Adenosine 5'-Triphosphate Synthesis Energized by an Artificially Imposed Membrane Potential in Membrane Vesicles of *Escherichia coli*

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Adenosine 5'-triphosphate (ATP) synthesis driven by an artificially imposed membrane potential in right-side-out membrane vesicles of *Escherichia coli* was investigated. Membrane vesicles prepared in the presence of adenosine diphosphate were loaded with K⁺ by incubation with 0.5 M potassium phosphate. Addition of valinomycin resulted in the synthesis of 0.2 to 0.3 nmol of ATP/mg of membrane protein, whereas no synthesis was observed after addition of nigericin. Addition of K⁺, dicyclohexylcarbodiimide, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, or azide to the assay buffer inhibited ATP synthesis. Adenosine diphosphate and Mg²⁺ were found to be required. Ca²⁺, which can replace Mg²⁺ for the hydrolytic activity of the Mg²⁺-adenosine triphosphatase (ATPase) (EC 3.6.1.3), could not replace Mg²⁺ in the synthetic reaction and, in fact, inhibited ATP synthesis even in the presence of Mg²⁺. Strain NR-70, a mutant lacking the Mg²⁺-ATPase, was unable to synthesize ATP using an artificially imposed membrane potential. Additionally, the Mg²⁺-ATPase was found to contain tightly bound ATP.

Mitchell has proposed that an electrochemical gradient of protons across the membrane is the driving force for oxidative phosphorylation (25). In this hypothesis, the electron transport chain acts as a proton pump during the oxidation of substrates. The extrusion of protons generates a membrane potential (negative inside) and a chemical gradient of protons (alkaline inside). The sum of these two components, called the protonmotive force, is the most likely driving force for adenosine 5'-triphosphate (ATP) synthesis in oxidative and photosynthetic phosphorylation (9, 10) and for some active transport systems (3, 10). Moreover, ATP hydrolysis by the membrane-bound Mg²⁺-adenosine triphosphatase (ATPase) (BF₁,F₃) is believed to cause proton translocation across the membrane (13, 36). Thus, the BF₁,F₃ catalyzes a reversible reaction between ATP and the protonmotive force.

Membrane potentials can be established not only by the physiological flux of protons, but also by electrogenic movement of ions across the membrane. A flux of potassium mediated by valinomycin generates a membrane potential and has been shown to cause proton translocation both in whole cells (20) and membrane vesicles (2) of bacteria. An artificially imposed potassium gradient has also been shown to drive ATP synthesis in mitochondria (5), chloroplasts (32), and bacterial cells (24) upon the addition of valinomycin. Similar results have been found for the energization of active transport in bacteria (19) and bacterial membrane vesicles (14).

Bacterial membrane vesicles are a useful model system for the study of energy-linked membrane processes such as active transport (18). Previous attempts to demonstrate ATP synthesis in right-side-out membrane vesicles have been unsuccessful (22, 33). In such experiments, adenosine 5'-diphosphate (ADP) was added to the outside of the vesicles, although the catalytic portion of the Mg²⁺-ATPase (BF₁) is located on the inner surface of the cytoplasmic membrane (6). On the other hand, ATP synthesis has been observed in everted membrane vesicles (4, 13), where the BF₁ is exposed to its substrates.

We have recently shown that right-side-out membrane vesicles are capable of phosphorylating ADP in the presence of oxidizable substrates or an artificially imposed chemical gradient of protons when the membranes are first loaded with ADP (37). In this paper, we report the synthesis of ATP driven by an artificially imposed membrane potential in right-side-out vesicles of *Escherichia coli*.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. *E. coli* stain 7 (12) and its derivative, strain NR-70 (28),...
which lacks the BF₁, were grown in basal salts medium (35) supplemented with 68 mM glycerol as a carbon source. Cultures were harvested in midexponential phase by centrifugation.

Chemicals. ADP, ATP, firefly lantern extract (FLE-50), deoxyribonuclease (DNase), lysozyme, and valinomycin were all purchased from Sigma Chemical Co. ADP and ATP were used as the sodium salts. Dicyclohexylcarbodiimide (DCCD) was purchased from Eastman Kodak Co. Carboxylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was the generous gift of P. G. Heytler of the E. I. Dupont de Nemours Co. Nigericin was the generous gift of L. H. Frank of this department. All other compounds were reagent grade and purchased from commercial sources.

Preparation of ADP-loaded membrane vesicles. Membrane vesicles were prepared by a modification of the procedure of Kack (17). Spheroplasts (1 g, wet weight) were suspended in 1 ml of 0.1 M sodium phosphate buffer (pH 6.6) containing 5% sucrose. The spheroplasts were lysed by dilution with 20 ml of 50 mM sodium phosphate buffer (pH 6.6) containing 5 mM ADP and dispersed by means of a syringe equipped with a 18-gauge needle. EDTA (ethylenediaminetetraacetic acid), MgSO₄, and DNase were added sequentially, and the suspension was shaken at 37°C, all according to the method of Kack (17). Cells and unbroken spheroplasts were removed by centrifugation at 3000 × g for 15 min, and the membrane vesicles were collected as a pellet by centrifugation at 48,000 × g for 30 min.

Preparation of potassium-loaded vesicles. ADP-loaded membrane vesicles were suspended in 10 ml of 0.5 M potassium phosphate (pH 7.3) and incubated for 30 min at 40°C (14). The suspension was then chilled in an ice bath, and MgSO₄ was added to 10 mM, followed by centrifugation at 60,000 × g for 20 min. The vesicles were washed once with 20 mM sodium phosphate (pH 8) containing 0.28 M sucrose and 5 mM MgCl₂, and resuspended in the same solution at a concentration of about 10 to 20 mg of membrane protein per ml, all at 4°C. Vesicles loaded with ADP and potassium phosphate were kept in an ice bath and used within 1 to 2 h of preparation.

ATP synthesis. Synthesis of ATP by membrane vesicles loaded with ADP and potassium phosphate was assayed in a reaction mixture consisting of 20 mM sodium phosphate (pH 4.6), 0.28 M sucrose, 5 mM MgCl₂, and 0.5 to 1.0 mg of membrane protein per ml, unless specified otherwise in the text. After preincubation for 10 min at 23°C, valinomycin was added to start the reaction. Samples (0.1 ml) were withdrawn at various time intervals and immediately diluted with 0.1 ml of cold 12% perchloric acid. ATP content was determined using a firefly lantern luciferin-luciferase assay by a published method (34). Relatively high endogenous levels of ATP in vesicles (0.13 to 0.19 nmol of ATP/mg of membrane protein) were observed. A portion of the endogenous ATP may derive from contamination of the ADP by ATP, or from myokinase activity, which is known to exist in crude firefly lantern extract (34). These possibilities are likely, since the background levels varied according to the ADP concentration used for preloading. In vesicles prepared from spheroplasts, and not loaded with ADP, the ATP level was about 0.12 nmol/mg of protein, compared with the value of about 0.16 nmol/mg of protein in ADP-loaded vesicles. As discussed below, the remainder of the endogenous ATP may be due to ATP bound to the BF₁. All values reported are corrected for endogenous ATP.

Treatment of BF₁-containing fractions with antiserum to the BF₁. Preparation of antiserum to BF₁ has been described previously (36). Everted vesicles, EDTA-treated everted vesicles, and EDTA extract (crude soluble BF₁) were prepared as described previously (30). Crude soluble BF₁ solutions were concentrated to about 1 mg of protein per ml by ultrafiltration (Amicon Corp., Lexington, Mass.).

The following method, devised from a suggestion by Y. Anraku, was used to determine the adenine nucleotide content of BF₁-containing fractions. Various amounts of antiserum against the BF₁ of control serum were added to a crude BF₁ fraction, and kept 90 min at 23°C. After centrifugation at 6,000 × g for 10 min, ATPase activity and ATP content of the supernatant were measured. The precipitate was washed once with a buffer consisting of 1 mM tris(hydroxymethyl)aminomethanehydrochloride (pH 8.0) containing 0.5 mM EDTA, 2 mM mercaptoethanol, and 10% glycerol, and ATP content was measured.

Other methods. Protein concentrations were determined by a modification of the method of Lowry et al. (23). ATPase activity was measured as described previously (36), with a unit defined as the release of 1 μmol of inorganic orthophosphate/min.

RESULTS

ATP synthesis energized by an artificially imposed membrane potential. The addition of valinomycin to membrane vesicles preloaded with both ADP and potassium phosphate resulted in the synthesis of 0.2 to 0.3 nmol of ATP per mg of membrane protein (Fig. 1). The initial rate of ATP synthesis was estimated to be approximately 0.8 to 1.2 nmol/min per mg of protein. Maximal ATP synthesis was observed approximately 30 s after the addition of valinomycin. At later times, ATP hydrolysis occurred. To demonstrate the necessity for electrogentic eflux of potassium as opposed to electroneutral eflux, similar assays were performed with nigericin replacing valinomycin. As can be seen in Fig. 1, nigericin was unable to elicit phosphorylation of ADP. Moreover, an actual gradient of potassium was necessary. When potassium was added to the external medium, ATP synthesis was reduced or eliminated, depending on the external potassium concentration (Fig. 2), demonstrating the necessity for an actual gradient of potassium.

Effect of inhibitors of the Mg₂⁺-ATPase and proton conductors. Table 1 shows the effect of (i) DCCD, which interacts with the membrane binding site of the BF₁, preventing both pro-
ton translocation and ATP hydrolysis; (ii) azide, an inhibitor of the BF; and (iii) FCCP, a proton conductor and uncoupler of oxidative phosphorylation (10). At 50 μM, DCCD inhibited ATP synthesis by about 75%. NaN₃ completely inhibited ATP synthesis at 10 mM. At similar concentrations, azide has been found to inhibit ATPase activity almost completely (21, 27). These results suggest that the observed ATP synthesis was catalyzed by the BF₆F₆.

FCCP likewise was a potent inhibitor of ATP synthesis (Table 1). Since FCCP acts as a proton conductor, it would be expected to provide a channel for protons to enter the vesicles in response to the membrane potential generated by the flow of potassium out of the vesicles. Since this proton flux does not occur via the BF₆F₆, it could not be expected to couple to ATP synthesis, as was observed.

Effect of ADP loading, pH, and divalent cations on ATP synthesis. ATP synthesis driven by a membrane potential was dependent on the loading of ADP within the vesicles (data not shown). Maximal ATP synthesis was observed when the loading concentration was 5 mM or greater. It should be pointed out that the concentrations of ADP in the preparation buffer may not be the actual concentrations of ADP within the vesicles during ATP synthesis.

Some ATP synthesis was observed in vesicles that had not been loaded with ADP. Similar results were found when an artificially imposed proton gradient was used to drive ATP synthesis (37). This suggests the existence of small amounts of ADP within the vesicles.

The optimum pH for ATP synthesis was found to be between 4 and 5 (Fig. 3), similar to the value reported by Maloney et al. (24) for ATP synthesis driven by a proton motive force in whole cells. The combination of a base-to-

![Graph](image1)

**Fig. 1.** ATP synthesis in ADP- and K⁺-loaded vesicles induced by valinomycin. The reaction, measured as described under Materials and Methods, was initiated by addition by either valinomycin (●) or nigericin (○), both at a concentration of 2 μg/ml.

![Graph](image2)

**Fig. 2.** Effect of external potassium ion concentration on valinomycin-induced ATP synthesis. Potassium chloride was added to the incubation mixture 10 min before the addition of 2 μg of valinomycin per ml. Symbols: (●) no KCl; (■) 5 mM KCl; (▲) 20 mM KCl.

**Table 1.** Effect of uncouplers and inhibitors of oxidative phosphorylation on ATP synthesis driven by an artificially imposed membrane potential

<table>
<thead>
<tr>
<th>Expt</th>
<th>Inhibitor *</th>
<th>ATP synthesis (nmol/min per mg protein)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.76</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50 μM DCCD</td>
<td>0.20</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>5 μM FCCP</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0.84</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 mM NaN₃</td>
<td>0.48</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>2 mM NaN₃</td>
<td>0.28</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>5 mM NaN₃</td>
<td>0.12</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10 mM NaN₃</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

* The vesicles were incubated with inhibitor at the indicated concentrations for 15 min at 23 °C before initiation of the assay by the addition of valinomycin.

* Values are the maximal synthesis observed, normalized to 1 min.
The combination does either alone has gradient synthesis vesicles over synthesis of potential membrane fold into buffers transition, acid concentrations varying EDTA, with start of the synthesis Ca\(^{2+}\) with Mg\(^{2+}\) the mixture not (data was shown). To investigate the procedure was modified to remove as much Mg\(^{2+}\) as possible after DNase treatment. When EDTA was used to chelate free Mg\(^{2+}\), followed by washing of the vesicles with Mg\(^{2+}\)-free buffers, ATP synthesis did not occur (data not shown). If Mg\(^{2+}\) was added to the incubation mixture 10 min before the start of the reaction, the resulting activity was about 50% that found with Mg\(^{2+}\) present throughout the preparation (data not shown). When Mg\(^{2+}\) was replaced with Ca\(^{2+}\) under the same conditions, no ATP synthesis was observed (data not shown).

Omission of Mg\(^{2+}\) from the buffer during the washing of the vesicles, but without chelation with EDTA, was by itself sufficient to reduce ATP synthesis by 50% (Fig. 4). Addition of varying concentrations of MgCl\(_2\) to the incubation mixture 10 min before initiation of the assay increased the maximal amount of ATP synthesized, with saturation of MgCl\(_2\) occurring at approximately 5 mM (Fig. 4). Addition of CaCl\(_2\), on the other hand, inhibited ATP production, both in vesicles prepared with 5 mM MgCl\(_2\) and in Mg\(^{2+}\)-depleted vesicles (Fig. 4). This effect is of considerable interest considering that the hydrolytic activity of the BF, is stimulated by either Mg\(^{2+}\) or Ca\(^{2+}\) in vitro.

**ATP synthesis in a BF, deficient mutant.** To confirm the involvement of the Mg\(^{2+}\)-ATPase in ATP synthesis driven by an artificially imposed membrane potential, synthesis was measured in strain NR-70, a derivative of strain 7 lacking the BF,. As shown in Fig. 5, right-side-out vesicles prepared from NR-70 were unable to synthesize ATP using a membrane potential. Similarly, NR-70 was found to be unable to use a proton gradient to drive the phosphorylation of ADP (37).

**Endogenous ATP in membrane vesicles.** As mentioned under Materials and Methods, right-side-out membrane vesicles contain about

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**Fig. 3. Effect of pH on membrane potential-induced ATP synthesis.** Membrane vesicles loaded with ADP and potassium phosphate were diluted 20-fold into buffers at various pH values, and preincubated for 10 min before addition of valinomycin to start the reaction. Values are expressed as described in footnote b of Table 1.

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**Fig. 4. Effect of magnesium ion and calcium ion on ATP synthesis.** Potassium-loaded vesicles were washed with a magnesium-free buffer consisting of 20 mM sodium phosphate (pH 8.0) containing 0.28 M sucrose and resuspended in the same buffer. Various concentrations of MgCl\(_2\) (○) or CaCl\(_2\) (△) were added to an assay mixture consisting of 20 mM sodium phosphate (pH 4.6) containing 0.28 M sucrose and vesicles. After 10 min of preincubation, valinomycin was added. In one experiment, vesicles were prepared with MgCl\(_2\) exactly as described under Materials and Methods, and CaCl\(_2\) was added to the assay mixture at the concentrations indicated on the abscissa (●). In this case, the assay mixture also contained 5 mM MgCl\(_2\). Values are expressed as described in footnote b of Table 1.
0.1 to 0.2 nmol of ATP/mg of membrane protein. As shown in Table 2, a similar value was found for everted membranes, prepared by lysis of cells with a French pressure cell. Treatment of everted vesicles with EDTA solubilized the ATP. It is possible that the ATP is bound to the BF₁, since the ratio of ATP contents between the EDTA extract and control membranes (ratio = 3.7; calculated from Table 1) and the ratio of ATPase activities between the two (ratio = 3.9) were very close. The Mg²⁺-ATPase of other organisms has been shown to contain tightly bound ATP (1, 11). If the enzyme would be approximately 2:1, if we assume that the molecular weight of the BF₁ is 300,000 (8) and the specific activity of the purified enzyme is approximately 100 U/mg of protein (8, 36).

Recently, Anraku and his co-workers have developed a method to determine the ATP content of the Mg²⁺-ATPase (Y. Anraku, personal communication; M. Maeda, H. Kobayashi, M. Futai, and Y. Anraku, Biochem. Biophys. Res. Commun., in press). To determine the exact molar contents of adenine nucleotides bound to the BF₁, they used antiserum against the BF₁ to specifically precipitate that enzyme. According to their results, the BF₁ of E. coli contains about 2 molecules of ATP and 0.5 molecule of ADP per molecule of BF₁. We have likewise found that the ATP observed in membrane vesicles and in crude BF₁ fractions is bound to this enzyme. Figure 6 shows that antiserum against BF₁ precipitates nearly all the ATP in the crude BF₁ fraction. Control serum did not precipitate any ATP from the BF₁ fraction. The amount of antiserum sufficient to precipitate most of the ATP was also sufficient to remove nearly all ATPase activity from the supernatant fraction after centrifugation at 6000 × g. These results indicate that the ATP which exists in the crude fraction is bound to the BF₁ itself.

**DISCUSSION**

The use of isolated membrane vesicles for the study of membrane-related phenomena has many advantages over the use of whole cells for the study of transport processes. One of the most advantageous aspects of the use of vesicles is the absence of cellular metabolism and metabolic pools. Thus, the development of a system using only isolated membranes for the study of

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**Figure 5.** Membrane potential-induced ATP synthesis by vesicles of strain 7 and strain NR-70. Symbols: (●) strain 7; (○) strain NR-70.

**Figure 6.** Precipitation of ATP present in the BF₁ fraction by antiserum against the BF₁. The BF₁ fraction (EDTA extract) was treated with antiserum as described under Materials and Methods. Symbols: (●) Mg²⁺-ATPase activity in supernatant fraction after precipitation with antiserum; (△) ATP content in supernatant fraction after precipitation with antiserum; (○) ATP content in the immune precipitate; (■) ATP content in supernatant fraction after treatment with control serum. Ordinate expresses percentage of ATP or activity present before addition of serum.

**Table 2.** Endogenous ATP content of membrane vesicles and EDTA-soluble fraction

<table>
<thead>
<tr>
<th>Source</th>
<th>ATP content (nmol/mg protein)</th>
<th>Mg²⁺-ATPase activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Everted vesicles</td>
<td>0.21</td>
<td>2.5</td>
</tr>
<tr>
<td>EDTA-treated</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>everted vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA-soluble</td>
<td>0.77</td>
<td>9.7</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ATP synthesis would be of value in determining the mechanism of this process.

The major difficulty with measuring ATP synthesis in right-side-out vesicles is the inaccessibility of the BF,F, to its substrate, ADP, since the catalytic site of that complex is located primarily on the inner surface of the cytoplasmic membrane (6). Kaback (16) has reported a procedure for loading right-side-out vesicles with phosphorylated substrates. Futai (7) modified this method in an elegant demonstration that nicotinamide adenine dinucleotide, reduced form, was capable of driving transport reactions when generated inside of the vesicles. Our modifications of these procedures have allowed for the preparation of right-side-out vesicles containing ADP within the intravesicular space.

As we have shown previously (37) and in this report, right-side-out membrane vesicles loaded with ADP are capable of synthesizing ATP utilizing energy derived from a proton gradient or membrane potential. The amount of ATP synthesized upon the application of a membrane potential was dependent on the concentration of ADP present during the loading procedure. The maximal observed synthesis was about 0.2 to 0.3 nmol/mg of protein. If we assume an intravesicular space of 1 μl/mg of protein (14), these values correspond to ATP concentrations of 0.2 to 0.3 mM. Maloney et al. (24) reported a value of about 1 mM in whole cells of E. coli. Thus, our values compare favorably with the in vivo synthesis of ATP.

More important is the fact that our data demonstrate the involvement of a membrane potential in ATP synthesis, where the membrane potential is derived from a potassium gradient in the absence of cellular metabolism. Although much of the evidence supporting the importance of a chemiosmotic mechanism in active transport has come from studies of bacterial systems (14, 19, 20), the involvement of such a mechanism in ATP synthesis has been supported mainly through the use of mitochondria (5, 26) and chloroplasts (15, 32) of eukaryotic organisms. Our intention is to demonstrate the utility of bacterial systems in the study of ATP synthesis.

It is clear from our data that ATP synthesis in right-side-out vesicles is catalyzed by the same enzyme as is used physiologically for oxidative phosphorylation, the BF,F, complex, since (i) ATP synthesis was sensitive to BF,F, inhibitors DCCD and azide and anó (ii) membrane vesicles from a BF,-deficient mutant were unable to catalyze phosphorylation of ADP. It is also clear that ATP synthesis is related to proton fluxes, as predicted by the chemiosmotic hypothesis (25). When ATP synthesis was driven by K+ efflux, the amount of ATP produced was proportional to the magnitude of the K+ gradient (Fig. 2). The effects of nigericin (Fig. 1) and FCCP (Table I) support the concept of proton movement being involved in ATP synthesis. More directly, we have shown that ATP synthesis can be driven by a proton gradient alone in right-side-out vesicles of E. coli (37), just as Jagendorf and Uribe (15) showed for chloroplasts.

Ca2+ has been shown to be able to replace Mg2+ in the hydrolytic activity of the BF, (21). But in the system reported in this communication, Ca2+ not only did not substitute for Mg2+, but was actually antagonistic, inhibiting ATP synthesis in the presence of Mg2+. It may be that Ca2+ stimulates the Mg2+-ATPase when the enzyme acts in its hydrolytic mode but inhibits during its synthetic mode. More likely, we believe, is the possibility that the potential is utilized for calcium transport, reducing the amount of energy available for ATP synthesis. Although calcium is normally transported into everted vesicles but not right-side-out vesicles (29), we have observed calcium accumulation in both everted and right-side-out vesicles concomitant with K+ efflux (unpublished data). Inhibition of ATP synthesis during calcium transport has been observed in mitochondria (39).

It has been stated repeatedly, entirely from negative data, that the energy for active transport in membrane vesicles of E. coli could not be derived from ATP hydrolysis (18). Yet, it is clear that ATP hydrolysis is an effective source of energy for active transport in whole cells (3). We have previously shown that the calcium transport system in everted membrane vesicles of E. coli can be energized by ATP hydrolysis (29). Although ATP-driven transport has not yet been observed in right-side-out membrane vesicles, the ability to load the vesicles with ADP suggests that it may be possible to load with ATP. If the appropriate conditions can be found, the question of the ability of ATP hydrolysis to drive active transport may be resolved.

We reported that calcium transport could be driven by a proton gradient in membrane vesicles (38). Hirata et al. have shown that a membrane potential could drive the transport of amino acids and sugars in membrane vesicles (14). Previously, we reported the phosphorylation of ADP induced by a proton gradient (37). In this paper, we have reported the synthesis of ATP by a membrane potential. Thus a proton-motive force, as proposed by Mitchell, has been
shown to drive both transport and ATP synthesis in membrane vesicles of *E. coli* in the absence of cellular metabolism.

**ACKNOWLEDGMENTS**

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