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Protonmotive Force as the Source of Energy for Adenosine 5'-Triphosphate Synthesis in *Escherichia coli*

DOROTHY M. WILSON,* JOHN F. ALDERETE,[1] PETER C. MALONEY, AND T. HASTINGS WILSON

Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

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Net synthesis of adenosine 5'-triphosphate (ATP) in energy-depleted cells of *Escherichia coli* was observed when an inwardly directed protonmotive force was artificially imposed. In wild-type cells, ATP synthesis occurred whether the protonmotive force was dominated by the membrane potential (negative inside) or the pH gradient (alkaline inside). Formation of ATP did not occur unless the protonmotive force exceeded a value of 200 mV. Under these conditions, no ATP synthesis was found when cells were exposed to an inhibitor of the membrane-bound Ca^{2+}- and Mg^{2+}-stimulated adenosine triphosphatase (EC 3.6.1.3), dicyclohexylcarbodiimide, or to a proton conductor, carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone. Adenosine triphosphatase-negative mutants failed to show ATP synthesis in response to either a membrane potential or a pH gradient. ATP synthesis driven by a protonmotive force was observed in a cytochrome-deficient mutant. These observations are consistent with the chemiosmotic hypothesis of Mitchell (1961, 1966, 1974).

The chemiosmotic hypothesis of Mitchell (25-28) has provided valuable insights into the coupling between electron transport and phosphorylation, especially in studies of mitochondria and chloroplasts (see Greville [7], Harold [11], and Skulachev [37] for reviews). The striking similarity between oxidative phosphorylation in microorganisms and animal cells (18) suggests that the analysis of bacterial systems will also profit from considerations of the chemiosmotic hypothesis (see Harold and Altendorf [12] for a review). According to this view, the oxidation of substrates by the respiratory chain is coupled to the extrusion of protons from the cell. Such active transport of the hydrogen ion results in the acidification of the medium and the generation of a pH gradient (alkaline inside) across the cell membrane. In addition, such hydrogen ion movements lead to the formation of a membrane potential (negative inside). In this way, energy dissipated by the oxidation of substrate is conserved as a "protonmotive force," a difference in the electrochemical potential for hydrogen ions across the cell membrane. Subsequent to this primary energy conservation step, the reentry of protons by way of the membrane-bound Ca^{2+}- and Mg^{2+}-stimulated adenosine triphosphatase (Ca^{2+},Mg^{2+}-ATPase) is coupled to the synthesis of adenosine 5'-triphosphate (ATP). Thus, the driving force for the synthesis of ATP is represented by an inwardly directed protonmotive force.

Several observations support this view of oxidative phosphorylation in microorganisms. The first experiments demonstrating proton extrusion associated with respiration were reported by Scholes and Mitchell (34), who studied *Micrococcus* (now *Paracoccus*) *denitrificans*. Similar results were obtained with intact cells of other bacteria (17, 20, 24, 38), as well as membrane vesicles from *Escherichia coli* (1, 14, 29). It was also shown that a membrane potential (inside negative) is present in respiring cells (9, 10) or membrane vesicles (1, 15) of *E. coli*. From the study of specific mutants of *E. coli*, it is known that the Ca^{2+},Mg^{2+}-ATPase is required for the synthesis of ATP during oxidative phosphorylation (see Cox and Gibson [5] for a review). West and Mitchell (39) and Hertzberg and Hinkle (14) have provided evidence that in *E. coli* this enzyme catalyzes proton translocation. Using everted membrane vesicles from *E. coli*, these authors showed that protons moved into the vesicle lumen as a result of ATP hydrolysis.

Recently, Maloney et al. (22) provided a direct demonstration that the membrane-bound ATPase of both *Streptococcus lactis* and *E. coli* catalyzes the synthesis of ATP in response to an inwardly directed protonmotive force. The studies reported here represent a continuation of that work and provide additional documentation of the observations made using *E. coli*. It is

[1] Present address: Department of Microbiology, University of Kansas, Lawrence, Kan. 66044.

shown here that ATP formation occurs whether the protonmotive force is composed of a membrane potential (negative inside), or a pH gradient (alkaline inside). In addition, the pathway that allows the coupling of proton entry to ATP synthesis is identified as the membrane-bound Ca^{2+},Mg^{2+}-ATPase, since mutants lacking this enzyme fail to form ATP in response to either a membrane potential or a pH gradient.

(A preliminary report of these studies has been presented [T. H. Wilson, J. F. Alderete, D. M. Wilson, and P. C. Maloney. Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, p. 159, K73).

Recently, Grinius and co-workers (8) have also shown that wild-type cells of *E. coli* synthesize ATP in response to an inwardly directed protonmotive force.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Several strains of *E. coli* K-12 were studied. Wild-type strain 1100 and its ATPase-negative derivatives, strains 72 and 45 (41), were provided by T. Yamamoto and R. Valentine. Wild-type strain AN180 and its ATPase-negative derivative, strain AN120 (2), were from F. Gibson. The *hemA* mutant, SASX76 (33), was from A. Sasarman. In addition to the K-12 strains listed above, *E. coli* ML308-225 (40) and its ATPase-negative derivative, strain DL54 (35), were also used; strain DL54 was a gift of R. Simoni. After completion of an experiment using an ATPase-negative mutant, the cell suspension that had been used was tested for the presence of revertants by streaking onto succinate minimal medium plates containing the necessary supplements. During the experiments reported here, no significant reversion occurred.

Unless otherwise specified, cells were grown in mineral medium 63 (3), which contained 0.2% glucose and 0.5 μg of thiamine per ml. In experiments using both ATPase-negative and wild-type strains, the growth medium also contained 0.1% Casamino Acids (Difco). Arginine (0.2 mM) was also present for growth of strains AN180 and AN120. Bacteria were grown in batches of 200 ml at 37 C with continuous shaking at 200 rpm. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter (no. 42 filter); at least three generations of exponential growth were allowed before harvesting. It was assumed that 1 ml of a suspension at a density of 100 Klett units contained 0.6 μl of intracellular water and 220 μg (dry weight) of cells (40).

Chemicals. Valinomycin and *N,N'*-dicyclohexylcarbodiimide (DCCD) were purchased from Calbiochem. Carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP) was a gift of E. P. Kennedy (Harvard Medical School, Boston, Mass.). Valinomycin, DCCD, and FCCP were added to cell suspensions as small volumes of stock solution in 95% ethanol. Final ethanol concentrations did not exceed 0.2%. [^{14}C]methylamine, [^{14}C]thiomethylgalactoside, and [^{3}H]inulin were obtained from New England Nuclear Corp. [^{3}H]inulin was purified by paper chromatography before use. Non-radioactive isopropylthiogalactoside was obtained from Sigma Chemical Co.

Depletion of endogenous substrates. Initial experiments employed the procedure reported earlier (22) for starvation of strains 1100, 72, and 45. The method finally adopted for routine use was that originally described by Koch (19). This procedure allowed rapid depletion of metabolizable reserves by exploiting a cyclic phosphorylation and dephosphorylation of α-methylglucoside. Cells were first harvested by centrifugation at 4 C and washed once with either medium 63 or 120 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8); no significant differences were found for cells washed in either way. Washed cells were then suspended at a density of about 800 Klett units in medium 63 containing 20 mM α-methylglucoside and 40 mM sodium azide. Incubation time at 37 C was 120 min for strains 1100, AN180, An120, and ML308-225; strains 72 and DL54 were incubated for 45 min. In all cases, depleted cells were centrifuged and washed first with a solution (pH 8) containing 100 mM potassium phosphate plus 100 mM sodium phosphate. The second wash was with one of two different solutions. For those experiments that examined the effect of a membrane potential, the second wash fluid contained 200 mM sodium phosphate (pH 6) and 1 mM sodium cyanide. These cells were then resuspended in this same medium as a concentrated stock. For experiments that examined the effect of pH gradient, the second wash contained 100 mM sodium phosphate (pH 8) plus 100 mM KCl. These cells were then resuspended in this same medium as a concentrated stock. Additional details will be found in the legends to the figures.

Measurement of ATP. ATP was measured by the method of Cole et al. (4). Intracellular ATP was extracted by placing 0.4 ml of a cell suspension (200 to 300 Klett units) onto 0.1 ml of 3 M perchloric acid at 0 C. After at least 10 min, the acid extract was neutralized with 0.3 ml of 1 M KOH. 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 neutralized cell extract was mixed with 0.9 ml of 45 mM glycylglycine buffer (pH 7.4) in a 1-dram (15 by 45 mm) glass vial (no. 7475, Rochester Scientific Co.). Firefly lantern extract (0.025 ml) was then 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 into the well of a liquid scintillation counter (Nuclear Chicago Mark I) that had been set for maximum sensitivity, with the coincidence circuit off. After 15 s, the sample was counted for 6 s. With no added ATP, background counts were about 2,000; when 25 pmol of ATP were added, about 60,000 counts were obtained. The experimental samples contained 0 to 25 pmol of ATP, and over this range there was a linear relationship between counts and ATP concentration. Control experiments showed that the valinomycin, salts, etc., used in these studies did not affect the assay of ATP.

Quantitative experiments. In some experiments (see Table 3), ATP synthesis was measured under conditions where the initial value of the protonmotive force was experimentally determined. The size of the membrane potential was calculated from the measured ratio of internal to external potassium, using the Nernst equation. The size of the pH gradient was determined from measurements of internal and external pH, and expressed in terms of millivolts (59 ΔpH). The protonmotive force represents the contributions made by both the membrane potential and pH gradient.

In these experiments, depleted cells were suspended (5,000 Klett units) in 100 mM potassium phosphate (pH 8) containing 100 mM NaCl. To measure internal potassium, a portion was removed and centrifuged. After the supernatant was decanted, the sides of the tube were carefully wiped free of adhering extracellular fluid, and the pellet was resuspended using medium of the same osmotic strength, but in which sodium replaced potassium; final cell density was about 200 Klett units. One-milliliter portions were then immediately filtered (0.65-μm pore size) but not washed. Intracellular potassium was extracted using the method previously described (23) and was measured by flame photometry. Values for internal potassium ranged from 190 to 230 mM, for both wild-type and ATPase-negative cells. External potassium (170 mM) was also measured by flame photometry.

Internal pH was calculated from the observed distribution of the weak base, methylamine (pK of 10.6), using inulin as a marker for extracellular water (23). Depleted cells were diluted 20-fold into the same medium, which also contained [^{14}C]methylamine (0.03 mM; 0.15 μCi/ml) and [^{3}H]inulin (4.2 μg/ml; 0.5 μCi/ml). Valinomycin was added after 5 min, and four 0.5-ml samples were taken at 45-s intervals for filtration (0.65-μm pore size) without washing. Filters were analyzed for both ^{14}C and ^{3}H. Under these conditions, the internal pH was constant in any one experiment, but varied between 7.3 and 7.9 in different experiments.

ATP synthesis was measured in a parallel tube. Depleted cells were diluted 25-fold into 100 mM potassium phosphate (pH 8) containing 100 mM NaCl. Other experimental details are given in the text and the legend of Fig. 8. As an index of ATP synthesis, it was convenient to compare the basal level of ATP to that found 45 s after the addition of an acid pulse. This usually corresponded to the peak level of ATP, and was never less than 80% of the peak value.

Measurement of intracellular thiomethylgalactoside. Cells were grown in medium 63 containing 1% tryptone (Difco), 0.4% glycerol, and 0.4 mM isopropylthiogalactoside. After washing twice with medium 63, cells were resuspended in this medium (1,000 Klett units) with or without 2 mM DCCD. Incubation at 25 C was for 30 min, after which an equal volume of medium 63 containing 0.2 mM [^{14}C]thiomethylgalactoside (0.2 μCi/ml) was added. Five minutes later, 0.2-ml samples were withdrawn and filtered (0.65-μm pore size); this was followed by a brief wash with 5 ml of medium 63. Radioactivity retained on the filter was then determined.

RESULTS

Hydrogen ion permeability of ATPase-negative mutants. Some ATPase-negative mutants of *E. coli* possess an abnormally high permeability to the hydrogen ion (1, 31, 32). As a consequence of this, such strains also show decreased capacity to accumulate substrates (31, 35) that enter by way of proton-coupled active transport systems. Pretreatment of these cells with DCCD lowers proton permeability and thus stimulates their capacity to carry out active transport (1, 31, 32). Of the ATPase-negative mutants examined in the studies reported here, one (strain 72) falls into that class which shows elevated permeability to protons, whereas another (strain AN120) shows no such defect. These identifications are supported by the following observations. Using the techniques developed by Scholes and Mitchell (34), we compared the ATPase-negative mutants and their corresponding parental strains with respect to the permeability of the cell membrane towards hydrogen ions. The experiment given in Fig. 1 shows that an immediate acidification of the medium occurred when a pulse of oxygen was introduced into suspensions of cells maintained under anaerobic conditions. This was followed by a less rapid return of the external pH to its initial value. Whereas rates of

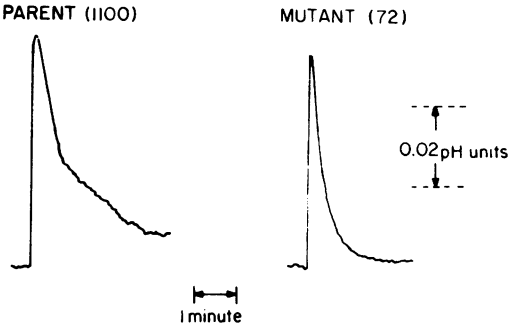

FIG. 1. *Proton extrusion and reentry after an oxygen pulse. Cells in the late exponential phase of growth were washed and then resuspended (3,000 Klett units) in 2 ml of a solution containing 1.5 mM glycylglycine, 200 mM KCl, 30 mM NaSCN, and 20 μg of carbonic anhydrase per ml. After a 60-min incubation under anaerobic conditions at 25 C, oxygen was introduced by injection of 50 μl of the same solution equilibrated with room air (34). The initial pH of the suspension containing the ATPase-negative mutant (strain 72) was 6.09; the initial pH of the parental cell (strain 1100) was 6.02. External pH was measured using a combination glass electrode (Radiometer, GK2321C). The output of the pH meter (Radiometer, PHM63) was amplified and displayed on a recorder (Linear Instruments) at 0.2 pH units, full scale.*

proton extrusion were similar for both ATPase-negative (strain 72) and wild-type (strain 1100) cells, the rate of reentry of protons was markedly increased for the mutant. For example, in this experiment the half-time for proton reentry into wild-type cells was 32 s, whereas for the mutant, half-time for proton reentry was only 14 s. In a number of such experiments it was observed that the half-time for proton reentry into strain 72 was reduced by about a factor of 2 when compared with its parent, strain 1100 (Table 1). However, the half-time for proton reentry into the ATPase-negative strain AN120 was not significantly different from that found for its parent, strain AN180 (Table 1). In addition, it was observed that galactoside accumulation was significantly reduced in one ATPase-negative mutant, strain 72, whereas both AN120 and its wild-type counterpart, AN180, showed about the same capacity to accumulate substrate (Table 2). The data in Table 2 also show that pretreatment with DCCD stimulated galactoside transport only in strain 72, and not in the other ATPase-negative strain, AN120.

ATP synthesis driven by manipulation of both the membrane potential and the pH gradient. Experiments were first carried out to determine optimal conditions for reducing ATP levels in *E. coli*. In our previous study (22), depletion of endogenous metabolizable reserves was based on a modification of the method developed by Koch (19), and required exposure of cells to sodium arsenate (10 mM) during starvation. This protocol severely depressed basal levels of cellular ATP (to less than 0.1 mM), and permitted the demonstration of ATP synthesis driven by an artificially imposed protonmotive force (22). Under these conditions, ATP synthesis could be observed in a wild-type strain, 1100, but not in two ATPase-negative mutants, strains 72 and 45, derived from 1100. However, in such experiments the yields of ATP in the wild-type strain were small. It was later found that incubation for suitable times in the presence of α-methylglucoside and sodium azide (the combination originally suggested by Koch), without arsenate, was the most effective technique for reducing basal levels of ATP while retaining full capacity for ATP synthesis catalyzed by the membrane-bound Ca^{2+},Mg^{2+}-ATPase.

TABLE 1. *Ratio of half-times ($T_{1/2}$) values for proton reentry into parental and ATPase-negative strains*[a]

Mutant/parent	No. of expt	Ratio of $T_{1/2}$
72/1100	9	0.51 ± 0.09
AN120/AN180	5	1.19 ± 0.12

[a] See legend for Fig. 1 for experimental details. The half-times for proton reentry were estimated from semilogarithmic plots of external pH versus time (34). In each experiment, a mutant was compared with its wild-type ancestor and the ratio of half-times for proton reentry was calculated. Values given are the means and standard errors.

TABLE 2. *Thiomethylgalactoside accumulation by parental and ATPase-negative strains*[a]

Strain	Intracellular thiomethylgalactoside (mM)	
	Control	+DCCD
1100 (parent)	4.8 ± 0.1	5.3 ± 0.6
72 (mutant)	1.3 ± 0.1	3.2 ± 0.4
AN180 (parent)	4.2 ± 0.3	3.8 ± 0.3
AN120 (mutant)	5.4 ± 0.7	4.7 ± 0.1

[a] Values given are the means and standard errors of four (strains 1100 and 72) or three (strains AN180 and AN120) separate determinations at equilibrium after 5 min.

A second important consideration was the method of establishing an inwardly directed protonmotive force. An electrical gradient favorable to proton entry was established by suspending cells in a potassium-free medium and then exposing them to the potassium ionophore, valinomycin. Under these conditions the membrane potential (negative inside) results from the outward diffusion of the positively charged potassium ion, by way of valinomycin. A chemical gradient favorable to proton entry was established simply by acidification of the external medium.

For the experiment shown in Fig. 2, depleted cells were treated with valinomycin 10 s after dilution from buffer at pH 8 into buffer at pH 5. This resulted in a rapid increase in intracellular ATP (filled circles) to a peak value of about 1.1 mM at 1 min. This was followed by a less rapid decay during the next 20 min. When this experiment was repeated in the presence of 1 mM sodium cyanide (filled triangles), there was a similar rapid net synthesis of ATP. However, the presence of cyanide appeared to accelerate the subsequent decay of ATP levels from their peak value. This effect of cyanide may result from inhibition of the oxidation of small amounts of substrates retained by depleted cells. Nevertheless, it is apparent that most of the ATP formed after imposition of a protonmotive force results from reactions that do not require the presence of a functional respiratory chain. This experiment also shows that cyanide effectively blocks respiration by starved cells; net synthesis of ATP after the addition of D-lactate was not found for

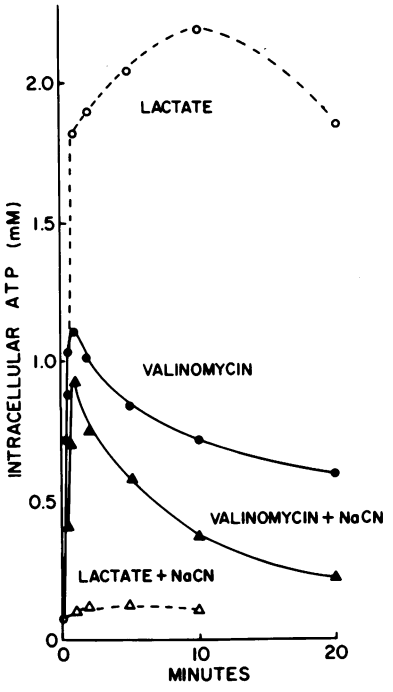

FIG. 2. *ATP synthesis in depleted cells. Depleted cells of strain 1100 were suspended as a concentrated stock (6,500 Klett units) in 200 mM sodium phosphate (pH 8). One portion of this stock was exposed for 10 min to 1 mM NaCN; the remainder served as a control. For measurement of ATP synthesis driven by an artificially imposed protonmotive force, NaCN-treated cells were diluted 25-fold into 200 mM sodium phosphate (pH 5) containing 1 mM NaCN. Ten seconds later, valinomycin was added (10 μM final concentration). Control cells were treated in the same way, except that NaCN was not present. For measurement of ATP synthesis coupled to* D*-lactate oxidation, cells were diluted 25-fold into 200 mM sodium phosphate (pH 8), which contained 10 mM* D*-lactate, with or without 1 mM NaCN.*

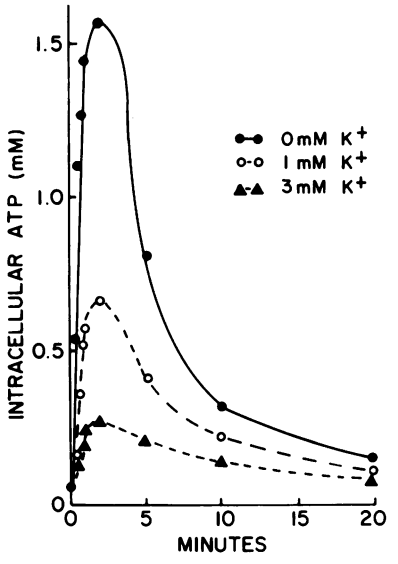

FIG. 3. *Effect of external potassium on valinomycin-induced ATP synthesis. Depleted cells of strain 1100 were suspended (5,000 Klett units) in a solution containing 200 mM sodium phosphate (pH 6) and 1 mM NaCN; they were then diluted 25-fold into this same medium, which also contained either 10 mM NaCl (●), 9 mM NaCl plus 1 mM KCl (○) or 7 mM NaCl plus 3 mM KCl (▲). Valinomycin (10 μM final concentration) was added immediately after dilution.*

cells exposed to cyanide (Fig. 2). For this reason, in many of the following experiments cyanide was used to minimize the possibility that some part of the observed synthesis of ATP reflected the oxidation of residual substrates.

ATP synthesis driven by a membrane potential. In the following experiments, the artificially imposed protonmotive force was composed primarily of a membrane potential (negative inside). During their final wash and resuspension, starved cells were exposed to a medium buffered at pH 6 and then diluted into this same medium just before the addition of valinomycin. As shown by the experiment given in Fig. 3, when cells were equilibrated at pH 6 the addition of valinomycin resulted in the transient net synthesis of ATP. If the driving force for ATP synthesis under these conditions were due to a potassium diffusion potential, one would expect inhibition of ATP synthesis when the membrane potential is reduced by the presence of external potassium. When valinomycin was added to cells suspended in 1 or 3 mM external potassium, marked reductions in the yields of ATP were found (Fig. 3). In other experiments (not shown) no net synthesis of ATP was observed when potassium in the medium was present at concentrations of 10 mM or above.

Net synthesis of ATP under these conditions was also blocked when the experiment was performed in the presence of the proton conductor, FCCP (Fig. 4). This observation supports the idea that such ATP synthesis is driven by an electrochemical potential difference for protons, since the necessary protonmotive force would not be maintained in the presence of such a proton conductor.

We concluded from the results of earlier experiments (21, 22) that ATP synthesis under these conditions was catalyzed by the membrane-bound Ca^{2+},Mg^{2+}-ATPase. This possibility was more carefully examined in the work reported here. The first approach was to study

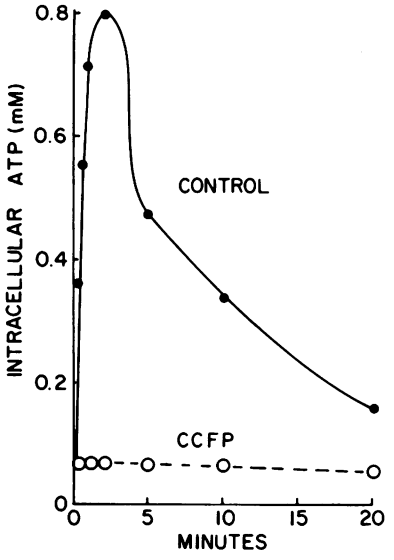

FIG. 4. *Effect of FCCP on valinomycin-induced ATP synthesis. Depleted cells of strain 1100 were suspended (5,900 Klett units) in a solution containing 200 mM sodium phosphate (pH 6) and 1 mM NaCN. To one portion of this stock, FCCP (10 μM final concentration) was added. These cells were then immediately diluted 25-fold into this same medium, without additional FCCP. Valinomycin (10 μM final concentration) was added 10 s later. Control cells were treated in the same way except that a corresponding volume of ethanol was used, instead of FCCP.*

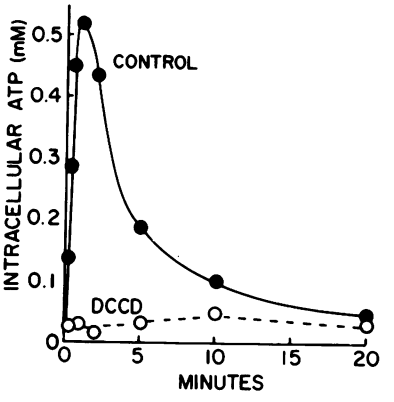

FIG. 5. *Effect of DCCD on valinomycin-induced ATP synthesis. Depleted cells of strain 1100 were suspended (7,500 Klett units) in a solution containing 200 mM sodium phosphate (pH 8) containing 1 mM NaCN. One milliliter of this suspension received 1 μl of 1 M DCCD (in ethanol); a second 1-ml aliquot received 1 μl of ethanol. After 30 min at 25 C, cells were centrifuged and resuspended at their original density in 200 mM sodium phosphate (pH 6) containing 1 mM NaCN but without DCCD or ethanol. After an additional 15 min, cells were diluted 25-fold into this same medium (pH 6). Valinomycin (10 μM final concentration) was added after dilution.*

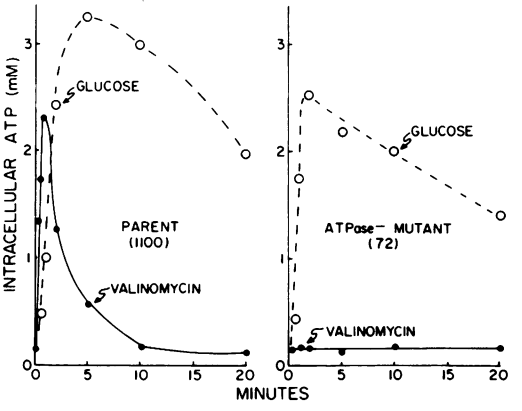

FIG. 6. *Absence of valinomycin-induced ATP synthesis in an ATPase-negative mutant. Depleted cells of strain 1100 or its ATPase-negative derivative, strain 72, were suspended (6,700 Klett units) in a solution containing 200 mM sodium phosphate (pH 6) and 1 mM NaCN. They were then diluted 25-fold into this same medium before addition of valinomycin (closed symbols) or 10 mM glucose (open symbols).*

the effect of DCCD, an inhibitor of this enzyme (6, 13). As shown in Fig. 5, pretreatment of cells with 1 mM DCCD completely blocked the ATP synthesis normally observed after the addition of valinomycin. A second approach was to examine the behavior of mutant strains that lack a functional ATPase. We have studied two types of such ATPase-negative mutants. One mutant, strain 72, is representative of that class of ATPase-negative mutants which show an abnormally high permeability to protons (Fig. 1 and Table 1). Strain 72 also shows a defect in active transport, as has been reported for other mutants of this type (1, 31, 32, 35), and this defect is largely repaired by pretreatment of cells with DCCD (Table 2). When depleted cells of strain 72 were exposed to valinomycin at pH 6, no net synthesis of ATP was observed (Fig. 6), whereas the parent, strain 1100, responded normally to this treatment. However, both mutant and wild-type cells showed similar capacity to form ATP using glycolytic reactions. Strain 72 differed from its parent in one other respect. Only 45 min of incubation with β-methylglucoside and azide was required for the depletion of the mutant, rather than the 120 min required for the parent. Depletion times shorter than and longer than 45 min were tested for the mutant; under no conditions was ATP synthesis observed after addition of valinomycin.

The second ATPase-negative mutant selected

for these studies was strain AN120. This mutant shows no defect in either proton permeability or galactoside accumulation (Tables 1 and 2). The experiment given in Fig. 7 shows that no net synthesis of ATP was found after the addition of valinomycin to depleted cells of AN120. However, ATP synthesis was observed when the corresponding parental cells (AN180) were tested. Both cell types formed ATP from the metabolism of glucose (Fig. 7).

ATP synthesis driven by a pH gradient. In this series of experiments, an inwardly directed protonmotive force was established by imposing a pH gradient (alkaline inside) across the cell membrane. The membrane potential was maintained at a low value by exposing cells to valinomycin in the presence of 100 mM potassium. An example of this type of experiment is shown in Fig. 8. Depleted cells were suspended at pH 8 in the presence of 100 mM KCl. The addition of valinomycin (first arrow) did not lead to elevation of ATP levels above the basal value. However, when a small volume of acid was added (second arrow), lowering the external pH from 8 to 3, there was transient net synthesis of ATP; internal levels of ATP rose from the basal value of about 0.1 mM to a peak value of about 2.1 mM. In the same experiment, a smaller pH jump, from 8 to 3.9, gave reduced yields of ATP; shifting the external pH down to 5.3 did not result in significant net synthesis of ATP. These results indicate that under these conditions ATP synthesis is sensitive to the ratio of internal to external hydrogen ion concentration, and that net synthesis of ATP occurs only after imposition of a suitably large protonmotive force.

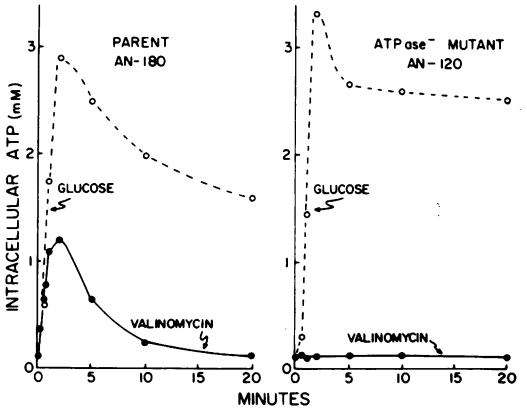

FIG. 7. *Absence of valinomycin-induced ATP synthesis in an ATPase-negative mutant. Strain AN180 and its ATPase-negative derivative, strain AN120, were used. See legend to Fig. 6 for experimental details.*

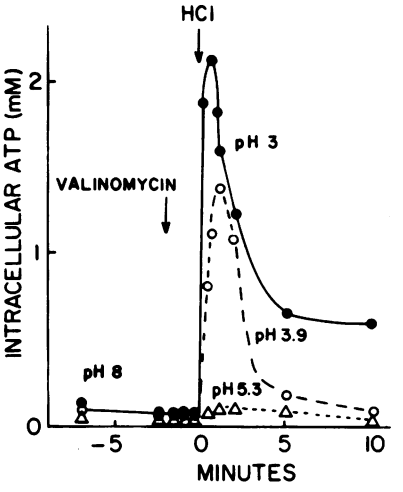

FIG. 8. *ATP synthesis driven by a pH gradient. Depleted cells of strain AN180 were suspended (4,900 Klett units) in a solution containing 100 mM sodium phosphate (pH 8) and 100 mM KCl. Cells were then diluted 25-fold into this same buffer. After 5 min, valinomycin (5 μM final concentration) was added; after an additional 2 min, varying amounts of 2 N HCl were added to lower the external pH. External pH was measured after sampling for ATP levels had been completed.*

In agreement with this reasoning, ATP synthesis after a pH jump did not occur in the presence of the proton conductor, FCCP (Fig. 9). It is expected that the addition of FCCP makes the cell membrane permeable to protons, and so reduces the size of the protonmotive force that can be maintained across that membrane.

Two kinds of observations support the idea that the membrane-bound Ca^{2+},Mg^{2+}-ATPase catalyzes the synthesis of ATP observed after a pH jump. Such ATP synthesis was markedly reduced when cells were pretreated with the inhibitor DCCD (Fig. 10). In addition, mutants that lack a functional Ca^{2+},Mg^{2+}-ATPase did not form ATP after an acid pulse, although transient net synthesis of ATP was found in the respective parental strains. The data in Fig. 11 illustrate this for the mutant, AN120, and its parent, AN180. Data given in a later section (Table 3) demonstrate this for the mutant, strain 72, and its parent, 1100. Similar observations (not shown) were made when the behavior of the mutant DL54, was compared with its parent, ML308-225.

ATP synthesis in a cytochrome-deficient mutant. The *hemA* mutant SASX76 is defective in the biosynthesis of heme (33), and cytochromes that function in electron transport are not formed unless the cells are grown in the

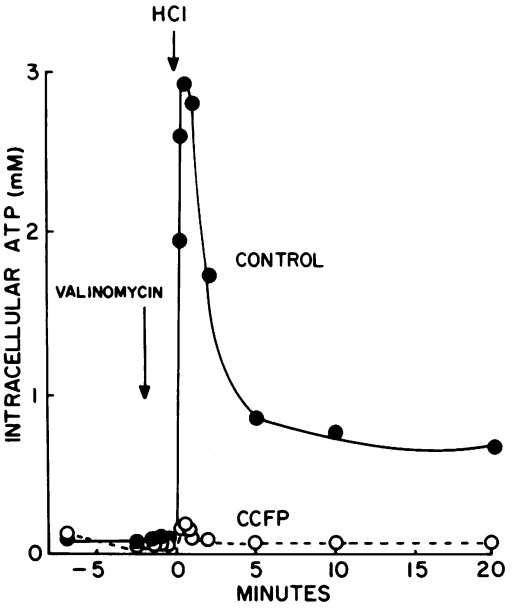

FIG. 9. *Effect of FCCP on ATP synthesis driven by a pH gradient. Strain AN180 was used. The procedure described in the legend to Fig. 8 was followed, except that 1 min before dilution, cells were exposed to either 20 μM FCCP (in ethanol) or an equivalent amount of ethanol. The final external pH for both suspensions was 3.1.*

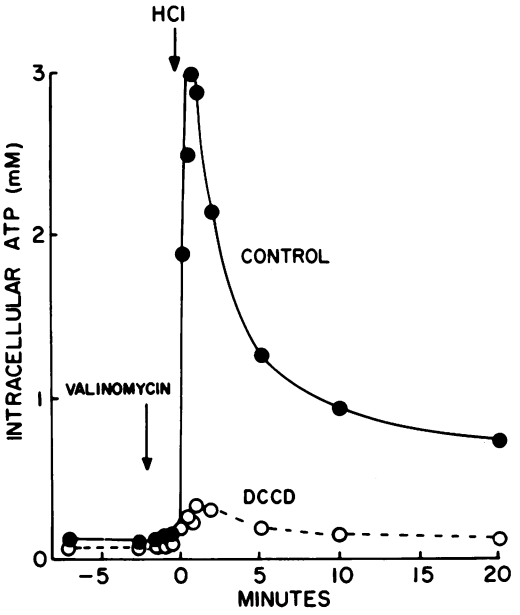

FIG. 10. *Effect of DCCD on ATP synthesis driven by a pH gradient. Strain AN180 was used. The procedure described in the legend to Fig. 8 was followed, except that 30 min before dilution cells were exposed to either 1 mM DCCD (in ethanol) or an equivalent amount of ethanol. The final external pH was 3.05 for both samples.*

presence of δ-aminolevulinic acid (36). For the experiment shown in Fig. 12, cells of SASX76 were grown overnight in a rich medium (Penassay broth [Difco]) without added δ-aminolevulinic acid. Because washed cells of SASX76 contained low levels of ATP, it was possible to avoid exposure to α-methylglucoside and azide. Thus, fresh cells could be examined directly under conditions where the protonmotive force took the form of either a membrane potential or a pH gradient. As shown in Fig. 12, transient net synthesis of ATP was found in both instances. It was noted that after the addition of valinomycin to cells suspended in medium without added potassium, the appearance of ATP was slower and less marked than that usually observed (Fig. 2-7). The reason for this difference is not completely understood, but may reflect poor access of valinomycin to the cell membrane under these conditions. This possibility has not yet been tested by the appropriate experiments.

Quantitative experiments. In a number of experiments, similar to that shown in Fig. 8, we have correlated ATP synthesis with the size of the total protonmotive force. The value of the membrane potential in the presence of valinomycin was estimated from the Nernst equation,

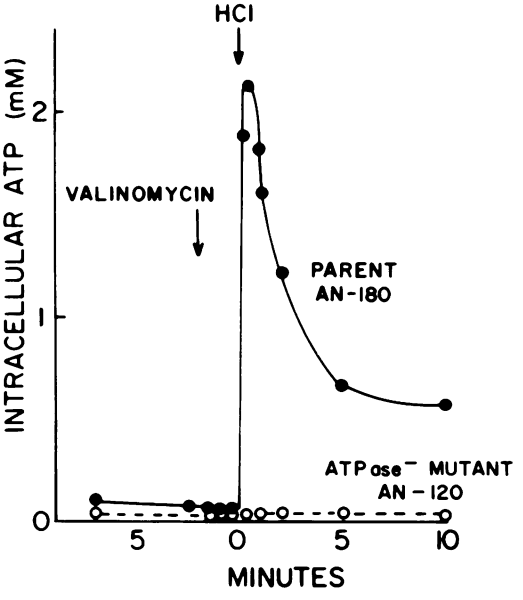

FIG. 11. *Absence of ATP synthesis driven by a pH gradient in an ATPase-negative mutant. Strain AN180 and its ATPase-negative derivative, strain AN120, were used. See legend to Fig. 8 for experimental details. The final external pH was 3.0 for both cell types.*

TABLE 3. *Correlation between ATP synthesis and the protonmotive force*[a]

ATP synthesis (45-s ATP/initial ATP)	Δp (mV)
Strain 1100 (parent)	
1.1	104
1.3	129
1.5	145
1.4	148
1.7	149
5.0	199
5.3	205
10.0	237
14.8	237
18.6	241
38.2	251
67.8	276
44.0	282
19.6	285
13.2	294
Strain 72 (ATPase-negative mutant)	
1.1	282
1.0	285
1.0	287

[a] Experimental details found in Materials and Methods. The size of the total protonmotive force was varied by changing the pH gradient. Internal pH was 7.3 to 7.9; external pH, measured using a glass electrode, varied between 5.6 and 3.0.

using measured values for the ratio of internal (190 to 230 mM) to external (170 mM) potassium. This calculation assumes that valinomycin-treated cells are very much more permeable to the potassium ion than to any other ion. Under these conditions the membrane potential was low, since internal and external potassium levels were similar. Consequently, additions of differing amounts of hydrochloric acid served to vary the size of the total protonmotive force. The distribution of methylamine was used to estimate the initial value of intracellular pH. Knowing the value for internal pH, the size of the pH gradient could be calculated from measurement of external pH, after the addition of acid. The results of these experiments are given in Table 3. Significant synthesis of ATP over the basal value was observed only when the total protonmotive force attained a value of 200 mV. For wild-type cells, the apparent decline in ATP synthesis at the higher values of Δp (>280 mV) is not significant, but reflects higher basal levels of ATP in these few samples. As noted previously, no synthesis of ATP was observed in an ATPase-negative mutant, even though the total protonmotive force at zero time was 280 to 290 mV, well above the threshold required for demonstration of ATP formation in normal cells.

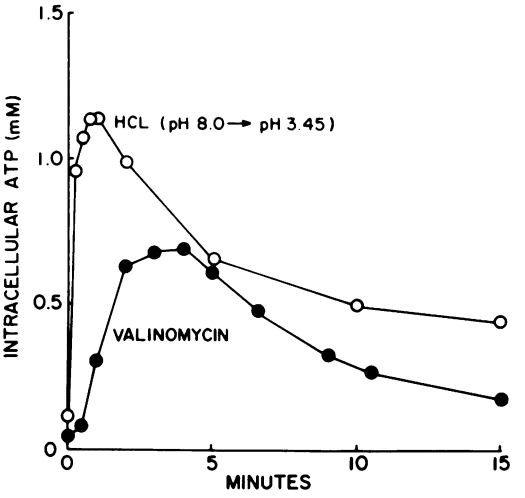

FIG. 12. *ATP synthesis driven by a protonmotive force in a cytochrome-deficient mutant, strain SASX76. Washed cells were suspended (9,000 Klett units) in either 200 mM sodium phosphate (pH 6) or 200 mM potassium phosphate (pH 8). To demonstrate ATP synthesis driven by a membrane potential (●), cells suspended at pH 6 were diluted 25-fold into the same medium before the addition of valinomycin (10 μM final concentration). To demonstrate ATP synthesis driven by a pH gradient (○), the cells suspended at pH 8 were exposed to 10 μM valinomycin for 60 min before a 25-fold dilution into this same medium; HCl was then added to lower the external pH.*

DISCUSSION

According to the chemiosmotic hypothesis, the membrane-bound Ca^{2+},Mg^{2+}-ATPase couples the inward movement of protons to the phosphorylation of adenosine diphosphate (ADP) (25-28). Thus, during oxidative or photosynthetic phosphorylations, the driving force for the synthesis of ATP is the electrochemical potential difference for the hydrogen ion. This protonmotive force Δp represents the contributions made by both the electric potential difference ($\Delta\psi$) and the chemical potential difference (ΔpH) across the membrane. When expressed in electrical units (millivolts), $\Delta p = \Delta\psi - Z\,\Delta pH$, where Z is equal to 2.3 RT/F and has a value of about 59 mV at 25 C.

One important implication of the chemiosmotic hypothesis is that the electron transport chain and the ATPase may be considered as separate and distinct complexes, systems that are connected only by way of the circulation of protons. Thus, in the absence of oxidative reactions the synthesis of ATP would occur if the necessary protonmotive force was artificially imposed. Experiments that verify this prediction were first performed using chloroplasts (16)

and mitochondria (30) and have recently been reported for bacterial systems as well (8, 21, 22).

The studies reported here lend strong support for the chemiosmotic view of oxidative phosphorylation in *E. coli*. These experiments demonstrate that in the absence of oxidative reactions the membrane-bound Ca^{2+},Mg^{2+}-ATPase of *E. coli* catalyzes the net synthesis of ATP in response to an inwardly directed protonmotive force. By choosing appropriate initial conditions, the relative proportion of the two components of the protonmotive force was manipulated. ATP synthesis was observed whether the protonmotive force was dominated by either a membrane potential (e.g., Fig. 3) or a pH gradient (e.g., Fig. 8). In both instances, net synthesis of ATP was transient. This undoubtedly reflects decay of the protonmotive force over the experimental time period, as has been previously shown for similar experiments using *S. lactis* (21, 22).

Strong evidence supporting the idea that ATP synthesis driven by a protonmotive force is catalyzed by the membrane-bound Ca^{2+}, Mg^{2+}-ATPase comes from the studies reported here, which examined a number of ATPase-negative mutants. Two such mutants were examined in some detail (Fig. 6, 7, and 11; Table 3), since they were representative of two classes of ATPase-negative strains. One (strain AN120) shows normal permeability to protons, whereas the other (strain 72) shows an abnormally high permeability to protons. Both mutant types failed to synthesize ATP in response to either a membrane potential or a pH gradient. Two additional ATPase-negative mutants (strains 45 and DL54) were also unable to form ATP in response to a protonmotive force. When considered in conjunction with the finding that such ATP synthesis was not observed in normal cells exposed to DCCD (Fig. 5 and 10), a chemical inhibitor of the Ca^{2+},Mg^{2+}-ATPase, these results strongly suggest that the membrane-bound Ca^{2+},Mg^{2+}-ATPase couples the synthesis of ATP to the entry of protons.

We have also observed ATP synthesis driven by a protonmotive force in a mutant of *E. coli* SASX76, which lacked functional cytochromes (Fig. 12). These results are of interest for several reasons. They indicate that such experiments can be performed without subjecting cells to possible trauma during depletion or other pretreatments. In addition, these findings appear to exclude the possibility that such ATP synthesis requires interaction (direct or indirect) between the Ca^{2+},Mg^{2+}-ATPase and cytochromes.

Quantitative measurements of the size of the protonmotive force required to drive ATP synthesis suggest a minimum value of approximately 200 mV (Table 3). This corresponds well with that estimated previously for the ATPase of *S. lactis* (21), and compares favorably with the value of 210 mV, calculated by Mitchell (26), which would be required to maintain the ATP/ADP ratio at 1.

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