Energetics of *Helicobacter pylori* and Its Implications for the Mechanism of Urease-Dependent Acid Tolerance at pH 1

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In the presence of urea the neutrophilic human pathogen *Helicobacter pylori* survives for several hours at pH 1 with concomitant cytoplasmic pH homeostasis. To study this effect in detail, the transmembrane proton motive force and cytoplasmic urease activity of *H. pylori* were determined at various pH values. In the absence of urea, the organism maintained a close-to-neutral cytoplasm and an internally negative membrane potential at external pH values greater than 4 to 5. In the presence of urea, *H. pylori* accomplished cytoplasmic pH homeostasis down to an external pH of 1.2. At this external pH, the cytoplasmic pH was 4.9 and the membrane potential was slightly negative inside. The latter finding is in contrast to the situation in acidophiles, which develop inside-positive membrane potentials under similar conditions. Measurements of the time course of the membrane potential confirmed that addition of urea to the cells led to hyperpolarization. Most likely, this effect was due to electrogenic export of ammonium cations from the cytoplasm. The urease activity of intact cells increased nearly exponentially with decreasing external pH. This activation was not due to enhanced gene expression at low external pH values. In cell extracts the pH optimum of urease activity was dependent on the buffer system and was about pH 5 in sodium citrate buffer. Since this is the cytoplasmic pH of the cells at pH 1 to 2, we propose that cytoplasmic pH is a factor in the in vivo activation of the urease at low external pH values. The mechanism by which urease activity leads to cytoplasmic pH homeostasis in *H. pylori* is discussed.

As a neutrophilic bacterium capable of growth at pH values of >5.5 (21), *Helicobacter pylori* is unique with respect to its acid tolerance and long-term persistence in the human stomach. Mechanisms enabling *H. pylori* to cope with fluctuating pH must be essential, particularly during primary infection, in order to overcome the gastric acid barrier. Recently, we have shown that in the presence of urea and without any previous adaptation, growing cells of *H. pylori* are capable of survival and cytoplasmic pH (pH_{cyt}) homeostasis for several hours after a shift of the medium pH (pH_{out}) to pH 1 (30), a physiologically relevant condition frequently found in the gastric lumen. At this pH, acidophiles exhibit a positive inside membrane potential (ΔΨ) (2, 22, 33). For *H. pylori*, the sign and value of ΔΨ at low pH_{out} values is still a matter of debate, and data are lacking for pH_{out} values of <3. An inside positive ΔΨ has been reported at pH 3, which was, however, not sensitive to addition of a protonophore (14). In other studies ΔΨ remained negative inside down to a pH of 3 (28). Because of these conflicting data, we reexamined the ΔΨ of *H. pylori* cells at low pH_{out} values and extended the study to pH 1 to 2. We observed that in the presence of urea ΔΨ remained inside negative at all pH_{out} values between 1.2 and 7. Therefore, we propose that this phenomenon is associated with the electrogenic export of ammonium cations from the cytoplasm.

Urease is a virulence factor of *H. pylori*, and this enzyme is thought to confer acid resistance to *H. pylori* by cleavage of urea and elevation of the microenvironmental pH (12). However, the mechanism by which urease contributes to survival under acidic conditions is highly controversial. Originally, it was assumed that the enzyme activity is extracytoplasmic and that in the stomach protection occurs due to the creation of a cloud of ammonia around the cells (9). This hypothesis, that external urease activity protects *H. pylori* from acid stress, was recently put forward again, based on the observation that in a nonstirred solution urease exhibits some residual activity at pH 3 (8). However, it has been shown that urease is cytoplasmatic (28) and that the urea porter UreI influences urease activity by mediating acid-triggered urea uptake (24, 27, 36). According to the authors of these papers the NH₃ product of the urease reaction leaves the cytoplasm in its neutral form and neutralizes the periplasmic pH by binding protons in that environment. Finally, our preliminary data suggest that urease activity leads to cytoplasmic rather than periplasmic pH homeostasis of *H. pylori* cells and that this process is sufficient for survival at pH 1 (30). The results of the experiments reported here fully support this notion, and a hypothesis for the mechanism of this process (30, 37) is discussed.

A further controversial issue concerns the pH optimum of the *H. pylori* urease. In cell extracts diluted with citrate-phosphate buffer the enzyme exhibits a pH optimum of 7.4 (28). In whole cells urease activity is maximal at a low pH_{out} (28). This phenomenon is attributed to a controlled urea supply determined by acid activation of UreI. However, at a pH_{out} of 3 urease activity in the cytoplasm is expected to be severely inhibited, since under these conditions the pH_{cyt} is only 5.5 to 5.7 (30), a value substantially different from the pH optimum of the urease (28). The urease activities of *Yersinia enterocolitica* and *Morganella morganii* have a pH optimum of 5.5 in citrate-based buffers (37). Moreover, the activity of the urease from *Klebsiella aerogenes* has been reported to be inhibited by acid forms of phosphate (31). These conflicting data led us to...
reexamine the pH optimum of urease activity in *H. pylori* cell extracts. Various buffer systems were used, since it is known that at acidic pH values the pH optimum of an enzyme may depend strongly on the buffer used (15). We observed that in sodium citrate buffer the pH optimum of *H. pylori* urease is around pH 5. This finding was combined with the results of determinations of pHin and cytoplasmic urease activity of cells suspended at pHout values between 1.2 and 7. We concluded that lowering the pHin to values around 5 after an acid shift of *H. pylori* cells may contribute to the activation of urease activity observed under these conditions.

**MATERIALS AND METHODS**

**Strain and growth conditions.** *H. pylori* wild-type strain DSM 4867 was cultivated as described before (30).

**Buffers.** Citrate-phosphate buffers were prepared at 37°C as described previously (16, 18). For pH 3 to 7 buffers 100 mM citric acid and 200 mM Na2HPO4 were mixed to obtain the appropriate pH. For the pH 1 buffer, the pH of 100 mM citric acid was adjusted with concentrated HCl. For pH 2, the 100 mM citric acid buffer was used without any further addition. Buffer containing 100 mM MES [2-(N-morpholino)ethanesulfonic acid] or 100 mM citric acid was titrated with concentrated NaOH to obtain the appropriate pH. Sodium phosphate buffer was composed of a mixture of 100 mM Na2HPO4 and 100 mM NaH2PO4.

**Urease activity.** The in vivo urease activity of *H. pylori* cells was monitored by determining the decrease in the urea concentration in APM (acid-precipitated medium) prepared at 5,400 × g for 10 min at 5°C, separated from the medium by centrifugation in a 12-ml conical polypropylene centrifuge tube (Sarstedt) through a 0.2-μm filter. After centrifugation, the supernatant was determined as described by Rahmatullah and Boyde (23). Subsequently, samples were thawed in a sonication bath for 3 min and centrifuged at 16,000 × g for 5 min, the urease activity in the supernatant was determined by measuring the released ammonia by the phenol-hypochlorite assay (35). The assay was started by addition of 1 mg of protein in 1 ml of buffer supplemented with 10 mM urea. After 5, 10, and 15 min of incubation at 37°C, a 20- to 40-μl aliquot was added to 400 μl of 100 mM sodium phosphate buffer (pH 7.4) plus 200 μl of 3% phenol-0.003% sodium nitroprusside; this was followed by addition of 200 μl of 2% NaOH-0.05% NaClO. After incubation for 45 min in the dark at room temperature, absorption was determined at 635 nm. NH3 was used as a standard.

**Protein concentration determination.** Protein concentrations in cell extracts were determined by the method of Bradford (5) by using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) and bovine serum albumin as a standard.

**Determination of pHin.** For the acid shock experiments, cells were first collected by centrifugation (10 min, 5,000 × g, 37°C), resuspended to an optical density at 578 nm (OD578) of about 2 in 150 mM NaCl, and diluted 10-fold with 20 mM Tris-HCl (pH 8.0). The cell pellet was frozen in liquid N2. After thawing, the cells were resuspended in 500 μl of 20 mM Tris-HCl (pH 8.0) and sonicated three times (30 s each) on ice by using a microtip (Branson cell disrupter B 15; output control 3, 50% pulsed). After centrifugation at 16,000 × g for 5 min, the urease activity in the supernatant was determined by measuring the released ammonia by the phenol-hypochlorite assay (35). The assay was started by addition of 1 mg of protein in 1 ml of buffer supplemented with 10 mM urea. After 5, 10, and 15 min of incubation at 37°C, a 20- to 40-μl aliquot was added to 400 μl of 100 mM sodium phosphate buffer (pH 7.4) plus 200 μl of 3% phenol-0.003% sodium nitroprusside; this was followed by addition of 200 μl of 2% NaOH-0.05% NaClO. After incubation for 45 min in the dark at room temperature, absorption was determined at 635 nm. NH3 was used as a standard.

**Determining the pHin in the presence of urea.** Figure 1 shows the profiles for pHin (Fig. 1A and B) and ΔpH (Fig. 1C) of *H. pylori* cells suspended in citrate-phosphate buffers having pH values between 1 and 7.2. The data were obtained by measuring the pH values in the same sample and using the exponential mean method (38). These changes in pHout were taken into account for calculation of all data. Figure 1A shows that the apparent pHin of urea-degrading cells decreased from 7.5 to 5.4 when the pHout decreased from 7.2 to 1.2. At a pHout of 2 the apparent pHin (pH 5.5) was the same as the value previously determined after 1 h of incubation in the same buffer (30), indicating that after 8 min of acid stress a steady-state pHin was reached. In the presence of 1 mM 2,4-DNP (a protonophore) at a low pHout, the apparent pHin decreased to around 4 (Fig. 1A). A residual pHin of 4 has also been reported for nonenergized acidophilic organisms (19, 39) and has been attributed to several phenomena (13, 19), including nonspecific binding of the radiochemical to cellular components (11, 19). Support for the notion that the latter phenomenon also occurs in *H. pylori* comes from the observation...
that permeabilization of the cells by treatment with 2% n-butanol gave values virtually identical to those obtained after treatment with 1 mM 2,4-DNP (data not shown). By using data obtained in the presence of 2,4-DNP, pH in values were corrected for binding by the exponential mean method (Fig. 1B) (38). At pH in values of 1.2 and 2.4, a corrected pH in of 4.9 was maintained with urea, whereas the pH in was 5.2 for a pH out of 3.3. At pH out values of >4 and in the presence of urea, ΔpH decreased continuously to zero at a neutral pH out (Fig. 1C).

**pH in the absence of urea.** In the absence of urea, pH out remained constant regardless of the starting pH of the buffer used. At pH out values of ≥4 the cells maintained an alkaline inside ΔpH across the cytoplasmic membrane, indicating that they performed pH homeostasis (Fig. 1). In contrast, at pH out values between 1 and 3 the cells did not accomplish pH homeostasis, since the pH in was close to that of cells treated either with the protonophore 2,4-DNP (Fig. 1A) or with n-butanol (data not shown). Hence, these cells did not maintain a ΔpH across the cytoplasmic membrane at pH in values of <4 (Fig. 1C). At pH out values of 4 to 7, the pH in was lower in the presence of urea than in its absence. Possible explanations for this effect are discussed below.

Taken together, the data in Fig. 1 indicate that urease activity is essential for pH homeostasis at pH out values of >4 and that at pH out values of >4, at which urease activity apparently was not essential for the pathogen, the pH in increased gradually from 5.8 to 7.5 with increasing pH out, irrespective of the presence of urea.

**Continuous fluorimetric determination of ΔΨ.** The potentiometric fluorescent probe DiSC3(5) can be used for qualitative, continuous detection of ΔΨ of H. pylori (18, 28). This cationic fluorophore accumulates on hyperpolarized membranes and is translocated into the lipid bilayer, resulting in a decrease in fluorescence and absorption shifts (6). At pH 7, addition of H. pylori cells (final OD 578, ~0.03) led to a 75% quenching of fluorescence intensity caused by uptake of the fluorescent dye driven by ΔΨ (Fig. 2A). Addition of 5 mM urea resulted in a marginal further decrease in fluorescence (i.e., increase in ΔΨ). The ΔΨ was stable until its collapse was induced by the addition of 150 mM TCS, a protonophore. For determination of ΔΨ of H. pylori cells at lower pH out values, the fluorometric method turned out to have limited value. At pH 3, addition of urea did result in a significant quenching of fluorescence, suggesting that there was hyperpolarization (internally negative) (Fig. 2B). However, fluorescence intensity was not stable and increased with time to a value hardly lower than that prior to addition of urea, whereas the pH out remained constant at the low cell density used. Collapse of ΔΨ was implemented with 2% butanol, since TSC was not suitable for uncoupling the cells at a pH of <4 (data not shown). n-Butanol caused only a small further increase in fluorescence intensity. Similar results were obtained at pH out values of 4 and 5, whereas at pH out values of 1 to 2 no reproducible data were obtained (results not shown). As a control, cells of the acidophilic gram-positive bacterium Alicyclobacillus acidocaldarius were used, which do not possess significant urease activity. Addition of urea to these cells at a pH of 3 had no effect on ΔΨ when the fluorimetric method was used (results not shown). Taken together, these data confirm that addition of urea to H. pylori cells at a low pH out leads to rapid and significant hyperpolarization (28). We discuss this effect below.

**Determination of ΔΨ via distribution of the lipophilic ions TPP+ and SCN−.** A more quantitative analysis of ΔΨ was performed by using the radioactive lipophilic ions [14C]TPP+ and S35SCN− (19, 25, 33). The principle for this measurement is the movement of a permeable ion across the membrane in

**FIG. 1.** pH in of H. pylori measured after 8 min of incubation in citrate-phosphate buffer at the appropriate pH out. (A) Apparent pH in; (B) pH in after correction for binding as described by Zaritsky et al. (38); (C) ΔpH (corrected values). Open circles, cells in the absence of urea; solid circles, cells in the presence of 20 mM urea; gray circles, cells with urea and 1 mM 2,4-DNP. The ratio of accumulation of 14C in the cells to accumulation of 14C in the medium [(14C)in/(14C)out] was 280 ± 67 for pH 1 in the presence of urea, while it was only 10 ± 1 at pH 1 in the presence of urea and 2,4-DNP. At pH 7 [(14C)in/(14C)out] was around 2 ± 0.5, irrespective of the presence of urea or 2,4-DNP, indicating that the pH in, approximately matched the pH out.
response to $\Delta \Psi$ until electrochemical equilibrium is established. An additional advantage over the fluorimetric method is the possibility of obtaining simultaneous measurements for several samples, which is especially important for *H. pylori* as aging of cells rapidly leads to a loss of viability.

Figure 3 shows that when $[\text{14C}]\text{TPP}^+$ was the $\Delta \Psi$ probe, *H. pylori* maintained a negative internal $\Delta \Psi$ in the presence of urea over the whole pH out range used, pH 1 to 7. Binding of the radiochemical was estimated after addition of $n$-butanol, which permeabilizes cells. By using the data obtained, corrected $\Delta \Psi$ values were calculated (Fig. 3B) (38). At a pH out of 1, $\Delta \Psi$ was $-26 \text{ mV}$. $\Delta \Psi$ increased to $-64 \text{ mV}$ at a pH out of 3 (Fig. 3B), remained approximately constant at pH out values between 3 and 5, and increased further to $-140 \text{ mV}$ at a pH out of 7. In the absence of urea, $\Delta \Psi$ was close to zero at pH out values between 1 and 5. At pH out values of $>5$, $\Delta \Psi$ increased steeply to values similar to those observed in the presence of urea (Fig. 3B).

The data obtained with the permeant anion $\text{S}^{14}\text{CN}^-$ confirmed that in the presence of urea *H. pylori* does not invert its $\Delta \Psi$ to an internally positive value. $\text{S}^{14}\text{CN}^-$ uptake by the cells was not diminished by $n$-butanol (Table 1), indicating that this uptake reflects binding rather than accumulation inside the cytoplasm. Moreover, addition of 10 mM KSCN, which uncouples cells of *A. acidocaldarius* (19), an observation which we confirmed, did not have any effect on the pH in of *H. pylori* at a pH out of 2 (results not shown). Taken together, these data indicate that down to a pH out of 1 *H. pylori* maintains an internally negative $\Delta \Psi$, presumably because of the electrogenic extrusion of $\text{NH}_4^+$ cations from the cytoplasm (see below).

PMF. PMF can be calculated from $\Delta \text{pH}$ and $\Delta \Psi$ with the equation: $\text{PMF} = \Delta \Psi - 61.5(\Delta \text{pH})$ (in millivolts at 37°C) (20). By using the data for $\Delta \text{pH}$ and $\Delta \Psi$ corrected for probe binding (Fig. 1B and 3B, respectively), cells suspended in the presence of urea had negative PMF values ($-254$ to $-181 \text{ mV}$) at pH out values of 1.2 to 3.3. These values dropped to $-96$ and $-62 \text{ mV}$ at pH out values of 4.8 and 5.8, respectively, and increased to $-143 \text{ mV}$ at a pH out of 7.2. In the absence of urea the PMF was also low at pH out values of 4.8 and 5.8 and increased to about $-130 \text{ mV}$ at pH out values of $\geq 6.3$, conditions under which *H. pylori* grows (Table 2).

Urease. The urease activity of intact cells was measured after acid shock by determining the decrease in the urea concentration in the medium, which was 20 mM at the moment of the acid shock. When the pH out increased from 1 to 7, the urease activity of...
activity of a cell suspension at an OD578 of ~0.2 decreased approximately exponentially from 0.66 mM urea/min at pH 1 to 0.08 mM urea/min at pH 7 (Fig. 4). Urease activity was also measured in extracts of H. pylori cells which had been exposed to pHout values of 1 to 7 for 30 min in the presence of the concentrations of urea indicated (Table 3). Control experiments showed that these concentrations were saturating and not inhibitory. Irrespective of the preincubation conditions, the urease activities in H. pylori cell extracts were similar, and the average value was 57 ± 7.5 μmol min⁻¹ mg of protein⁻¹ (Table 3). These data indicate that the increase in cell urease activity at a low pHout was due to activation of an enzyme-transporter function rather than to an increase in gene expression during incubation of the cells at low pH.

### Table 2. ΔpH, ΔΨ, and PMF determined for H. pylori at different pHout values

<table>
<thead>
<tr>
<th>pHout</th>
<th>ΔpH (mV)</th>
<th>ΔΨ (mV)</th>
<th>PMF (mV)</th>
<th>ΔpH (mV)</th>
<th>ΔΨ (mV)</th>
<th>PMF (mV)</th>
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<tbody>
<tr>
<td>1.2</td>
<td>-228</td>
<td>-26</td>
<td>-254</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.4</td>
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<td>-53</td>
<td>-207</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.3</td>
<td>-117</td>
<td>-64</td>
<td>-181</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.8</td>
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<td>-96</td>
<td>-86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.8</td>
<td>-6</td>
<td>-56</td>
<td>-62</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6.3</td>
<td>0</td>
<td>-108</td>
<td>-108</td>
<td></td>
<td></td>
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<tr>
<td>7.2</td>
<td>0</td>
<td>-143</td>
<td>-143</td>
<td></td>
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</table>

### Table 3. Urease activities of H. pylori cell extracts after 30 min of acid shock in the presence of urea

<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Urease activity (μmol of urea min⁻¹ mg of protein⁻¹)</th>
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</thead>
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<tr>
<td>pH 1 + 50 mM urea</td>
<td>57.5</td>
</tr>
<tr>
<td>pH 2 + 20 mM urea</td>
<td>50.8 ± 9.3</td>
</tr>
<tr>
<td>pH 3 + 20 mM urea</td>
<td>51.4 ± 11.8</td>
</tr>
<tr>
<td>pH 4 + 20 mM urea</td>
<td>68.3 ± 6.5</td>
</tr>
<tr>
<td>pH 5 + 20 mM urea</td>
<td>49.11</td>
</tr>
<tr>
<td>pH 6 + 20 mM urea</td>
<td>66.4 ± 5.5</td>
</tr>
</tbody>
</table>

*Urease activity was measured by determining ammonia release with cell extracts in 100 mM sodium phosphate buffer (pH 7.4) supplemented with 10 mM urea using the phenol-hypochlorite assay (35).
FIG. 5. pH optima of \( H. pylori \) urease from cell extracts in different buffer systems. Gray bars, 100 mM citrate-NaOH; solid bars, 100 mM MES-NaOH; cross-hatched bars, 100 mM sodium phosphate. Urease activity was determined by the phenol-hypochlorite assay (35).

the cells, since at low pH\(_{in}\) values cytoplasmic enzymes become inactive, as experiments with protonophores at low pH\(_{in}\) values with both acidophiles (19) and \( H. pylori \) (24, 30) have shown. Urease has been proposed to play the following role in this homeostasis of pH\(_{in}\) (29, 30, 37). In the cytoplasm NH\(_4^+\), a product of the urease reaction, binds protons leaking in from the acidic medium. This process occurs because all prokaryotes, including acidophiles, possess a cytoplasmic membrane with some permeability for protons (33, 34). The NH\(_4^+\) produced is then removed from the cytoplasm via a hypothetical NH\(_4^+\) efflux system. The present study was carried out to obtain more information about this mechanism. To do this, cells were suspended in the presence or absence of urea in citrate-phosphate buffers at a wide range of pH values. It has been shown previously that at a pH\(_{out}\) of 2 cells do not lose viability rapidly in this type of buffer (30). At pH\(_{out}\) values of <4, pH homeostasis of \( H. pylori \) depended on urease activity (Fig. 1), which increased exponentially with the inverse of pH\(_{out}\) (Fig. 4). This observation is consistent with the fact that \( H. pylori \) relies on the availability of urea for survival at pH\(_{out}\) values of <4 (17). A urea-dependent rise in cellular pH has previously been observed for \( H. pylori \) suspended in buffer at pH 3 to 5 and was attributed to changes in the periplasmic pH (18). Several points argue against the notion that the changes in cellular pH\(_{in}\) observed in the present study reflect changes in the periplasmic pH: (i) the pH\(_{in}\) values reported here for energized and nonenergized \( H. pylori \) cells are very similar to those of a gram-positive organism and several archaeal acidophiles (2, 19, 26, 33) which do not possess a periplasmic space; (ii) protons are expected to move rapidly through the outer membrane portins of gram-negative bacteria, thereby restricting the possibility of building up a large pH gradient across this membrane; (iii) if the \( H. pylori \) outer membrane had limited permeability, the probes used for measuring the \( \Delta V \) and \( \Delta pK \) of \( H. pylori \) (Fig. 1, 2, and 3) (14, 18) were not expected to reach the cytoplasmic membrane, contrary to what was observed; and (iv) the anionic form of \(^{14}C\)salicylic acid is expected to move rapidly across the \( H. pylori \) outer membrane, making this probe unsuitable for detection of a \( \Delta pK \) across this membrane. Since this probe did, however, detect a urea-dependent and protonophore-sensitive \( \Delta pK \), we concluded that it detects pH differences across the cytoplasmic membrane and that we measured pH\(_{in}\) rather than the periplasmic pH of \( H. pylori \) in this study and previously (30).

The exact pH\(_{in}\) in \( H. pylori \) during survival at pH 1 is not known. It is between 5.8, the apparent value determined in growth medium (30), and 4.9, the value corrected for probe binding reported here (Fig. 1B). The latter value is close to pH 5.0, the value for the pH optimum of urease activity in cell extracts assayed in sodium citrate buffer (Fig. 5), and it is higher than pH 4.6, the value reported for the extremely acidophilic thermophilic archaeon \( Picrophilus oshimae \) (33). This suggests that urease is active under conditions under which it is most needed by the cells (i.e., at a pH\(_{out}\) of 1) and that this effect contributes to the survival of the organism under extremely acidic conditions. However, the pH optimum of urease activity was strongly dependent on the type of buffer used for the assay (Fig. 5), and at present it is not known how the ionic composition of the \( H. pylori \) cytoplasm affects the enzyme activity and pH optimum of its urease.

Recently, Ha et al. (8) have reported that in pH 3 buffer \( H. pylori \) urease is inactive. However, in nonbuffered, nonstirred solutions these authors observed some residual activity of the enzyme at pH 3 (8). This observation was taken to support the altruistic autolysis hypothesis for the survival of \( H. pylori \) at low pH (10). According to this hypothesis, some of the \( H. pylori \) cells lyse, thereby releasing their urease into the medium, where it binds to the surface of other, still viable \( H. pylori \) cells (8, 10). At a low medium pH and in the presence of urea this surface-bound urease is thought to create a cloud of ammonia around the cells, which protects the cells by binding protons, thereby increasing the local pH just outside the cells to a more neutral value (8, 10). We criticize this concept on several grounds. First, the data of Ha et al. do not support the conclusions of these authors. In their nonbuffered solutions, urease activity was completely nonproportional to enzyme concentration, reflecting ill-defined conditions. Moreover, from their data it can be calculated that at the highest protein
concentration the turnover rate of the urease at pH 3 was only about 2.5% of the maximal turnover rate at neutral pH, raising doubt concerning the contention that at low pH the urease activity of the enzyme sticking to the surface of nonlysed cells is sufficient for creating and maintaining the cloud of neutralizing ammonia around the cells (8). Second, survival of 

*Helicobacter pylori* at pH 1 has been observed (30). At pH 1 to 1.3 the stomach contents are well buffered, since it takes about 99 to 29 mM OH− ions to raise the pH to 3. Hence, any results specifically obtained in nonbuffered solutions at pH 3, such as those reported by Ha et al. (8), are irrelevant for the survival of 

*Helicobacter pylori* at pH 1. Finally, we contend that the altruistic autolysis hypothesis does not apply to the low-pH conditions under which we observed survival of 

*Helicobacter pylori* cells. This argument is based on the observation that addition of a protonophore to cells in either buffer or growth medium at pH 1 to 4 eliminates all urease activity as pH_in reaches the same value as pH_out (24, 30), suggesting that at these low pH values there is no external urease that is still active. Nevertheless, cells do not lose viability at pH_out values of 1 to 4 before the protonophore is added, suggesting that the presence of surface-bound urease is not essential for survival of 

*Helicobacter pylori* at low pH.

At pH 4 to 7, the pH_in of 

*Helicobacter pylori* cells was slightly lower in the presence of urea than in the absence of urea (Fig. 1B). Moreover, in the presence of urea the cells did not develop as negative a ΔΨ as other prokaryotes develop in this pH range (Fig. 3B) and therefore had a low PMF (Table 2). At present, the reason for this result is not clear. It may be an artifact of the methods used. In the case of pH, salicylate may not have been completely trapped within the cells due to the formation of a pH gradient ranging from the cytoplasm to the external medium rather than a steep pH change at the cytoplasmic membrane under these conditions. However, it is noteworthy that this effect has nothing to do with neutralization of the microenvironment or the periplasm, particularly at an extremely low pH, since proton and ammonia diffusion through the outer membrane is expected to be some orders of magnitude greater than the production of ammonia by urease activity. In addition, it is also important to mention that 

*Helicobacter pylori* contains several drug efflux systems (1, 32), and it may very well be that some of the probes used in the present study for measurement of ΔpH and ΔΨ are exported from the cells by these systems.

A remarkable result of the present study was that unlike acidophiles (2, 33), at pH_out values of <3 

*Helicobacter pylori* did not reverse the sign of its ΔΨ from internally negative to internally positive (Fig. 3 and Table 1). This conclusion disagrees with that of previous work, in which an internally positive ΔΨ was postulated for 

*Helicobacter pylori* at pH 3 (14). We agree with Matin et al. that at a low pH the cells take up the lipophilic anion SCN−. However, SCN− uptake was not diminished by a protonophore or by permeabilization of the cells with n-butanol (Table 1), indicating that it reflects binding to the cells rather than accumulation in the cytoplasm. The question that then arises is, by which mechanism is the internally negative ΔΨ generated. It depends on the presence of urea in the medium. Moreover, addition of urea to the cells causes immediate hyperpolarization (internally negative) of the cells at low pH (28) (Fig. 2). We propose that this hyperpolarization is caused by the electrogenic efflux of NH4+ cations by a hypothetical transporter. However, further work, including identification of such a transport system, is needed to show whether this proposal is correct.

Finally, our finding that even at pH 1 the urease activity of intact cells was only one-third of the urease activity in cell extracts prepared from the equivalent number of cells (Fig. 4 and Table 3) has important implications for the mechanism(s) by which urease activity is regulated. It shows that besides the well-documented pH-dependent activation of the urea carrier UreI (24, 27, 36) and the possible direct activation of urease activity by low pH (this study), other still unknown factors may regulate urease activity in 

*Helicobacter pylori* cells.

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