Adenosine 5'-Triphosphate Synthesis Driven by a Protonmotive Force in Membrane Vesicles of *Escherichia coli*

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Adenosine 5'-triphosphate (ATP) synthesis energized by an artificially imposed protonmotive force (Δp) in adenosine 5'-diphosphate-loaded membrane vesicles of *Escherichia coli* was investigated. The protonmotive force is composed of an artificially imposed pH gradient (ΔpH) or membrane potential (Δψ), or both. A ΔpH was established by a rapid alteration of the pH of the assay medium. A Δψ was created by the establishment of diffusion potential of K⁺ in the presence of valinomycin. The maximal amount of ATP synthesized was 0.4 to 0.5 nmol/mg of membrane protein when energized by a ΔpH and 0.2 to 0.3 nmol/mg of membrane protein when a Δψ was imposed. Simultaneous imposition of both a ΔpH and Δψ resulted in the formation of greater amounts of ATP (0.8 nmol/mg of membrane protein) than with either alone. The amount of ATP synthesized was roughly proportional to the magnitude of the artificially imposed Δp. Although p-chloromercuribenzoate, 2-heptyl-4-hydroxyquinoline-N-oxide, or NaCN each inhibits oxidation of d-lactate, and thus oxidative phosphorylation, none inhibited ATP synthesis driven by an artificially imposed Δp. Membrane vesicles prepared from uncA or uncB strains, which are defective in oxidative phosphorylation, likewise were unable to catalyze ATP synthesis when energy was supplied by an artificially imposed Δp.

Ion-translocating adenosine triphosphatases (ATPases) are ubiquitous in nature. At least three different types have been shown to exist in a wide range of organisms. The Na⁺/K⁺-ATPase, found in many eukaryotic membranes, pumps Na⁺ out of the cell and K⁺ inwards, utilizing directly the energy of adenosine 5'-triphosphate (ATP) (25). The Ca²⁺-ATPase of sarcoplasmic reticulum catalyzes the movement of Ca²⁺ across the membrane with energy derived from ATP hydrolysis (3). In mitochondria and bacteria, the Mg²⁺-ATPase complex (F₀F₁, in the case of the mitochondrial complex; BF₀F₁, for the bacterial) acts as a hydrogen ion translocating system (16). Each of these three ATPases has been shown to act reversibly; that is, they establish ion gradients in one direction with concomitant ATP hydrolysis, and they form ATP when the electrochemical ion gradient is of sufficient magnitude to allow for the formation of a high-energy phosphate bond. In erythrocytes, high external Na⁺ and high internal K⁺ induced the synthesis of ATP from adenosine 5'-diphosphate (ADP) and Pᵢ (4). The reversal of the sarcoplasmic reticular Ca²⁺ pump likewise resulted in ATP synthesis (13). In mitochondria (20), chloroplasts (9), and bacteria (5, 15, 35), a chemical gradient of proton, acid outside, has been shown to induce synthesis of ATP. Likewise, a membrane potential, which is capable of driving protons electrogenically through the F₀F₁, has been shown to elicit ATP formation (2, 14, 24). The demonstration of proton-coupled ATP synthesis lends strong support to Mitchell's proposed chemiosmotic mechanism of energy coupling (16).

We have shown previously that ATP synthesis from ADP and Pᵢ, can be energized by an artificially imposed pH gradient (33) or membrane potential (32) in ADP-loaded membrane vesicles of *Escherichia coli*, prepared by the method of Kaback (10). Oxidative phosphorylation in those vesicles could be driven by the oxidation of substrates such as d-lactate, reduced phenazine methosulfate, or succinate (31). This communication reports further characterization of the coupling of the BF₀F₁ to an artificially imposed protonmotive force. The protonmotive force, Δp, has been defined by Mitchell as the electrochemical proton gradient, composed of an electrical component, the membrane potential, and a chemical component, ΔpH (16). The relationship between these is expressed as Δp = Δψ - 59ΔpH. Thus, the protonmotive force can be composed solely of a membrane potential or solely of a pH gradient,
depending on the circumstances. In this paper, the $\Delta p$ has been formed by application of a $\Delta pH$ alone or by the sum of $\Delta pH$ and $\Delta \psi$.

**MATERIALS AND METHODS**

**Growth of cells.** *E. coli* K-12 strain 7 (6) and its derivative, strain NR70, which lacks the BF, (22), were grown aerobically in a basal salts medium (28), with 68 mM glycerol as a carbon source. Strains AN180 (wild type) and AN120 (uncA) and AN382 (uncB) (1), derivatives of K-12, were obtained from F. Gibson and were grown as described above with a supplement of 0.2 mM l-arginine. All experiments were done with cells in the exponential phase of growth.

**Chemicals.** ADP, ATP, firefly lantern extract (FLE-50), 2-heptyl-4-hydroxyquinoline-N-oxide (HQQNO), valinomycin, and nigericin were purchased from Sigma Chemical Co. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was the generous gift of P. G. Heytler of the E. I. DuPont de Nemours Co. N,N'-dicyclohexylcarbodiimide (DCCD) was obtained from Eastman Kodak Co. The other chemicals used in these studies were reagent grade and purchased from commercial sources.

**Preparation of membrane vesicles.** Membrane vesicles were prepared as described previously (31). Vesicles used for the experiments described in Fig. 2B and 5 were prepared in sodium phosphate buffer, and vesicles used in other experiments were prepared in potassium phosphate buffer. The preparation is essentially that of Kackab (10), in which spheroplasts are prepared by treatment with lysozyme and ethylenediaminetetraacetic acid (EDTA), followed by osmotic lysis. The procedure is modified to allow preloading with ADP (31, 33) or K$^+$ (8, 32). When the vesicles were preloaded with both ADP and K$^+$, ADP-loaded vesicles were incubated with 0.5 M potassium phosphate buffer, pH 7.3, containing 5 mM ADP for 30 min at 40°C. The vesicles were then washed once with a buffer consisting of 10 mM sodium phosphate, pH 8.2, containing 0.28 M sucrose and 5 mM MgCl$_2$.

In the past these vesicles have been termed "right-side-out" (31, 33). However, the recent report of Adler and Rosen (L. W. Adler and B. P. Rosen, J. Bacteriol., submitted for publication) has shown that the vesicles are functionally mosaic. For that reason, the vesicles utilized in these experiments have been termed simply "EDTA-lysozyme vesicles."

**Measurement of ATP synthesis.** Oxidative phosphorylation was assayed with n-lactate (lithium salt) as described previously (31). Phosphorylation driven by artificially imposed pH gradients and membrane potentials were performed as described previously (32, 33). An alternate method of producing a pH jump was performed by preincubation of vesicles for 10 min at 23°C in a buffer consisting of 10 mM potassium phosphate, pH 8.2, containing 0.28 M sucrose and 5 mM MgCl$_2$. The reaction was then initiated by the rapid addition of enough 1 N HCl to lower the external pH to 2.5. Nearly identical results were obtained using either method. It should be pointed out that a white aggregate formed in the vesicle suspension after about 15 s at pH 2.5. This presumably contains denatured vesicles.

As shown previously (32), membrane vesicles contain some ATP bound to the BF. Moreover, commercially obtained ADP contains trace amounts of ATP. These background amounts of ATP have been subtracted from the data, which are expressed as net ATP synthesis.

**Other methods.** Oxygen consumption was measured by a published method (22). ATP levels were determined as described previously (27, 31). Protein concentrations were determined by the method of Lowry et al. (12), using bovine serum albumin as a standard.

**RESULTS**

**Effect of ionophores and permeant ions on $\Delta pH$-driven ATP synthesis.** We have previously reported that a pH gradient artificially imposed across the membrane could drive ATP formation from ADP and P, (33). The electrogenic influx of protons would be expected to establish a membrane potential, interior positive, which could act as a back pressure for the further influx of protons. Thus, the formation of a reverse membrane potential may reduce the overall magnitude of the protonotive force driving ATP synthesis. Dissipation of this membrane potential should lead to higher levels of ATP synthesis. Addition of valinomycin, an ionophore for K$^+$ which mediates translocation of K$^+$ according to the Nerst expression, should reduce the membrane potential established by electrogenic proton influx. Valinomycin stimulated the ATP synthesis energized by a pH gradient about 60% (Fig. 1). On the other hand, nigericin, which mediates an electrically neutral exchange of H$^+$ for K$^+$ and dissipates proton gradients, inhibited proton-coupled synthesis in the presence of potassium (Fig. 1), with about 90% inhibition at 2 $\mu$g of nigericin per ml.

Previously, we reported the optimum pH for ATP synthesis energized by an artificial pH gradient as being about 2.5 in the absence of valinomycin (33). Similar results were obtained when valinomycin was used (data not shown). However, $\Delta p$H-driven ATP synthesis in the presence of valinomycin and potassium has a broader pH range than in the absence of valinomycin. The significance of this observation is not known.

Figure 2 shows the synthesis of ATP driven by a $\Delta p$H in the presence or absence of valinomycin in vesicles prepared with or without K$^+$. Valinomycin stimulated in K$^+$ vesicles (Fig. 2A) but not in Na$^+$ vesicles (Fig. 2B). Valinomycin partially inhibited oxidative phos-
agents such as SCN\(^-\) and ClO\(_4\)- at very high concentration caused an inhibition of lactose transport in EDTA-lysozyme vesicles. This inhibition would be, most likely, caused by the removal of BF\(_3\). The treatment of EDTA-lysozyme vesicles with high concentration of chaotropic reagent makes vesicles more permeable to protons (17). Hasan and Rosen showed that rebinding of BF\(_3\) to everted membrane vesicles treated with chaotropic reagent restores proton-coupled processes in such vesicles (S. M. Hasan and B. P. Rosen, submitted for publication). However, low concentration of such chaotropic reagents did not show the inhibition of active transport in membrane vesicles (17).

![Graph](image)

**FIG. 1.** Effect of valinomycin and nigericin on \(\Delta p\)H-induced ATP synthesis. ADP-loaded membrane vesicles were preincubated in a buffer consisting of 10 mM potassium phosphate, 0.28 M sucrose, and 5 mM MgCl\(_2\) (pH 8.2) at room temperature for 10 min. Valinomycin (○) or nigericin (■) was added at the indicated concentrations. The reaction was initiated by the addition of 10 \(\mu\)l of 1 N HCl to 0.5 ml of reaction mixture. For the measurement of ATP synthesis, samples were taken at 5 s, and the ATP content was determined as described in the text. The control value is 0.30 nmol of ATP per mg of membrane protein.

Phosphorylation in K\(^+\) vesicles (31), but it did not inhibit this process in Na\(^+\) vesicles (data not shown). Similarly, nigericin had no significant effect on either oxidative or \(\Delta p\)H-driven phosphorylation in Na\(^+\) vesicles (data not shown).

It was of interest to test the effect of the other permeant ions on \(\Delta p\)H-driven ATP synthesis. SCN\(^-\) did not show any stimulatory effect at 5 to 20 mM; on the contrary, it strongly inhibited the reaction (Table 1). The use of HClO\(_4\) instead of HCl did not have a significant effect. Even in the presence of ClO\(_4\)-, valinomycin plus K\(^+\) showed a stimulatory effect (data not shown). Perhaps ClO\(_4\)- is not an effective permeant ion in E. coli membranes. The effect of SCN\(^-\) on oxidative phosphorylation was also tested. SCN\(^-\) partially inhibited respiratory-driven phosphorylation at 5 to 20 mM (Table 1). This partial inhibition may be caused by the dissipation of membrane potential, similar to the effect of valinomycin plus K\(^+\) on the oxidative phosphorylation (31). However, if that is the case, then the observed inhibition of \(\Delta p\)H-driven ATP synthesis by SCN\(^-\) cannot be explained, since dissipation of a \(\Delta \psi\) would be expected to stimulate. Thus, the effects of SCN\(^-\) and ClO\(_4\)- require additional investigation.

Patel et al. (17) reported that chaotropic re-

![Graph](image)

**FIG. 2.** Effect of valinomycin on K\(^+\) vesicles and Na\(^+\) vesicles. Membrane vesicles prepared in potassium phosphate buffer (K\(^+\) vesicles) or sodium phosphate buffer (Na\(^+\) vesicles) were loaded with ADP and preincubated in a buffer consisting of either 10 mM potassium phosphate or sodium phosphate, each containing 0.28 M sucrose and 5 mM MgCl\(_2\) at pH 8.2. To initiate the reaction, vesicles were diluted 20-fold into the buffers of the same composition, except that the pH was 2.5. At the time points indicated, samples were taken and ATP content was determined. (A) K\(^+\) vesicles; (B) Na\(^+\) vesicles: control (○), + 2 \(\mu\)g of valinomycin per ml (■).

**TABLE 1.** Effect of SCN\(^-\) on ATP synthesis

<table>
<thead>
<tr>
<th>Addition</th>
<th>(\Delta p)H driven*</th>
<th>(\Delta \psi) driven*</th>
</tr>
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<tbody>
<tr>
<td>HCl</td>
<td>0.45</td>
<td>100%</td>
</tr>
<tr>
<td>HCl + 5 mM</td>
<td>0.04</td>
<td>9%</td>
</tr>
<tr>
<td>KSCN</td>
<td>-0.01</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>HCl + 20 mM</td>
<td>&lt;0.01</td>
<td>0.32%</td>
</tr>
</tbody>
</table>

* HCl was added to the reaction mixture to establish the pH gradient.

\(\Delta \psi\) driven ATP synthesis was initiated by the addition of 20 mM \(\psi\)-lactate.
changing the base stage pH from 5.0 to 8.7. Preparation of the vesicles in buffer of pH less than 5 caused denaturation, whereas use of buffers of pH 9 or above caused precipitation of a salt that was presumably a magnesium phosphate complex. The yield of ATP was roughly proportional to the size of the pH change (Fig. 3). Similar results were obtained with ΔpH-driven Ca\(^{2+}\) transport in everted membrane vesicles (34).

**Effect of electron transport chain inhibitors.** The effect of inhibitors of the electron transport chain on ATP synthesis driven by an artificial ΔpH is shown in Fig. 4. p-Chloromercuribenzoate, HOQNO, and NaCN all inhibited oxygen consumption and oxidative phosphorylation in membrane vesicles when D-lactate was utilized as an energy source. However, these compounds had no significant effect on ΔpH-driven phosphorylation. At higher concentrations, HOQNO caused some inhibition. High concentrations of HOQNO have been reported to inhibit \(\Delta\psi\)-driven proline transport in similar vesicles (8). Thus, HOQNO may have a secondary action on the membrane system in addition to its primary action on the quinone component of the electron transport chain. These effects of respiratory-chain inhibitors support the idea that the respiratory chain is not involved in the phosphorylation energized by an artificial proton flux. We cannot exclude

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**Fig. 3.** ATP synthesis as a function of the magnitude of ΔpH. ADP-loaded membrane vesicles were washed once with buffer consisting of 10 mM potassium phosphate containing 0.28 M sucrose, and 5 mM MgCl\(_2\) at various pH values (pH 5.0, 6.0, 7.0, 8.0, or 8.7) and suspended in the same buffer. After preincubation at 23°C for 10 min, the vesicles were diluted 20-fold into buffer consisting of 10 mM potassium phosphate containing 0.28 M sucrose, 5 mM MgCl\(_2\), and 2 μg of valinomycin per ml (pH 2.5). After 5 s, samples were withdrawn and ATP content was measured.

**Fig. 4.** Effect of inhibitors of the respiratory chain on oxidation, oxidative phosphorylation, and ΔpH-induced phosphorylation. Oxygen consumption (■) and oxidative phosphorylation (▲) were measured as described in the text, with 20 mM D-lactate as an oxidizable substrate. ΔpH-induced phosphorylation (●) was measured as described in the legend to Fig. 1, with 2 μg of valinomycin per ml. p-Chloromercuribenzoate (A), HOQNO (B), or NaCN (C) was added to the assay mixture at the indicated concentration and was preincubated with vesicles for 10 min. The values for the controls were: 82-ng atoms of O/min per mg of protein for oxygen consumption, 1.42 nmol of ATP/min per mg of protein for oxidative phosphorylation, and 0.52 nmol of ATP/8 s per mg of protein for ΔpH-induced phosphorylation.
the possibility that cyanide may not work at very low pH because of the formation and volatility of HCN.

\( \Delta p \)-Driven phosphorylation in BF\textsubscript{0}F\textsubscript{1} mutants. Previously, we reported (31-33) that membrane vesicles from a BF\textsubscript{1} mutant, NR70, are incapable of ATP synthesis when energy is supplied by respiration, a \( \Delta p \text{H} \), or a \( \Delta \psi \). This strain lacks the BF\textsubscript{1}, and its membrane is very permeable to proton (21). Strain AN120 is also a BF\textsubscript{1} mutant lacking ATP hydrolyzing activity. However, this strain is believed to have an inactive BF\textsubscript{1}, so that the membrane is able to maintain normal proton impermeability.

Butlin et al. (1) reported the isolation of an uncB mutant, which lacks ATP-linked membrane processes although it has normal ATP hydrolyzing activity. Other mutant strains that have characteristics similar to those of uncB mutants have been isolated (11, 26). Mutants of the uncB type are most likely defective in the BF\textsubscript{0} or intrinsic membrane protein portion of the ATP synthetase complex.

Membrane vesicles from either AN120 or AN382 failed to synthesize ATP coupled to either respiration or an artificially imposed \( \Delta p \text{H} \) (Table 2). Wilson et al. (35) reported similar results using whole cells of AN120. It is very likely that the BF\textsubscript{0}F\textsubscript{1} of AN382 cannot mediate proton movement, so that an electrochemical proton gradient cannot couple to ATP synthesis, nor can hydrolysis of ATP generate a proton motive force.

Simultaneous imposition of \( \Delta \text{pH} \) and \( \Delta \psi \). Simultaneous imposition of both a \( \Delta \text{pH} \) and a \( \Delta \psi \) has been reported to elicit greater ATP synthesis than either alone in mitochondria (29) and chloroplasts (24). Figure 5 shows ATP synthesis by both a \( \Delta \text{pH} \) and a \( \Delta \psi \), imposed simultaneously, with a maximal level of 0.8 to 0.9 nmol of ATP per mg of membrane protein synthesized. In the absence of a K\textsuperscript{+} gradient across the membrane, valinomycin stimulated the phosphorylation by \( \Delta \text{pH} \) about 60\% (Fig. 1 and 2). In K\textsuperscript{+}-loaded vesicles, the stimulation by valinomycin was more than 100\% at the maximum level (Fig. 5). Although externally added KCl does not significantly inhibit ATP synthesis energized by a \( \Delta \text{pH} \), 20 mM KCl inhibited ATP synthesis energized by the combination of a \( \Delta \text{pH} \) and \( \Delta \psi \) about 30\% (data not shown). As reported previously (32), this concentration of external KCl inhibits \( \Delta \psi \)-driven ATP synthesis almost completely. On the other hand, nigerin markedly inhibited phosphorylation. Thus, under the conditions described in this paper, a \( \Delta \text{pH} \) is more effective than a \( \Delta \psi \) in energizing phosphorylation.

FCCP, a proton conductor, DCCD, an inhibitor of the BF\textsubscript{0}, or NaN\textsubscript{3}, an inhibitor of the BF\textsubscript{1}, all inhibited phosphorylation under the imposition of both a \( \Delta \text{pH} \) and \( \Delta \psi \) (Table 3).

### DISCUSSION

According to Mitchell's chemiosmotic postulate of oxidative and photosynthetic phosphorylation, the membrane-bound F\textsubscript{0}F\textsubscript{1} complex is a reversible electrogenic proton pump. Likewise, respiratory and photoredox chains act as primary proton pumps (16). Most of the experimental evidence supporting the chemiosmotic coupling mechanism have derived from the mitochondrial and chloroplast literature. Some of the more recent data, however, have been ob-

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**TABLE 2. Absence of ATP synthesis in membrane vesicles prepared from uncA and uncB mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \Delta \text{pH} ) driven*</th>
<th>( \Delta \text{lactate} ) driven</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nmol/5 s per mg of protein %</td>
<td>nmol/min per mg of protein %</td>
</tr>
<tr>
<td>AN180</td>
<td>0.30</td>
<td>0.90</td>
</tr>
<tr>
<td>AN120 (uncA\textsuperscript{+})</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AN382 (uncB\textsuperscript{-})</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Reaction was initiated by diluting vesicles into the buffer containing 2 \( \mu g \) of valinomycin per ml at pH 2.5.
tained using bacteria. Scholes et al. (23) reported that proton translocation occurs under the illumination of light or during respiration in both whole cells and membrane vesicles of several species of photosynthetic bacteria. Reeves (19) showed oxidation-driven proton extrusion from EDTA-lysosome membrane vesicles of E. coli. Hertzberg and Hinkle (7) showed respiration- and ATP-driven proton uptake in everted membrane vesicles. Maloney et al. (14, 15) recently reported ATP synthesis energized by an artificially imposed membrane potential or proton gradient in whole cells of Streptococcus lactis. Similar results have been obtained using E. coli cells (5, 35). Recently, an artificially imposed membrane potential (32) or proton gradient (33), in addition to oxidation, was shown to drive the synthesis of ATP in isolated membrane vesicles of E. coli. These results all demonstrate the utility of bacterial systems in the study of bioenergetics. In the system of ATP synthesis driven by an artificial proton motive force, net synthesis of ATP is a transient one. This is reasonable since the protons motive force was a transient one. The artificially imposed proton gradient and potassium gradient dissipate after a short time. If the protons motive force becomes lower than the threshold level, supposedly about 200 mV (15, 35), there would be no further detectable synthesis of ATP. Under the conditions described in this paper, acid hydrolysis of ATP might take place, since the pH of assay mixture is very low. Actually, the decrease of ATP level was observed 20 to 30 s after the pH transition.

The pH optimum for the phosphorylation driven by Δψ in E. coli cells (14) and E. coli vesicles (32) seems to be around 5. The pH transition from 8.2 to 5.0 produced a small amount of ATP (33). The reason for this low level of ATP formation would be that the initial proton motive force, approximately 190 mV, is too small. According to thermodynamic calculations, a force of 210 mV is necessary to obtain an ATP/ADP ratio of 1 with 10 mM phosphate (16). To establish ΔpH of sufficient magnitude we must lower the outer pH, since it is technically very difficult to raise the inner pH.

By lowering the pH of the reaction mixture, we can obtain a larger ΔpH, but the BF₆ complex is relatively unstable at very low pH. Thus, it is very difficult to observe the theoretical ATP/ADP ratio under the conditions used in this study. Thayer and Hinkle studied the kinetics of the artificially driven ATP synthesis by sub mitochondrial particles and observed a rate of ATP synthesis of 4 to 5 nmol/s per mg of protein (29, 30). For this measurement they used a continuous-flow mixing technique, measuring the initial 100 ms. In the experiments described in this report, the earliest time point taken is 5 s, so that true initial rates cannot be calculated. Still, a comparison of their values with ours suggests that the levels of ATP synthesis we observe are of the same order of magnitude.

The effect of respiratory-chain inhibitors on ΔpH-driven phosphorylation demonstrates that there is no direct coupling between the respiratory chain and ATP synthesis. Likewise, Wilson et al. (35) showed ATP synthesis energized by an artificial proton motive force in intact cells of a mutant that lacks functional cytochromes. Such findings eliminate the possibility of the involvement of cytochromes in ATP synthesis. Reconstitution experiments reported by Racker and Stoeckenius (18) and by Yoshida et al. (36), using mitochondrial oligomycin-sensitive ATPase or bacterial DCCD-sensitive ATPase, completely eliminated the possibility of the primary interaction of respiratory components and ATPase in ATP formation.

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LITERATURE CITED

ATP SYNTHESIS IN E. COI VESICLES


