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Hydrolysis of Urea by *Ureaplasma urealyticum* Generates a Transmembrane Potential with Resultant ATP Synthesis

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When urea is added to *Ureaplasma urealyticum*, it is hydrolysed internally by a cytosolic urease. Under our measuring conditions, and at an external pH of 6.0, urea hydrolysis caused an ammonia chemical potential equivalent to almost 80 mV and, simultaneously, an increase in proton electrochemical potential (Δp) of about 24 mV with resultant de novo ATP synthesis. Inhibition of the urease with the potent inhibitor flurofamide abolished both the chemical potential and the increase of Δp such that ATP synthesis was reduced to ~5% of normally obtained levels. Uncouplers of electrochemical gradients had little or no effect on these systems. The electrochemical parameters and ATP synthesis were measured similarly at three other external pH values. Any change in Δp was primarily via membrane potential ($\Delta\psi$), and the level of de novo ATP synthesis was related to the increase in Δp generated upon addition of urea and more closely to the ammonia chemical potential. Although the organisms lack an effective mechanism for internal pH homeostasis, they maintained a constant ΔpH. The data reported are consistent with, and give evidence for, the direct involvement of a chemiosmotic mechanism in the generation of around 95% of the ATP by this organism. Furthermore, the data suggest that the ATP-generating system is coupled to urea hydrolysis by the cytosolic urease via an ammonia chemical potential.

Ureaplasma urealyticum is a small, wall-less, free-living prokaryote which has been associated with infections of the human urogenital tract (2) and, more recently, with disease of preterm low-birth-weight neonates (4). The organism possesses enough genetic material for around 500 gene products (11), and it requires a very complex medium to support growth. Since it has no cytochromes and apparently lacks quinones (17), oxidative phosphorylation does not occur or is severely limited; it also lacks enzymes to utilize a number of substrates (15, 16, 27) and has an incomplete spectrum of tricarboxylic acid cycle enzymes (3). Thus, many mechanisms of substrate-level phosphorylation can also be ruled out.

Although one investigation has shown that an F_0F_1 proton-translocating ATPase is conserved in mycoplasmas (30), *U. urealyticum* was not included in that study. Others have shown, however, that the organism has membrane-bound ATPase with similarities to the F_0F_1 ATPases of other bacteria (22), and it has been reported that ATP determination represents a reliable and accurate means of measuring growth (28). It is therefore attractive to propose that an F_0F_1 membrane ATPase in *U. urealyticum* plays an important role in energy generation. Such a proposal could rely upon a chemiosmotic mechanism (10), as suggested elsewhere (9), whereby generation of a transmembrane electrochemical potential would provide the electromotive force for protons to enter the cell via the F_0F_1 ATPase to generate ATP. Such electrochemical potentials may also be utilized in transport processes. It has been suggested that since *U. urealyticum* has a potent cytosolic urease, urea hydrolysis to ammonium ions and carbon dioxide generates a transmembrane potential which drives ATP synthesis (9). While it has been reported that urea and urease activities are essential for ATP synthesis (19–21), the precise nature of any interrelationship remained uncertain.

In this study, we have examined the influence of urea hydrolysis upon electrochemical parameters and the influence of these parameters upon ATP synthesis.

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MATERIALS AND METHODS

Ureaplasma strain. *U. urealyticum*, serotype 8 (T960), was a gift of D. Taylor-Robinson (Clinical Research Centre, Harrow, England).

Chemicals. Radiolabelled chemicals were obtained from Du Pont, U.K., Ltd., Soluscint O and Solusol were from National Diagnostics, flurofamide was a gift of I. Kahane (Hebrew University, Hadassah Medical School, Jerusalem, Israel), and, except where stated, all other chemicals were from Sigma.

Cell culture and harvest. *U. urealyticum* was cultured (500-ml cultures) and harvested as described previously (18). After cell harvest, the pellets obtained by centrifugation (25,000 × *g*, 20 min) were washed with 0.25 M NaCl, and the final pellets were resuspended in an appropriate buffer (see below) at ~1 mg of protein ml^{-1} (estimated by the method of Lowry et al. [8]). On the basis of the determination of color-changing units per milliliter (18), no apparent alteration in viability of the cells was apparent after this treatment.

Determination of intracellular volume. After washing, the pellet was suspended in 150 mM choline chloride–80 mM Na_2SO_4–50 mM MES [2-(*N*-morpholino) ethanesulfonic acid] buffer (pH 6.0) containing tritiated water (1.0 μCi ml^{-1}; specific activity of stock, 2.5 mCi ml^{-1}) or tritiated inulin (1.0 μCi ml^{-1}; specific activity of stock, 100 mCi g^{-1}). Aliquots of suspension (200 μl) were layered onto 100 μl of silicone oil (DC 550 [BDH]; bis-3,5,5-trimethyl hexyl phthalate [Fluka], 60:40 [vol/vol]) in Eppendorf tubes (in triplicate), incubated (37°C, 15 min), and then centrifuged (12,000 × *g*, 5 min). Aliquots (100 μl) of aqueous phase were

removed and added to scintillant (Soluscint O–Triton X-100, 2:1 [vol/vol]), and the remaining pellets were solubilized in Solusol and placed in a scintillation vial to which scintillant was then added (Soluscint O–Triton X-100, 9:1 [vol/vol]). The internal water volume was calculated as described previously (14).

Measurement of membrane potential ($\Delta\psi$) and transmembrane pH difference (ΔpH). Washed cell pellets were suspended in buffer (150 mM choline chloride, 80 mM Na_2SO_4, and either 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] or 50 mM MES). Buffer with MES was adjusted to pH 5.5, 6.0, or 6.5, and buffer with HEPES was adjusted to pH 7.0 with 1 M NaOH. Aliquots of the suspension (100 μl) were added to Eppendorf tubes containing 100 μl of silicone oil overlaid with 100 μl of buffer containing radiolabelled probe and, where required, urea to give a final concentration of 10 mM. $\Delta\psi$ was monitored by using tritiated tetraphenyl phosphonium bromide (0.1 μCi ml^{-1}; concentration in assay, 2.2×10^{-12} mol ml^{-1}), and ΔpH was monitored by using a variety of probes (tritiated acetic acid, 0.4 μCi ml^{-1} [concentration in assay, 1×10^{-7} mol ml^{-1}]; [^{14}C]chloroacetic acid, 0.2 μCi ml^{-1} [concentration in assay, 0.6×10^{-7} mol ml^{-1}]; [^{14}C]methylamine, 1.0 μCi ml^{-1} [concentration in assay, 2.6×10^{-7} mol ml^{-1}]; or [^{14}C]ethanolamine, 1.0 μCi ml^{-1} [concentration in assay, 2.1×10^{-7} mol ml^{-1}]). Suspensions with probes were incubated (37°C, 30 s) and then centrifuged as described above. The aqueous phase and cell pellet were treated and radioactivity was determined as described before. Corrections for the nonspecific binding of probes to cellular components were carried out by inclusion of controls with suspensions of cells lysed by sonication (three times, 10 s each time, on ice; MSE Soniprep 150) at the same protein concentrations (mg ml^{-1}). These controls contained no viable cells upon culture, and we had demonstrated previously by electron microscopy (data not shown) that sonicated cells were disrupted. They typically bound ~10% of the total radioactivity of viable cells in the assays. Each time, assays were carried out in triplicate. Calculations of $\Delta\psi$ and ΔpH were described elsewhere (23).

Measurement of internal and external ammonia (NH_3 and NH_4^+) concentrations. The basis of the method for measuring internal and external ammonia concentrations was essentially the same as that used for determination of $\Delta\psi$ and ΔpH, and again, on each occasion, the assay was carried out in triplicate. To an Eppendorf tube containing 100 μl of silicone oil layered over 50 μl of trichloroacetic acid (TCA; 15% [wt/vol]), first 100 μl of ureaplasma suspension in buffer was added, and then 100 μl of buffer containing 20 mM urea was added. After incubation (37°C, 30 s), the tubes were centrifuged as described above. Under these conditions, ureaplasmas were pelleted through the oil into the TCA and lysed, enzymes were inactivated, and intracellular ammonia was released. Aliquots (10 μl) of the upper aqueous phase were removed and added to 90 μl of solution A (0.5 M NaOH, 3.3% [wt/vol] TCA) for measurement of external ammonia. After the remainder of the aqueous phase and the oil were carefully aspirated, aliquots (20 μl) of the lower TCA phase were removed and added to 80 μl of 0.56 M NaOH for measurement of internal ammonia. Thus, all samples contained a final concentration of 0.45 M NaOH and 3% (wt/vol) TCA. This procedure was necessary because we found that the Bertholet assay for determination of ammonia was pH sensitive (data not shown). The Bertholet assays were carried out in a microtiter plate, and, to each well, 50 μl of phenol nitroprusside and 50 μl of alkaline hypochlorite were added (Sigma urea nitrogen colorimetric kit for the Bertholet reaction). After incubation (20°C, 20 min), A_{600}s were determined (Titertek Multiscan). Ammonia concentrations were determined from a standard curve prepared similarly from appropriate concentrations of NH_4Cl.

Determination of intracellular ATP. Measurements of intracellular ATP were carried out in duplicate by using ureaplasma cell pellets suspended in MES or HEPES buffer as described above but containing 2 mM $MgCl_2$ and 10 mM NaH_2PO_4. Assays were performed in the presence and absence of exogenous 40 mM urea at external pH values of 5.5, 6.0, 6.5, and 7.0. In addition, assays were performed at an external pH of 6.0 in the presence and absence of exogenous 40 mM urea and in the presence of a range of concentrations of uncouplers which included those usually employed against other bacteria (200 μM 2,4-dinitrophenol [DNP]; 200 μM gramicidin; 10.0, 40.0, 160.0, and 640.0 μM carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]; or urease inhibitor [flurofamide, 25 μM] or the F_0F_1 ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide [DCCD, 0.01 and 0.001 mM]). Appropriate ureaplasma suspensions were incubated (37°C, 30 s [except where stated]) and then adjusted to 10% (vol/vol) with respect to TCA. After dilution (1:50 [vol/vol] in 100 mM Tris-acetate [pH 7.5] containing 2 mM EDTA), ATP was measured by using a luciferin-luciferase kit (Bio-Orbit) on a model 1250 luminometer (Bio-Orbit). ATP was calculated by using standard ATP solutions and expressed as nanomoles of ATP per milligram of cell protein.

RESULTS

Determination of intracellular water volume. Under our assay conditions, the internal volume of *U. urealyticum* was 3.8 ± 0.2 μl mg of cell $protein^{-1}$. This is comparable to reported volumes of 1.6, 2.5, and 4.8 μl mg of cell $protein^{-1}$ for *Mycoplasma gallisepticum*, *Acholeplasma laidlawii*, and *Mycoplasma mycoides* var. capri, respectively (6, 23, 24). This value was then used in the calculation of internal concentrations of probes and of ammonia.

$\Delta\psi$ and ΔpH determinations. Throughout the assays, all ureaplasma cells appeared to pass through the silicone oil mixture. There was no visual evidence of residual material in the aqueous phase, and no evidence of viable cells could be found in the aqueous phase by culture. At the density of the oil mixture used, even membranes would be expected to pass through the oil. Neither methylamine nor ethanolamine (accumulated by cells with a more acidic internal pH than the external environment) was accumulated by ureaplasmas at any of the four external pH values investigated. Ethanolamine was also used as a control in case methylamine could be transported by an ammonia carrier. This indicated that the internal cellular pH was higher than that of the external buffers used. Confirmation of this was obtained by uptake of acetic acid and chloroacetic acid at each external pH, giving reproducible ΔpH values over a large number of repeat assays, each carried out in triplicate. Results (Table 1) show that both $\Delta\psi$ and ΔpH values not only varied with external pH but also were elevated in cells catabolizing urea. These effects are discussed later. Under these circumstances, the values of $\Delta\psi$ rose between an external pH of 5.5 to 6.5 and then fell at pH 7.0. The differential in $\Delta\psi$ values with and without added urea, however, was greatest at external pH 6.0, showing decreased values below and above this pH. Conversely, the values for ΔpH in these cells displayed a narrower range and were raised only minimally in the presence of urea. Assays carried out with 100 mM phosphate

TABLE 1. Determinations of ureaplasma Δψ and ΔpH in the presence and absence of exogenous 40 mM urea and in the presence of 40 mM urea plus 25 μM flurofamide at four external pH values

External pH	Δψ (mV)[a]			ΔpH (mV)[a]		
	A[b]	B	C	A	B	C
5.5	50.0 ± 0.2	46.9 ± 0.1	49.7 ± 0.5	52.7 ± 0.4	53.6 ± 0.2	52.6 ± 0.2
6.0	33.5 ± 0.1	53.6 ± 0.3	33.4 ± 0.1	53.2 ± 0.3	56.8 ± 0.3	53.2 ± 0.4
6.5	62.0 ± 0.4	66.0 ± 0.5	61.8 ± 0.4	47.1 ± 0.2	50.3 ± 0.3	47.1 ± 0.5
7.0	57.5 ± 0.3	63.5 ± 0.4	57.4 ± 0.3	47.3 ± 0.2	49.3 ± 0.3	47.4 ± 0.2

[a] Values are means ± standard errors of the means.
[b] A, in the absence of urea; B, in the presence of exogenous 40 mM urea; C, in the presence of 40 mM urea plus 25 μM flurofamide.

buffer, as applied elsewhere (19, 21), gave similar values within the standard error. Inclusion of uncouplers (CCCP, DNP, gramicidin) over a range of concentrations, or of uncoupler solvent (ethanol), generally failed to alter the values obtained for either Δψ or for ΔpH with or without added urea (Table 2). This was peculiar to the ureaplasmas since the ionophores were active in parallel assays with a strain of *Escherichia coli* (data not shown). A significant reduction in Δψ values was seen, however, with higher concentrations (160.0 and 640.0 μM) of the proton ionophore CCCP, although at these concentrations its effect may be nonspecific. On the other hand, inclusion in the assays of the potent urease inhibitor flurofamide (5) at 25 μM totally abolished any increase in either Δψ or ΔpH observed in the presence of exogenous urea (Tables 1 and 2). This showed that at least the increased Δψ and ΔpH values observed in the presence of urea, compared with those in its absence, were directly related to urease activity.

Internal pH of *U. urealyticum*. From the values of ΔpH, the intracellular pH can be calculated. This was shown to vary directly with extracellular pH and found to be 0.78 to 0.95 pH unit higher than the pH of the external buffer. This finding was reproducible in many assays and suggests that, like other mycoplasmas (1, 7), *U. urealyticum* lacks an effective mechanism of internal pH homeostasis but maintains a relatively constant ΔpH.

TABLE 2. Δψ and de novo ATP synthesis determined for *U. urealyticum*, at an external pH of 6.0, in the presence and absence of uncouplers, or the urease inhibitor flurofamide, or the F_0F_1 ATPase inhibitor DCCD[a]

Uncoupler or inhibitor	Δψ (mV)[b]	ATP synthesized (nmol mg of cell protein^{-1})[c]
None (control)	−53.8 ± 0.4	9.84 ± 0.27
Ethanol (inhibitor or ionophore solvent)	−54.2 ± 0.2	9.84 ± 0.05
Flurofamide (25 μM)	−33.5 ± 0.4	0.49 ± 0.02
CCCP		
10 μM	−53.7 ± 0.3	9.84 ± 0.26
40 μM	−53.6 ± 0.5	6.55 ± 0.29
160 μM	−44.8 ± 0.4	5.33 ± 0.23
640 μM	−42.8 ± 0.2	1.70 ± 0.10
Gramicidin		
50 μM	−53.2 ± 0.5	ND[d]
200 μM	−53.0 ± 0.4	9.81 ± 0.29
DNP		
50 μM	−53.7 ± 0.3	ND
200 μM	−53.8 ± 0.2	9.77 ± 0.25
1,000 μM	−52.9 ± 0.4	ND
DCCD		
0.01 mM	ND	3.34 ± 0.40
0.001 mM	ND	4.72 ± 0.61

[a] De novo ATP synthesis was measured in the presence of 40 mM urea minus the value determined in the absence of added urea. The assay period was 30 s. In the presence of DCCD, cells were incubated for 3 min at 20°C prior to the addition of urea for the assay of ATP.
[b] Values are means ± standard errors of the means.
[c] Values are means, with ranges indicated.
[d] ND, not done.

Transmembrane chemical potential of ammonia. The concentrations of ammonia internally and externally were measured during urea hydrolysis under the same conditions (30-s incubation) as those used for the measurement of Δψ and ΔpH (see Materials and Methods). Although the internal ammonia concentrations varied with external pH under the conditions applied, the external ammonia concentration was 3.1 ± 0.1 mM irrespective of external pH or of internal ammonia concentration. A concentration difference of ammonia (inside to outside the cells) of ~21-fold, equivalent to 78.9 mV (calculated as described previously [14]), was seen at an external pH of 6.0. At external pH values of 5.5, 6.5, and 7.0, the values were equivalent to 69.9, 74.2, and 58.6 mV, respectively. These differences were abolished if the urease inhibitor flurofamide was added at a concentration of 25 μM in the assays.

Intracellular ATP synthesis. A time course study of ATP synthesis was carried out at an external pH of 6.0. A minimum assay period of 30 s was used to correlate these data with electrochemical data (see Discussion). The results (Table 3) indicated that a 30-s incubation gave maximal values over the time course investigated, that any endogenous urea in the cells was rapidly depleted, and that with a 30-s incubation, ATP synthesis from endogenous urea hydrolysis and/or substrate-level phosphorylation made a contribution of only ~5% to the values determined in the presence of exogenous urea. The decline in ATP values determined with time of incubation in both the presence and

TABLE 3. Time course of ATP production by *U. urealyticum* at an external pH of 6.0 in the presence and absence of exogenous 40 mM urea[a]

Assay incubation time (s)	ATP produced (nmol mg of cell protein^{-1})[b]	
	+Urea	−Urea
30	10.40 ± 0.37	1.00 ± 0.02
60	8.40 ± 0.34	0.69 ± 0.02
120	7.61 ± 0.25	ND[c]
180	4.80 ± 0.20	ND
240	3.79 ± 0.19	ND

[a] At time zero, cells were added to urea-containing or to non-urea-containing buffer.
[b] Values are means, with ranges indicated.
[c] ND, not detectable.

TABLE 4. De novo synthesis of ATP by *U. urealyticum* in the presence of exogenous 40 mM urea at four external pH values[a]

External pH	ATP synthesized (nmol/mg of cell protein)[b]
5.5	6.35 ± 0.33
6.0	9.54 ± 0.40
6.5	6.10 ± 0.27
7.0	4.78 ± 0.18

[a] De novo ATP synthesis is the value determined in the presence of 40 mM urea minus the resting ATP level measured in the absence of urea.
[b] Values are means, with ranges indicated.

absence of exogenous urea presumably is a consequence of the rate of ATP utilization being greater than the rate of synthesis as the available urea decreases through hydrolysis and as the ammonia chemical potential dissipates.

De novo ATP synthesis occurred at all four external pH values in the presence of exogenous urea (Table 4). The amount of ATP measured is consistent with the electrochemical data, in particular, with the magnitude of the differentials with respect to Δp in the presence and absence of urea (Table 5) and the ammonia chemical potential.

The effect of uncouplers on ATP synthesis at an external pH of 6.0 (Table 2) paralleled the effect of the same uncouplers on measured $\Delta\psi$ (and hence Δp) values. Synthesis of ATP was, however, reduced to ~5% of its maximum value when 25 μM flurofamide was included in the assays. The F_0F_1 ATPase inhibitor DCCD also significantly inhibited ATP synthesis (Table 2).

DISCUSSION

It has been suggested that the concomitant activity of ureaplasma urease and an ATPase must occur to permit ATP synthesis (21). Others have hypothesized that urea hydrolysis produces an electrochemical gradient to generate ATP by a chemiosmotic mechanism (9, 21). In these experiments, we have attempted to unravel the mechanism(s) which may play a role in such a system.

All electrochemical parameters were determined by using iso-osmotic buffers and careful sedimentation and suspension because of the known osmotic fragility of these cells. With these precautions, variations in both $\Delta\psi$ and ΔpH values were minimized. Moreover, for the measurement of $\Delta\psi$ and ΔpH, it is necessary to maintain a stable external pH. Although ammonia is released extracellularly by ureaplasmas after hydrolysis of urea by cytosolic urease, no increase in external pH was measurable during the 30-s period of the assays. The parameters were all maximal at ~30 s after the addition of urea, the minimum assay period possible with our protocol. This reflects the pulsed nature of the experimental system and may correlate with the in vivo situation where not only would cells receive an intermittent nutrient supply but also released metabolites would be quickly dissipated.

TABLE 5. Determination of ureaplasma proton electrochemical potential (Δp) in the presence and absence of exogenous 40 mM urea and in the presence of 40 mM urea plus 25 μM flurofamide at four external pH values[a]

External pH	Δp (mV)			Differential Δp(B−A)
	No urea (A)	+40 mM urea (B)	+25 μM flurofamide	
5.5	102.7	100.5	101.7	−2.2
6.0	86.7	110.4	86.6	23.7
6.5	109.1	116.3	108.9	7.2
7.0	104.8	112.8	104.8	8.0

[a] Values are means.

The data showed that the addition of urea to viable ureaplasmas produced an increase in both $\Delta\psi$ and ΔpH. The reported increase in Δp may be too low to account for the ~3 mM concentration increase in ATP determined. However, since the relationship between Δp and ATP synthesis is nonlinear, this rise may increase the energization of ureaplasmas sufficiently to initiate ATP synthesis dependent upon atypical H^+- or monovalent cation$^+$-to-ATP stoichiometry as observed in other microorganisms. In addition, the Δp values determined in the absence of urea suggest generation by urea-independent "housekeeping" metabolic processes. Variation in these Δp values with changing external pH suggests that these processes are pH dependent and may reflect an energy requirement for maintenance of cellular viability under suboptimal conditions. Although very difficult to prove experimentally, we suggest that urea hydrolysis down-regulates some or all housekeeping processes (perhaps in a pH-dependent manner) either directly by urea or via the ammonia chemical potential. If this was the case, the magnitude of Δp determined in the presence of exogenous urea and not the differential in Δp values determined (in the presence or absence of urea) would be expected to relate closely to the urease activity of the cells, which is indeed the case to some extent. The fact that Δp (in the presence of urea) does not follow precisely the de novo ATP synthesis may suggest that energy is still required for housekeeping systems at suboptimal pH values for growth. ATP synthesis, however, correlated to some extent with the differential in Δp (in the presence or absence of urea; see below). The predominant component in the elevation of Δp values is $\Delta\psi$, which contrasts with previous observations that ΔpH is the more significant factor (19, 21).

These data extend a previous report that gave qualitative information that the addition of urea to a suspension of *U. urealyticum* increased membrane potential (19). The increase in both $\Delta\psi$ and ΔpH was totally abolished by the addition of flurofamide, thus demonstrating that urea hydrolysis is, at a minimum, related to the increase in Δp.

Since we (13) and others have shown that the urease is entirely cytosolic and since only whole cells were used in our short assays, our studies show that cytosolic urea hydrolysis resulted in a concentration gradient of ammonia with an internal concentration up to 21-fold greater than the external concentration. This suggests that passive diffusion of ammonia is limited. We have reported that some of the ammonia is utilized in citrulline synthesis (26). The most logical source of at least the increase in Δp is by conversion of the ammonia concentration difference into a chemical potential, i.e., the ammonia chemical potential would equate approximately to the whole of the $\Delta\psi$ and not just to the increase in $\Delta\psi$ observed in the presence of urea. Such a conversion would require that the membrane is permeable to ammonia, as NH_4^+, via a saturatable uniporter (which would thus be a relatively slow process). It was noted that the external ammonia concentration remained constant despite variation of the internal ammonia concentration after the 30-s incubation at each pH. The question then arises that if the membrane has a finite permeability for NH_4^+ ions, why is an uncoupling cycle not set up with ammonia? This would be unlikely because the ratio of concentration of ammonia to

NH_4^+ ions will be very low in the assays, and, at the relatively acidic pH for optimum growth (25), to uncouple, ammonia would have to reenter the cell against a high concentration gradient induced by urea hydrolysis. Although as the external pH increases, dissipation of the ammonia chemical potential by passive diffusion of ammonia could increase, such a situation should have a minimal effect (which cannot be calculated at present because the overall kinetics of the system remain to be clarified) at the external pH values used in our assays.

Although effective in abolishing electrochemical gradients in other mycoplasmas (1, 7) and in our parallel studies with *E. coli*, several ionophores and uncouplers failed to affect either $\Delta\psi$ or ΔpH in *U. urealyticum*. There are several possible explanations for the inefficacy of these compounds. (i) The large flux of ammonia anticipated could swamp their effect. They would not be expected to affect ammonia chemical potential, but they should abolish Δp (proton) by equating $\Delta\psi$ and ΔpH terms to be equal and opposite. (ii) Ureaplasma membranes may be less susceptible as a result of a peculiar (yet-unknown) structure associated with the organisms being wall-less. (iii) A cation(s) other than protons is involved in the energy transduction system. Or, (iv) that the ion-to-ATP stoichiometry is greater than that usually observed. The answer to these questions is the basis of future studies, particularly with other cations in combination with other ionophores.

Higher concentrations of CCCP were effective in giving some reduction in $\Delta\psi$ and in ATP synthesis, and this is in agreement with other data. Thus, other workers (19, 21) reported a 75% reduction in ATP synthesis in the presence of 660 μM CCCP (a concentration at which CCCP may lose specificity and which could be inhibitory to other metabolic processes). This is consistent with only a small drop in Δp, assuming that Δp is the driving force for ATP synthesis, since, as stated, the relationship is nonlinear.

In contrast to the relative inefficacy of ionophores, inhibitors of both urease (flurofamide) and of F_0F_1 ATPase (DCCD) were effective in reducing ATP synthesis. When viewed along with the inhibition of ammonia chemical potential and the inhibition of the rise in Δp by flurofamide, inhibition of ATP synthesis indicates that hydrolysis of urea is the central factor in the synthesis of most ATP by ureaplasmas and that the two processes are most likely coupled by a chemiosmotic mechanism which may not conform exactly to the standard system. In fact, over the pH range investigated, a closer correlation was observed between the ammonia chemical potential, urease activity, and ATP synthesis than between Δp (in the presence or absence of urea), urease activity, and ATP synthesis. This further supports the interrelationship between urease activity and ATP synthesis via ammonia chemical potential. Whether the ATP synthesis is directed by end-product (NH_4^+) chemical potential or via a counter-ion-dependent ATPase remains to be elucidated. The observed synthesis of ATP by the ATPase does not appear to be directly stimulated by ammonium ions alone. It has been shown (21) that NH_4^+ ions do not stimulate ATP hydrolysis, and, in this study (data not shown), NH_4^+ ions had no effect on ATP synthesis; thus, any mechanism would appear to be chemiosmotic. The F_0F_1 ATPase inhibitor DCCD inhibited only 67% of ATP synthesis, and this may well be due to slow uptake and reaction of DCCD during the relatively short preincubation period. Nevertheless, the inhibiting effect of DCCD appears to confirm that ureaplasmas possess an ATP synthetase analogous to F_0F_1 ATPase.

The internal pH of ureaplasmas was always ~0.85 pH unit higher than the external pH, in agreement with similar findings for other mycoplasmas (1, 7). However, internal pH does not depend significantly on ammonia since, in the presence of urea, only a small increase in internal pH was seen. This small increase most likely occurs as a result of scalar H^+ consumption as a consequence of urease action.

The influence of external pH on ATP synthesis in ureaplasmas has been reported (20), and the data presented here are in broad agreement. In our study, it was noteworthy that the effects of exogenous urea, ionophores, and flurofamide on ATP synthesis mirrored their effects on the measurement of electrochemical parameters. Although exogenous orthophosphate was included in the assays to potentiate ATP synthesis, this was not necessary since ATP synthesis was observed in its absence (data not shown). This is despite a report (20) that its presence was essential. In addition, use of phosphate buffer or of the standard buffer plus phosphate produced values for electrochemical parameters similar to those obtained with the standard buffer alone.

Throughout the work presented, it is our contention that any exogenous urea would be hydrolyzed within the short assay periods used. We had previously reported (18) that at its optimum pH, purified ureaplasma urease is ~100 times more active than jack bean urease. Assuming the known urease content as a proportion of total cellular protein (12), the maximum amount of urea used in the assays would, under optimal conditions, be hydrolyzed in ~7.5 s. Even assuming suboptimal urea hydrolysis at the various pH values used, all added urea should be completely hydrolyzed well within the 30-s assay period.

With respect to ureaplasmas, it is of interest that the pH of the urogenital tract is on the acid side of neutrality (29), similar to the pH values for optimum growth (25), maximum increase in Δp, maximum ammonia chemical potential, maximum urease activity, and maximum ATP synthesis. This may provide an explanation for the preferred host sites for colonization.

Overall, the data presented indicate that urease activity generates an ammonia chemical potential with concomitant increases in both Δp and ATP. Each of these were pH dependent and maximal at an external pH of 6.0, the optimal pH for ureaplasma growth. All were inhibited by flurofamide, supporting a link between these factors and confirming the hypothesis of Masover et al. (9) of a chemiosmotic mechanism for ATP generation in *U. urealyticum* resulting from urea hydrolysis. There are also indications that the metabolic processes of these simple organisms may be more complex than previously believed and that ureaplasmas differ significantly from other bacteria in this respect. However, the nature of the cation(s) species involved, whether an ammonia porter exists and whether the entire electrochemical potential generated during urea catabolism is due to an ammonia chemical potential, will form the basis of future work.

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