JOURNAL OF BACTERIOLOGY, Nov. 1980, p. 830-832
0021-9193/80/11-0830/03$02.00/0

Vol. 144, No. 2

Adenosine 5'-Triphosphate Synthesis Induced by Urea Hydrolysis in *Ureaplasma urealyticum*

NINO ROMANO,[1]* GIUSEPPE TOLONE,[2] FRANCESCA AJELLO,[1] AND ROSARIA LA LICATA[1]

Instituto di Igiene[1] and Instituto di Patologia Generale,[2] Palermo, Italy

Although considerable attention has been devoted to the urea-hydrolyzing activity of *Ureaplasma urealyticum*, there is as yet no firmly established function for this enzyme. Present results support the idea that its activity generates a chemical gradient across the membrane which drives adenosine 5'-triphosphate synthesis through a chemiosmotic type of mechanism.

The parasitic mycoplasmas are characterized by a truncate electron transport chain lacking quinones and cytochromes (5), which rules out oxidative phosphorylation as a major ATP-generating system in these organisms. The only proven ways so far for ATP generation in mycoplasmas are those based on substrate-level phosphorylation. In fermentative mycoplasmas, ATP is formed during glycolysis (8), whereas in nonfermentative mycoplasmas, the arginine dihydrolase pathway has been proposed as the major source of ATP (7). The possibility that ATP may be generated in some mycoplasmas, such as the ureaplasmas, through the formation of an ion gradient coupled to urea hydrolysis has recently been suggested (3). This hypothesis stems from the localization of urease and ATPase in the cytoplasm and membrane, respectively, of ureaplasmas (3, 6). At physiological pH, urea is uncharged and presumably permeates the cell membrane freely. When hydrolyzed inside the cell, it yields CO_2 and ammonia, which, at physiological pH, accepts a proton, becoming the ammonium ion (NH_4^+). It is supposed (3) that charged ammonium ions diffuse very slowly across the membrane, producing an ion gradient and a membrane potential which drives ATP formation through membrane-bound ATPase. We have subjected this hypothesis to experimental control by testing whether urease activity in ureaplasmas is actually coupled to ATP formation.

The strain of *Ureaplasma urealyticum* studied was P 108. The organisms were grown, harvested as previously reported (6), washed twice with 0.1 M sodium phosphate (pH 6.0), and suspended in a small volume of the same buffer. Protein was determined by the method of Lowry et al. (2). ATP was measured by use of firefly extract by the procedure of Cole et al. (1). Urease activity was assayed by the disappearance of [^{14}C]urea (60 mCi/mmol; Radiochemical Centre, Amersham, England) from the reaction mixture (6) and was not inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD), carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), valinomycin, nigericin, triphenylmethylphosphonium ion, or acetic acid. Washed *Ureaplasma* cells had no detectable intracellular ATP.

As shown in Fig. 1, the addition of urea to resting *Ureaplasma* cells resulted in its hydrolysis and in a rapid increase in the intracellular level of ATP, followed by a somewhat slower decline. The ATP peak level was attained after 60 s, and within 3 to 4 min, ATP concentration returned to the starting level, whereas urease activity still proceeded at a maximal rate. ATP formation requires the concomitant activity of both cytoplasmic urease and membrane-bound ATPase. Thus, in cells exposed to either DCCD, a specific inhibitor of ATPase, or acetohydroxamic acid, a specific inhibitor of urease, urea-induced ATP formation was almost completely suppressed (Fig. 2A and B).

According to the chemiosmotic hypothesis (4), the synthesis of ATP occurs only when there is a concomitant movement of protons into the cells via ATPase. Therefore, ATP synthesis is expected to be blocked or reduced by conditions that provide alternate routes for proton entry. In agreement with this prediction, it was found that the treatment of *Ureaplasma* cells with CCCP, which renders membranes highly permeable to protons, resulted in a consistent reduction of urea-induced ATP formation (Fig. 2C).

Since the proton motive force consists of an electrical component ($\Delta\psi$) and a chemical component (ΔpH), both of which can energize ATP synthesis, we investigated the role of each in urea-induced ATP formation by ureaplasmas. The electrical component can be studied with valinomycin, which mediates the electrogenic movement of K^+. In the presence of this substance, a countercurrent of potassium ions neutralizes the membrane potential without interfering with the pH gradient. Thus, if urea-in-

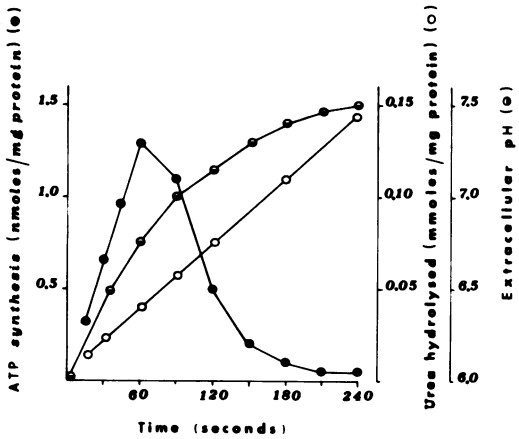

FIG. 1. *Effect of urea hydrolysis on ATP synthesis by U. urealyticum. Cells were washed, suspended in 0.1 M sodium phosphate (pH 6.0) at a final concentration of 500 μg of protein ml⁻¹, and incubated at 37°C. After samples were removed for measurement of zero-time ATP levels, urea (83.3 mM final concentration) was added. At the indicated times, samples were removed for the determination of ATP concentrations* (●), *urea hydrolysis* (○), *and extracellular pH* (◒).

duced ATP formation depends on a membrane potential produced by NH_4^+ diffusion across the membrane, the addition of valinomycin in the presence of a large amount of external K^+ should maintain the membrane potential of *Ureaplasma* cells at a low value, thereby blocking or reducing ATP synthesis. That is not the case, since urea-induced ATP formation by *Ureaplasma* cells exposed to 20 μM valinomycin in the presence of 100 mM external K^+ attained the same levels as in control (unexposed) cells. The same results were obtained by exposing *Ureaplasma* cells to 10 mM triphenylmethylphosphonium ion, a lipid-soluble cation whose uptake occurs in response to a $\Delta\psi$ (data not shown).

In contrast, severe reduction in ATP synthesis was observed when urea-hydrolyzing cells were incubated at higher pH, a condition which is expected to reduce the chemical potential difference across the membrane generated by ammonia production inside the cell. By raising the external pH from 6.0 to 7.5, ATP synthesis was almost completely suppressed in spite of a slight initial enhancement in urease activity (Fig. 3A). This result indicates that urea-induced ATP synthesis is very sensitive to the ratio of internal to external hydrogen ion concentration and that its optimum is attained when the external pH is rather acidic. By assuming that ATP synthesis is driven by a ΔpH, it should be expected that a

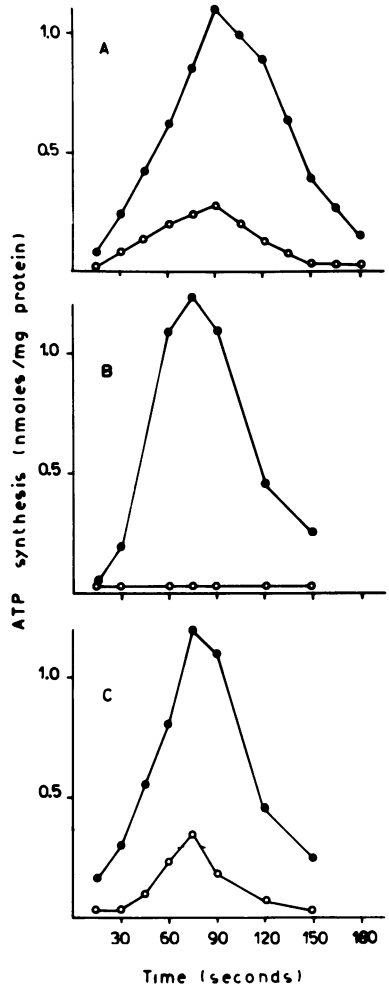

FIG. 2. *Effect of an ATPase inhibitor (DCCD), a urease inhibitor (acetohydroxamic acid), or a proton-conducting ionophore (CCCP) on urea-induced ATP synthesis by U. urealyticum. Ureaplasma cells (500 μg of protein ml⁻¹) were suspended in 0.1 M sodium phosphate (pH 6.0) and incubated at 37°C. After samples were removed for measurement of zero-time ATP levels, DCCD (0.1 mM final concentration) (A), acetohydroxamic acid (50 mM final concentration) (B), or CCCP (0.66 mM final concentration) (C) were added. DCCD-treated cells were kept at room temperature for 30 min before testing. Reaction was started by addition of urea (83.3 mM final concentration). At the indicated times, samples were removed for determination of ATP concentrations. DCCD and CCCP were added from solutions containing, respectively, ethanol and dimethyl sulfoxide so that the final concentration of both solvents was 0.1%. Control cells were treated in the same way, except that a corresponding volume of ethanol or dimethyl sulfoxide was used. Symbols:* ●, *control cells;* ○, *treated cells.*

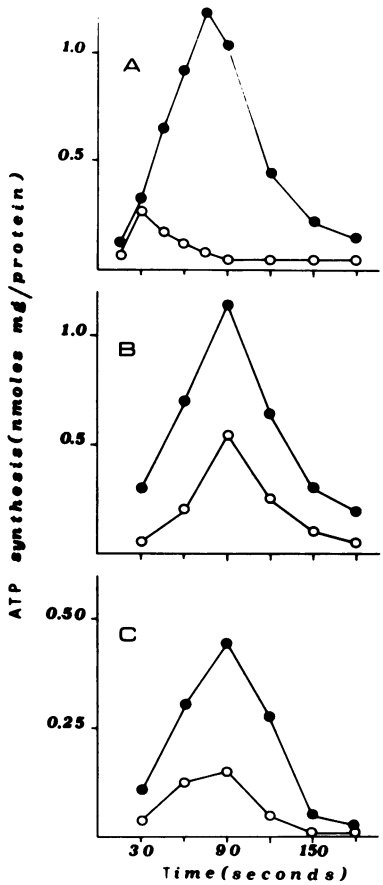

FIG. 3. *Influence of ΔpH on urea-induced ATP synthesis by U. urealyticum. (A) Effect of external pH. Cells (500 μg of protein ml^{-1}) were suspended in 0.1 M sodium phosphate at pH 6.0 (●) or pH 7.5 (○). (B) Effect of acetic acid. Cells (500 μg of protein ml^{-1}) were suspended in 0.1 M sodium phosphate (pH 6.0) with (○) or without (●) 200 mM acetic acid (final concentration). (C) Effect of nigericin. Ureaplasma cells were washed twice in 250 mM KCl containing 25 μM valinomycin (final concentration) and suspended in 0.1 M sodium phosphate (pH 6.0) and 20 mM acetohydroxamic acid and supplemented with (○) or without (●) 20 μM nigericin (final concentration). Valinomycin and nigericin were both in ethanol solution. The mixtures were incubated at 37°C, and the reaction was started by addition of urea (83.3 mM final concentration).*

weak acid which crosses the membrane in the uncharged form and accumulates in response to the pH (inside alkaline) will lower the chemical gradient and reduce ATP synthesis. Evidence supporting this statement comes from the finding that ureaplasmas exposed to 200 mM acetic acid underwent a 50% reduction in ATP synthesis (Fig. 3B). Additional evidence for linking ATP synthesis to ΔpH was obtained by the use of nigericin, which mediates electrically neutral K^+/H^+ antiport. As shown in Fig. 3C, *Ureaplasma* cells preloaded with K^+ and incubated with acetohydroxamic acid showed a significant reduction in ATP synthesis when exposed to 20 μM nigericin. Due to the high content of ammonia generated inside the cells after urea hydrolysis (about 0.08 mmol/mg of protein per min), reduction of urease activity by acetohydroxamic acid was required for observing nigericin effects.

Taken together, these results suggest that the chemical potential is the major driving force for ATP synthesis in urea-hydrolyzing *Ureaplasma* cells. It can be hypothesized that after urea hydrolysis, the intracellular pH increases sharply, creating a chemical potential difference across the membrane which enables the entry of protons via membrane-bound ATPase. This would also give an explanation for the finding that urea-hydrolyzing cells show only a transient increase in ATP levels. The rapid diffusion of ammonia outside the cell, by raising the external pH (Fig. 1), would reduce the chemical gradient and lower the proton motive force. This, in turn, would depress ATP levels since these are determined by the balance between synthetic and degradative reactions.

We thank S. Razin, V. P. Cirillo, and F. Azzone for helpful discussion, and R. L. Hamill of the Lilly Research Laboratories for the generous gift of nigericin.

LITERATURE CITED

1. **Cole, H. A., J. W. T. Wimpenny, and D. E. Hughes.** 1967. The ATP pool in *Escherichia coli*. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta **143**:445–453.
2. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
3. **Masover, G. K., S. Razin, and L. Hayflick.** 1977. Localization of enzymes in *Ureaplasma urealyticum* (T-strain mycoplasma). J. Bacteriol. **130**:297–302.
4. **Mitchell, P.** 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature (London) **191**:144–148.
5. **Razin, S.** 1973. Physiology of mycoplasmas. Adv. Microb. Physiol. **10**:1–80.
6. **Romano, N., and R. La Licata.** 1978. Cell fractions and enzymatic activities of *Ureaplasma urealyticum*. J. Bacteriol. **136**:833–838.
7. **Schimke, R. T., C. M. Berlin, E. W. Sweeney, and W. R. Caroll.** 1966. The generation of energy by the arginine dihydrolase pathway in *Mycoplasma hominis*. J. Biol. Chem. **241**:2228–2236.
8. **Smith, P. F.** 1971. The biology of mycoplasmas. Academic Press, Inc., New York.