive force resulted in inhibition of ATP formation. Curves *E* and *F* (right panel) give extensions of this protocol. In sample *E,* cells were shifted from buffer at pH 6 to buffer at pH 4.2 which contained 30 mM potassium. The comparison between curves *D* and *E* shows again that a reduced membrane potential may be supplemented by a new pH gradient so that ATP synthesis occurs. Finally, curve *F* gives data for cells which had been shifted from pH 6 into buffer at pH 4.2 which contained 100 mM potassium. Once again it is apparent that ATP synthesis can be largely blocked when the membrane potential is depressed by high extracellular potassium.

The bar graphs on the top of Fig. 8 show the initial value of the total protonmotive force (in millivolts) for each of the six samples examined. The contribution made by the pH gradient is indicated by the shaded area; the size of the membrane potential is given by the open area. These calculations were possible since measurements had been made of both intracellular pH (6.7) and intracellular potassium (480 mM). Using these data along with the known values for extracellular pH and extracellular potassium, the size of the total protonmotive force could be estimated (*see* Materials and Methods). These data illustrate several important points. For the three samples which showed net ATP synthesis (*A, C* and *E*) the total protonmotive force was similar, between 215 and 220 mV. However, the composition of the protonmotive force was quite different for each of these. For *A,* the membrane potential dominated, accounting for about 80% of the total. In *C* the membrane potential and pH gradient made about equal contributions, whereas in *E* the pH gradient was the larger, contributing about 70% of the total. These results further support the idea that ATP synthesis catalyzed by the membrane-bound ATPase can be driven by potential energy which is stored as either an electrical gradient or a pH gradient across the membrane. These data also show that net synthesis of ATP was not observed unless a sufficiently large total protonmotive force was imposed. In the three samples which gave a positive response (samples *A, C* and *E*) the total protonmotive force was 215–220 mV. In the other samples, which gave a negative response, the protonmotive force was about 160–190 mV.

ATP Synthesis Driven by a pH Gradient

Two procedures were used to demonstrate ATP synthesis driven by a pH gradient. The first of these is described in Fig. 9. To impose a large transmembrane pH gradient (alkaline inside), cells were first washed and suspended in 100 mM sodium borate at pH 9, and then diluted into 100 mM sodium phosphate at pH 4.3. This pH jump was followed by a temporary increase in the internal concentration of ATP. The peak level of ATP, attained after 3 min, represented a threefold increase over the basal level; both the rate of increase in ATP and the peak level of ATP found after the pH jump were considerably less than those observed when valinomycin was subsequently added to the cells. In other experiments (not given) an increase in ATP levels was not observed after such a pH jump when cells were pretreated with 0.5 mM DCCD (conditions as in Fig. 9).

The interpretation of such experiments is complicated. Although the transmembrane pH gradient can be readily determined, one cannot be certain of the size or polarity of the membrane potential under these conditions. In fact, hydrogen ions which enter the cell under the influence of the pH gradient probably generate a membrane potential which is positive inside, thus lowering the driving force for subsequent inward movement of protons. To overcome this problem, cells were subjected to a pH jump *after* they had been exposed to valinomycin. In this case,

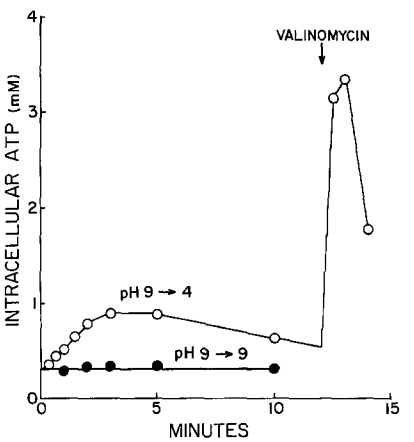

Fig. 9. ATP synthesis driven by a pH gradient. Cells were washed and resuspended in 0.1 M sodium borate, pH 9, at a final density of 8550 Klett units. At zero time, cells were diluted 35-fold with this same buffer (●) or with 0.1 M sodium phosphate, pH 4 (○). Valinomycin was added at a final concentration of 10 μM (arrow)

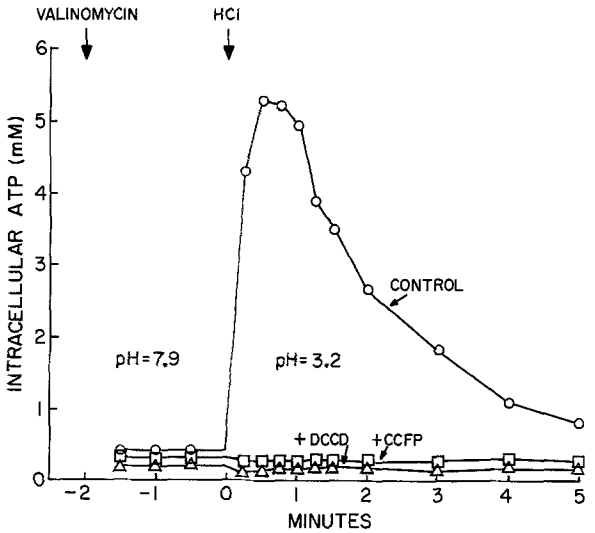

Fig. 10. ATP synthesis driven by a pH gradient in valinomycin-treated cells. Cells were washed and resuspended in 0.1 M sodium phosphate, pH 8. For ATP measurements, cells were diluted 50-fold (to 160 Klett units) with 0.1 M potassium phosphate, pH 7.9, which contained 0.125 M potassium chloride. The cells were then exposed for 35 min to 1 μM CCFP, 1 mM DCCD or ethanol. After this pretreatment 6.7 μM valinomycin was added (first arrow) and samples taken to measure basal levels of ATP. Two minutes later (second arrow) hydrochloric acid was added to lower the external pH

the inward movement of positively charged hydrogen ions could be balanced by the outward flow of potassium, and the size of the electrical gradient across the membrane could be ascertained from measurements of the potassium ratio in/out. High extracellular potassium was used so that in the presence of valinomycin the membrane potential would be low.

This kind of experiment is shown in Fig. 10. Cells were suspended at pH 7.9 in buffer containing 320 mM potassium. Valinomycin was then added (first arrow), and several samples taken to determine basal levels of ATP. After 2 min, hydrochloric acid was added (second arrow) to lower the external pH from pH 7.9 to pH 3.2. The response of three different preparations was examined in this experiment. For control cells, the pH jump was followed by an immediate rise in intracellular ATP. The maximal ATP level, found 30 sec after the addition of acid, represented a 12-fold increase over the level seen before the acid transition. This increase in ATP levels was not observed in cells exposed to DCCD or in cells exposed to CCFP.

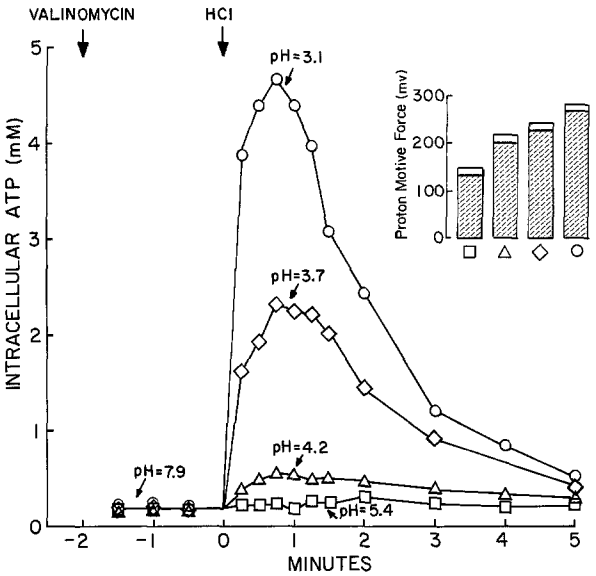

Fig. 11. ATP synthesis in response to pH gradients of varying size. Cells were washed and resuspended (8300 Klett units) in 0.1 M sodium phosphate, pH 8. Subsequently, cells were diluted 50-fold with 0.1 M potassium phosphate, pH 7.9, which contained 0.125 M potassium chloride. Five minutes later, 6.7 μM valinomycin was added (first arrow). Two minutes later (second arrow) varying amounts of hydrochloric acid were added. For measurement of initial internal pH, the final cell suspension (400 Klett units) also contained ^{14}C-methylamine and ^{3}H-D-sorbitol. After 5 min, valinomycin was added. Beginning 30 sec after valinomycin, four samples were removed at 45-sec intervals for filtration. The internal pH showed no significant variation during this time. For measurement of internal potassium, cells (400 Klett units) were placed in medium containing sodium rather than potassium. After 5 min ethanol, rather than valinomycin, was added and samples were withdrawn for filtration. The initial size of the total protonmotive force was calculated for each of the samples shown (bar graphs)

Using different conditions, it was found that net ATP synthesis was not observed unless the total protonmotive force attained an initial value of about 215 mV (*see* Fig. 8). It was of interest to determine whether this was also true for the ATP synthesis which followed a pH jump. To test this possibility, the experiment described in Fig. 11 was performed. As in the previous experiment, valinomycin was added to cells suspended in high potassium buffer at pH 7.9. This was then followed by the addition of varying amounts of hydrochloric acid, so that the final pH of the external medium ranged from pH 5.4 to pH 3.1. The initial value for the total protonmotive force (Δp) in each of these samples is indicated by the bar graphs (inset to Fig. 11). It is apparent that when the final pH was pH 5.4 ($\Delta p = 147$ mV), there was no net synthesis

of ATP following acidification of the medium. When the pH of the external medium was shifted to pH 4.2 ($\Delta p = 215$ mV) there was a slight increase in ATP levels. A more striking response was observed when the final pH was lowered to pH 3.7, ($\Delta p = 240$ mV). Finally, maximal increases in ATP were found when the pH jump was from pH 7.9 to pH 3.1 ($\Delta p = 280$ mV).

Correlation between ATP Synthesis and the Size of the Protonmotive Force

In a number of experiments it has been possible to measure changes in intracellular ATP under conditions where the initial value of the total protonmotive force was experimentally determined (*see* Figs. 8 and 11). These data are summarized in Table 1. As an index of ATP synthesis, the basal level of ATP was compared to the level found 1 min after the experiment was initiated; this usually corresponded to about the peak levels of ATP. For purposes of presentation, these samples are ranked (top to bottom) in order of increasing total protonmotive force. When treated in this way, the data fall into one of two categories, with only one exception. There was no net ATP synthesis (less than a twofold increase over basal levels) in the five cases in which the protonmotive force was 187 mV or lower. However, when the protonmotive

Table 1. Changes in ATP levels in response to protonmotive forces of varying size

ATP Synthesis $\left(\text{ratio: } \frac{\text{ATP at 1 min}}{\text{ATP at 0 min}}\right)$	Δp (mV)	$\Delta\Psi$ (mV)	$-Z\Delta$ pH (mV)
1.3	128	14	114
1.0	147	14	133
0.6	159	117	42
1.0	168	71	97
1.8	187	41	146
2.8	215	14	201
12.0	215	117	98
6.6	217	176	41
15.0	219	71	148
12.0	243	14	229
15.6	254	178	76
24.0	280	14	266
11.5	280	16	264

force was 215 mV or higher, there was net ATP synthesis (greater than a sixfold increase) in seven of eight instances. For the remaining sample, moderate (threefold) ATP synthesis was observed in response to a protonmotive force of 215 mV. These data suggest that a protonmotive force of about 215 mV represents a threshold at which the ATPase reaction is poised between net synthesis and net degradation. It is important to note that classification as to net synthesis or no net synthesis correlates with the *total* protonmotive force, and not with the size of the membrane potential or pH gradient alone. For example, a membrane potential of 117 mV was present in two samples, only one of which showed ATP synthesis. Similarly, in two cases the transmembrane pH gradient contributed about 145–150 mV to the total protonmotive force. Net ATP synthesis was observed only in the instance where a sufficiently large membrane potential brought the total above threshold. This conclusion is also apparent from inspection of the data given in Fig. 8.

Discussion

The chemiosmotic hypothesis proposes that energy derived from oxidation, or from the capture of light, is first conserved in the form of a protonmotive force, a difference in the electrochemical potential for protons across the coupling membrane. Subsequently, the inward movement of protons drives ATP synthesis catalyzed by the membrane-bound ATPase. This view predicts that ATP synthesis will occur in the absence of oxidative or light energy if adequate electrical potentials and/or pH gradients are supplied. The first experiments of this kind demonstrated ATP synthesis in response to a pH gradient, in both chloroplasts (Jagendorf & Uribe, 1966) and mitochondria (Reid, Moyle & Mitchell, 1966). In mitochondria, ATP synthesis can also be stimulated by the efflux of potassium in the presence of valinomycin (Cockrell, Harris & Pressman, 1966; Rossi & Azzone, 1970). Glynn (1967) has pointed out that these results are compatible with the idea that such ATP synthesis is driven by a potassium diffusion potential. In addition, Thayer and Hinkle (1975) have shown that ATP synthesis in submitochondrial particles can be driven by the appropriate combination of electrical potentials and pH gradients. Similar findings had been reported for intact mitochondria (Azzone & Massari, 1971), although the authors questioned the validity of a chemiosmotic interpretation. More recently, ATP synthesis catalyzed by the mitochondrial ATPase has been demonstrated with

an artificial system, in which the requisite protonmotive force arose from light-induced proton pumping by the "purple membrane" of *H. halobium* (Racker & Stoeckenius, 1974). As indicated above, ATP synthesis in chloroplasts may be driven by a pH gradient alone. In these organelles formation of ATP also occurs when an insufficiently large pH gradient is supplemented with the electrical potential generated by potassium movements in the presence of valinomycin. This has been shown in cases where the suboptimal pH gradient was established by either a limited acid-base transition (Schuldiner, Rottenberg & Avron, 1972; Uribe, 1973; Uribe & Li, 1973) or by prior illumination (Schuldiner *et al.*, 1972, 1973). Comparable results have recently been obtained using chromatophores from *Rhodospirillum rubrum* (Schuldiner *et al.*, 1973; Leiser & Gromet-Elhanan, 1974; Gromet-Elhanan & Leiser, 1975).

The experiments presented in this report and in earlier communications (Cole & Aleem, 1973; Maloney *et al.*, 1974) demonstrate that the membrane-bound ATPase of bacteria catalyzes the synthesis of ATP in response to a protonmotive force. One important objective of the experiments reported here was to establish that the driving force for such ATP synthesis could take the form of either a membrane potential, a pH gradient or the combination of these two. The data summarized in Table 1 clearly support this view. The conclusion that this ATP synthesis is catalyzed by the membrane-bound ATPase is based on the observation that ATP was not formed when cells were exposed to DCCD, a known inhibitor of this enzyme. Since the inhibitory effects of DCCD on energy-linked reactions in the related organism, *S. fecalis,* are not observed in mutants with a DCCD-resistant ATPase (Abrams *et al.*, 1972; Harold & Papineau, 1972*b*; Asghar *et al.*, 1973), it seems reasonable to assume that the primary target for DCCD is the ATPase. In addition, it is shown here that the activity of the ATPase, measured by its rate of hydrolysis of intracellular ATP, was sufficient to account for the rate of ATP synthesis observed when a protonmotive force was imposed (Fig. 3 and text).

In these experiments a transient net synthesis of ATP occurred when a protonmotive force was applied. The simplest explanation for the temporary nature of this response is that there is decay of the driving force for ATP synthesis. Direct evidence in support of this argument was obtained for experiments in which the protonmotive force was dominated by the membrane potential. In this case, net efflux of potassium was observed (Fig. 4) after the addition of valinomycin, leading to a change in the membrane potential from about 200 mV (zero time) to 145 mV

(4 min). Under these conditions potassium efflux is probably associated with proton entry (Harold & Baarda, 1967; Kashket & Wilson, 1973). This would make the inside more acid and thus further reduce the total protonmotive force. In addition to proton entry there may be a significant inward movement of sodium, since it has been reported that bacteria contain a system catalyzing the electroneutral exchange of protons and sodium ions (Harold & Papineau, 1972*b*; West & Mitchell, 1974*b*). In cases where ATP synthesis was driven by a pH gradient (Figs. 10 and 11), decay in the total protonmotive force would result from acidification of the internal space as protons enter under the influence of their chemical gradient. However, direct measurements of changes in internal pH have not yet been made under these conditions.

The observations reported here support the idea that the bacterial ATPase links ATP synthesis to the entry of protons. Evidence in favor of this view would be considerably strengthened by the demonstration that the observed value for the electrochemical potential difference for protons represents sufficient potential energy to maintain the observed poise of the ATPase reaction. For bacterial systems, arguments concerning this relationship are necessarily indirect, since the stoichiometry of protons translocated per ATP formed is unknown. Clearly, for an electrochemical gradient of given size, the potential energy made available for ATP formation will be in direct proportion to the number of protons which travel down this gradient during the synthetic reaction. A stoichiometry of $2H^+$/ATP has been found for the mitochondrial ATPase (Mitchell & Moyle, 1968; Thayer & Hinkle, 1973), and if this coupling ratio holds for the bacterial ATPase, then approximate calculations suggest that the data presented here are in agreement with the chemiosmotic hypothesis. In the experiment given in Fig. 6, a maximal ATP/ADP ratio of 3.4 was observed. It may be calculated that a protonmotive force of about 240 mV would be required to maintain this ratio. This assumes that ADP and ATP are present as their magnesium salts within the cell, giving a standard free energy of hydrolysis for ATP of about 7.6 Kcal/mole at 25 °C and pH 7 (Guynn & Veech, 1973); internal phosphate is taken as about 10 mM, and unit activity coefficients are assumed for reactants and products [*see* Greville (1969) for further examples and discussion]. This calculated value of 240 mV compares favorably with the experimental observation that ATP synthesis occurred only when the protonmotive force was 215 mV or greater. Such quantitative arguments are of somewhat limited value until the assumptions made have been verified by experiment. Nevertheless, these approximate calcula-

tions, taken together with the observations reported here, provide strong evidence supporting the idea that the bacterial ATPase operates as a reversible proton translocating pump.

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