Urea Protects *Helicobacter (Campylobacter) pylori* From the Bactericidal Effect of Acid

B. J. MARSHALL, L. J. BARRETT, C. PRAKASH,
R. W. McCALLUM, and R. L. GUERRANT
Divisions of Geographic Medicine and Gastroenterology, Department of Medicine, University of Virginia, Charlottesville, Virginia

Colonization of the stomach with *Helicobacter (Campylobacter) pylori* is common in patients with duodenal ulcer disease, which is known for its high acid secretion. Although the bacterium is usually isolated by culture of a gastric biopsy specimen, viable organisms may sometimes be found in the acidic gastric juice. It was postulated that urease, by generating ammonia, protected *H. pylori* from acid. To test this hypothesis, the pH susceptibility of *H. pylori*, *Proteus mirabilis*, and the urease-negative *Campylobacter jejuni* was examined in the presence and absence of urea. It was found that without urea the three bacteria were all highly susceptible to acid. In striking contrast, the addition of 5 mmol/L of urea completely protected *H. pylori* but not *P. mirabilis* or *C. jejuni* from pH values as low as 1.5. Furthermore, the protective effect of urea on *H. pylori* was found with urea concentrations as low as 0.05 mmol/L. It is concluded that the high urease activity of *H. pylori* enables it to survive in gastric acid.

For nearly a century researchers have noted the presence of urease in the gastric mucosa of animals, particularly dogs, cats, and humans (1–3). The isolation of *Helicobacter (Campylobacter) pylori* from the gastric mucosa of patients with peptic ulcer disease and gastritis (4) and the observation by Langenburg et al. (5) that the new bacterium produced urease has rekindled interest in this phenomenon. The isolation of similar urease-positive *Helicobacter*-like organisms from ferrets (6) and our own observations of urease-positive spiral organisms in cat gastric mucosa prompted this study. These other gastric *Helicobacter*-like organisms are ultrastructurally and antigenically similar to *H. pylori* (7,8).

Preliminary experiments with *H. pylori* indicated that it grew best on media with neutral pH (9); however, in some patients *H. pylori* organisms could be cultured from gastric juice of pH <2.0. This finding indicated that *H. pylori* could also survive in acid, perhaps by generating ammonia through the hydrolysis of urea normally present in the gastric juice (10).

Therefore, the survival rate of *H. pylori* in acidic solutions was studied with and without the addition of physiological concentrations of urea and compared with the survival rate of the morphologically similar but urease-negative *Campylobacter jejuni* and the weaker urease producer *Proteus mirabilis*.

Methods

Recently isolated *H. pylori* from human gastric biopsy specimens were cultured on horse-blood agar plates with GCHI enrichment (REMEL) incubated at 37°C for 3 days in an atmosphere of 10% CO_2, 5% oxygen, and 85% nitrogen ("campy gas"). *C. jejuni* and *Proteus mirabilis* were prepared in a similar manner after overnight culture on sheep-blood agar plates (BBL) at 42° and 37°C, respectively.

Bacteria were harvested from their respective agar plates and suspended in normal saline at pH 7 to give a final cloudy suspension of approximately 10^9 organisms/mL (turbidity = McFarland's no. 6). For each organism, 1.5 ml of this suspension was then added to 1.5 ml of sodium chloride solution or 1.5 ml of filter sterilized 100 mmol/L urea solution (6.2 g/L) in normal saline. After vigorous mixing, 0.25 mL of each of these suspensions was added to each of a set of tubes of phosphate buffered saline at pH values of 7, 5, 4.5, 4, 3.5, 3, 2.5, 2, and 1.5, with the final urea concentration being 5 mmol/L.

These tubes were then incubated for 30 minutes at 37°C. After 30 minutes, samples of each of these suspensions were taken and diluted in serial 10-fold dilutions to 1:10,000 for quantitative cultures. At the end of the experiment, the pH of the suspension in each of the acidified tubes was again measured with a glass pH electrode. The diluted suspensions were then inoculated onto horse-blood agar plates for *H. pylori*, sheep-blood agar plates for *C. jejuni*, and MacConkey for *P. mirabilis*, which were incubated for up to 5 days at 35°C in sealed plastic bags with campy gas for

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H. pylori, 48 hours at 42°C in campy gas for *C. jejuni*, and 24 hours at 37°C in air for *P. mirabilis*. Colony counts per plate were calculated as [no. of colonies × (1/dilution) × (1/0.01)] per mL. The lower limit of detection was 1 organism/0.01 mL, i.e., 10^2 organisms/mL.

Experiments were initially conducted using CP16 (*H. pylori*), C31 (*C. jejuni*), and LRA080173 (*P. mirabilis*). They were repeated on two further occasions using CP16. Subsequently, CP83, CP101, and CP105 were studied in single experiments.

To determine the lower limit of urea concentration for the "acid protection" effect, 1.4×10^7 viable *H. pylori* organisms were inoculated into tubes containing buffered saline at pH 2.0, with urea concentrations of 0.0, 0.05, 0.5, 1.0, 2.0, and 5.0. Tubes were incubated at 37°C and subcultured as described above at baseline, 10 minutes, and 30 minutes. Single experiments were performed on CP83 and two further *H. pylori* isolates, CP256 and CP239.

Results

There was very little difference in the survival rates of each of the three organisms in acidified normal saline. In the absence of urea, all three organisms survived well above pH 5, and there was a progressive 4- to 8-log or greater reduction in counts as the pH was decreased to 3 or lower; in this low-pH range there were often no viable organisms recovered (Figure 1). The curves for *H. pylori*, *C. jejuni*, and *P. mirabilis* were very similar, indicating that none of these organisms could survive at an acidic pH (less than 3) in the absence of urea (Figure 2). This effect was observed in eight of nine experiments on seven different strains of *H. pylori*. In one experiment using CP83, a 3-log kill rate (99.8%) was observed.

When 5 mmol/L urea was added to the solutions, there was less than a 2-log reduction in four *H. pylori* isolates incubated in acidified urea to pH 1.5 (Figure 3). In contrast, there was no change in the survival rates of *C. jejuni* and *P. mirabilis*. Both *C. jejuni* and *P. mirabilis* showed >4-log reduction in acidified urea (Figure 4).

When *H. pylori* was placed into decreasing concentrations of acidified urea, it remained viable for 30 minutes at all urea concentrations above 0.05 mmol/L (Table 1). Overall, substantial survival of *H. pylori* was observed in all nine experiments (on seven strains) in which the urea concentration was ≥0.05 mmol/L.

The rate of *H. pylori* survival could be explained in two ways (Figure 5). When the initial pH was above 2.5, the organism was able to generate enough ammonia to increase the pH of the suspension to approximately 6.5, at which level the organisms were fully viable. At pH values ≤2, however, *H. pylori* still survived even though the organism could not neutralize the suspension. This suggests that at low pH values intracellular urease generates enough ammonia from urea in the environment to maintain a viable pH within the *H. pylori* colony.

Discussion

Although *H. pylori* is a fastidious organism when cultured in vitro, it seems to be uniquely adapted to the environment of the stomach and of the mucous

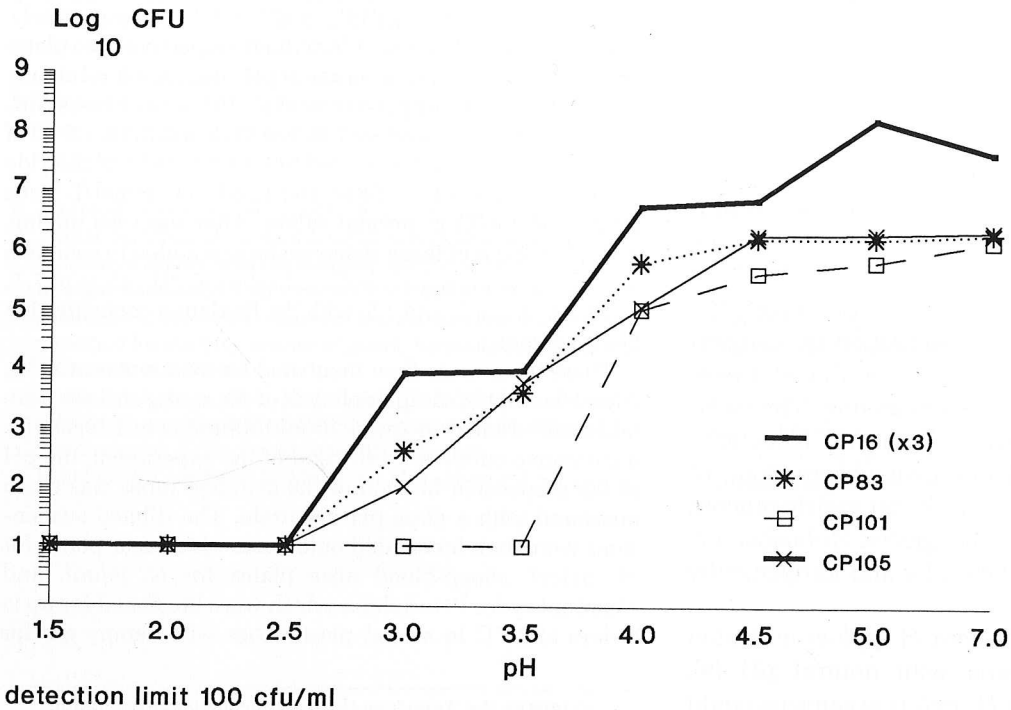

Figure 1. Survival rate of *H. pylori* in acid alone; x axis represents the starting pH of the test solution and y axis shows the number of viable organisms remaining after 30 minutes incubation at 37°C. *Thick line* (CP31) represents the mean of three experiments. Note poor survival rate of *H. pylori* in acidified saline at pH values <3.0.

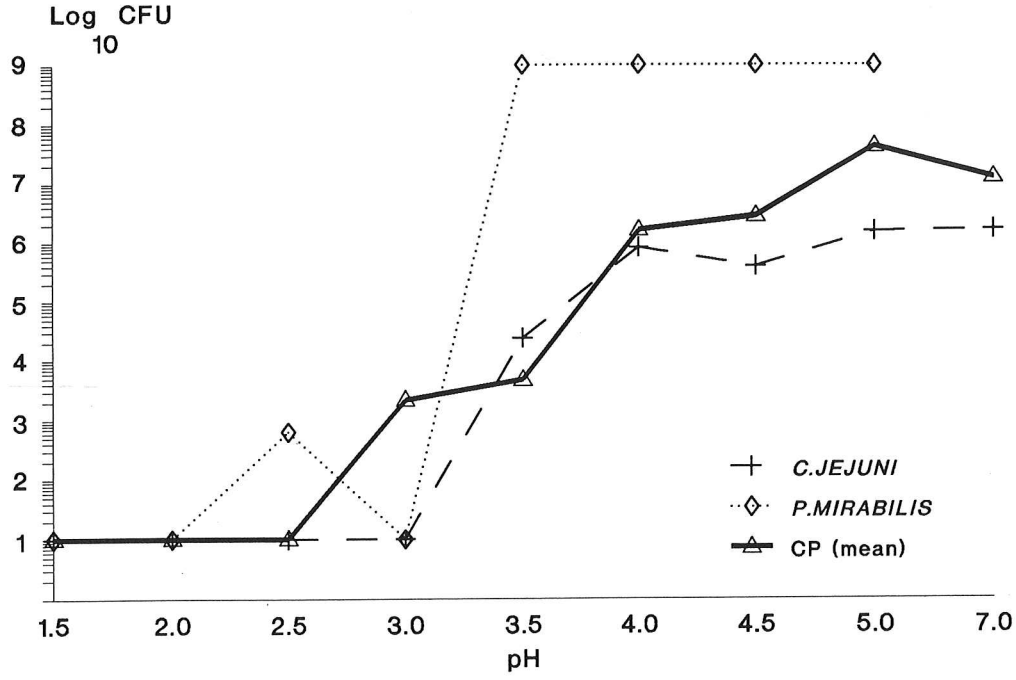

Figure 2. Survival rates of all bacteria in acid alone. *C. jejuni* and *P. mirabilis* behave similarly to *H. pylori*, showing poor survival in acid at pH values <3.0. *Thick line* represents the mean of all experiments on *H. pylori* (CP31 × 3, CP83, CP101, and CP105).

layer adjacent to the gastric mucus-secreting cells. Micropuncture studies indicate than in experimental animals the pH of this location is approximately 7.0 (11); therefore, acid-protecting mechanisms might not necessarily be required once a bacterium has attached to the epithelial cell. The *H. pylori* cell is spiral and flagellated, features enabling it to move in viscous solutions (12); it also has the ability to attach intimately to the gastric mucosal cells (13). However, other gastrointestinal pathogens have rarely, if ever, been reported in this submucous location. *H. pylori* and the gastric mucosal bacteria of cats, dogs, ferrets, and monkeys are all urease producers, suggesting that urease is essential for colonization of the acid-secreting stomach.

In a manner similar to that in which the gastric epithelial cells secrete bicarbonate, *H. pylori*, by means of its remarkable urease production, generates bicar-

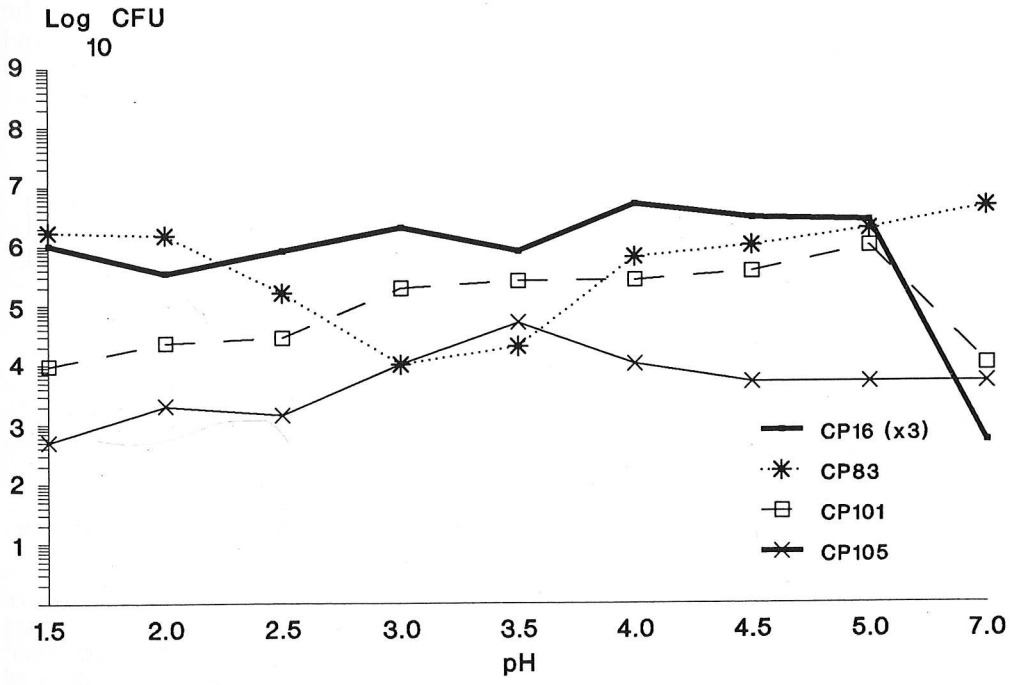

Figure 3. Survival rate of *H. pylori* in acid with urea. With the addition of urea, *H. pylori* survives well down to pH 1.5.

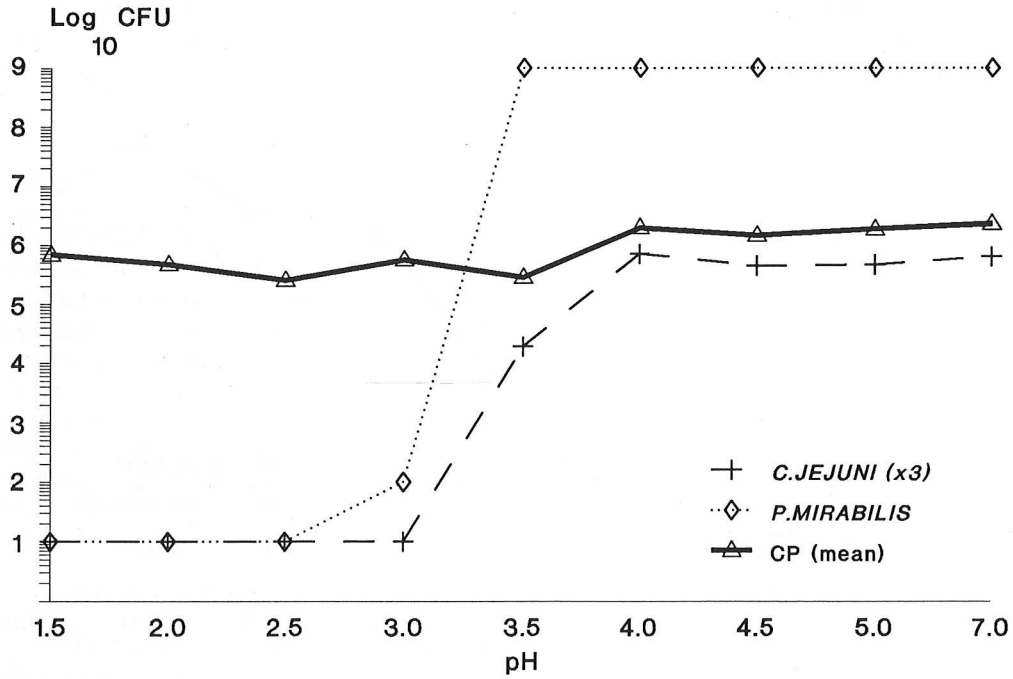

Figure 4. Survival rates of all bacteria in acid with urea. In contrast to *H. pylori* (thick line), *P. mirabilis* (a weak urease producer), and *C. jejuni* (urease negative) are not rescued by the addition of a physiological concentration of urea.

bonate and ammonia in its immediate environment and removes hydrogen ions.

$$\begin{array}{l}NH_2\\ |\\ C{=}O\\ |\\ NH_2\end{array} + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-$$

The ammonia is used by *H. pylori* as a nitrogen source for protein synthesis, and it may also impair the host defenses through inhibition of the tricarboxylic acid cycle of aerobic cells (14).

In patients with *H. pylori* infection, urea is sometimes undetectable in the gastric juice (10), whereas it is present at levels about half those in the blood in normal patients (normal range, ≈70–100 mg/dL or 1–2 mmol/L) (15). The present data show that *H. pylori* is able to utilize physiological concentrations of urea to neutralize the hydrogen ion present in gastric juice. This might be the cause of the hypochlorhydria (which may even be histamine fast) observed in epidemic hypochlorhydria (16), in a volunteer who developed acute *H. pylori* infection (17), and possibly also in patients with uremia (18). During basal acid secretion, *H. pylori* could obtain adequate amounts of urea from diffusion through the gastric epithelium and from the saliva to totally neutralize gastric juice. This could

Table 1. Effect of Decreasing Urea Concentrations on Survival of Helicobacter pylori *at pH 2.0*

Strain no.	Urea concentration (*mmol/L*)	Log_{10} survival after incubation 0 min	10 min	30 min
CP 256	0	5.204	NG	NG
	0.5	6.104	>5.0	>5.0
	1.0	5.892	>5.0	>5.0
	2.0	5.792	6.017	5.851
	5.0	6.0	6.013	5.934
CP 239	0	6.146	3.602	NG
	0.05	>7.0	6.447	5.602
	0.5	>7.0	>7.0	6.301
	5.0	>7.0	>7.0	>7.0
CP 83	0	6.663	5.114	3.74
	0.005	6.591	6.176	5.462
	0.05	6.732	6.633	6.462
	0.5	6.58	6.398	6.431
	5.0	6.398	6.505	6.415

NOTE. NG, no growth.

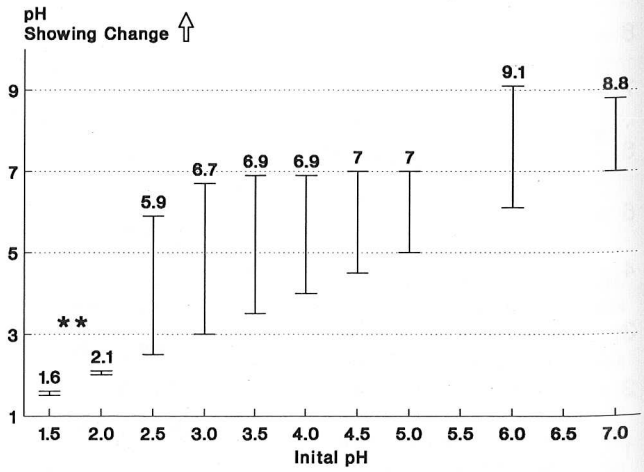

Figure 5. pH change during incubation of *H. pylori* in 5 mmol/L urea. *Vertical bars* show initial (lower limit) and final (upper limit) pH values of acidified urea solutions inoculated with *H. pylori*. **When pH is <2.5, *H. pylori* does not increase the pH of its macroenvironment but still survives well (Figure 3).

explain the basal hypochlorhydria observed in Morris' self-infection experiment. It is uncertain whether this mechanism would be enough also to neutralize secretion from the parietal cell glands during pentagastrin stimulation and cause pentagastrin-fast hypochlorhydria as observed in a Texas epidemic associated with *H. pylori* (16). Mossberg et al. (19) report that urea hydrolysis was not sufficient to neutralize gastric acid in hypochlorhydric patients with uremia. Ramsey et al. (16) maintain that the hypochlorhydria was caused by parietal cell failure. We suggest that ammonia production may be the cause of parietal cell failure in patients with *H. pylori* infection of the oxyntic gland mucosa. Ammonia depletes the tricarboxylic acid cycle of α-ketoglutarate and prevents the generation of adenosine triphosphate in aerobic cells (14).

It is remarkable that at pH values <2.5 *H. pylori* was still able to survive if urea was present. Because it was not able to increase the pH level of its macroenvironment in this situation, it is presumed that *H. pylori* was able to hydrolyze enough urea in its immediate microenvironment (perhaps intracellularly or in the pericellular space) to neutralize any hydrogen ion penetrating its cell wall. Its copious extracellular urease is probably immediately denatured at this pH, impairing the ability of *H. pylori* to increase the pH of its macroenvironment. Studies by McLean et al. (20) on the urease-producing bacteria of the bovine rumen indicate that urease is usually a cell wall-bound enzyme, an ideal location for such an action (Figure 6).

The bactericidal effect of gastric juice has been noted by Giannella et al. (21), who found that nearly all bacteria including salmonellae were killed by gastric acid and that this bactericidal effect was solely a result of the hydrochloric acid present. The present data show that the potent urease of *H. pylori* explains its capacity to colonize the acidic stomach, and, indeed, an acidic environment with urea present is an ideal ecologic niche for the organism. Certainly it explains why the gastric acid barrier does not kill *H. pylori* as it does most other cultivable bacteria. It can also be speculated that in patients not infected newly arriving *H. pylori* from the oropharnyx may survive in the gastric juice and easily colonize the mucous layer. This implies that premedication with cimetidine to render the stomach achlorhydric (22) or dosing of the organism in bicarbonate, as has been done in *C. jejuni* pathogenic studies (23), may not be necessary, especially when normal levels of urea are present (1–5 mmol/L). Conversely, consumption of antacids, H_2-receptor antagonist therapy, or the presence of gastric atrophy may not substantially alter the risk of *H. pylori* infection.

It is concluded that the potent urease of *H. pylori* enables it to select the acidic mammalian stomach as its unique ecological niche. The extent to which urease contributes (as a virulence factor) directly to achlorhydria or indirectly to cell damage (via cellular ammonia toxicity) and thus to pathology and disease with *H. pylori* infections awaits further study.

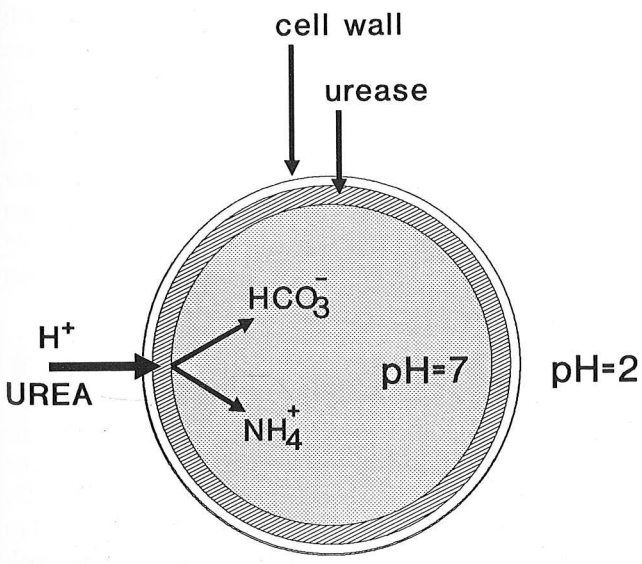

Figure 6. Proposed model of microenvironment pH maintenance by *H. pylori*. Urease, in the region of the cell wall, provides ammonium and bicarbonate by the hydrolysis of urea.

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Address requests for reprints to: B. J. Marshall, M.D., Box 145, Division of Gastroenterology, Department of Medicine, University of Virginia, Charlottesville, Virginia 22908.

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