BACTERIAL PATHOGENICITY

Factors affecting growth and antibiotic susceptibility of *Helicobacter pylori*: effect of pH and urea on the survival of a wild-type strain and a urease-deficient mutant

J. E. SJÖSTRÖM and H. LARSSON*

Departments of Cell Biology and *Gastrointestinal Pharmacology Preclinical Research and Development, Astra Hässle AB, Mölndal, Sweden

This study investigated how pH and the presence of urea affect the survival and growth of *Helicobacter pylori* and whether these factors affect susceptibility to antibiotics *in vitro*. The viability of a wild-type strain and a urease-deficient mutant of *H. pylori* was studied after incubation for 1 h in buffers at different pH values at 37°C under microaerophilic conditions. Viable counts were not affected at pH 5 and pH 7. In buffer at pH 3, there were no viable organisms, but urea (6.25 mM) protected the wild-type strain, which survived well. At pH 9, urea further reduced the viability of *H. pylori* and flurofamide almost abolished the effect of urea on the wild-type strain. Neither urea nor flurofamide affected the viability of the urease-deficient mutant under the same conditions. Growth was also pH dependent and was enhanced in shake-cultures. At pH 5, urea supported growth of the wild-type strain, but at pH 7 a toxic effect on the bacteria was observed. Growth of *H. pylori* at pH 5.9 was poor, and susceptibility to amoxycillin, erythromycin and clarithromycin was markedly less than at pH 7.2 and 7.9. The bactericidal activities of metronidazole and tetracycline were similar at the different pH values studied. At neutral pH the killing rates of amoxycillin and clarithromycin were growth rate dependent. Susceptibility to metronidazole was enhanced in stationary cultures. The interaction obtained between the proton pump inhibitor, omeprazole, and amoxycillin at pH 7 was of additive type. These results suggest that pH and growth conditions may be important in the antibacterial efficacy of different antibiotics *in vivo* and also provide a possible explanation for the potentiating effect of omeprazole with antibiotics in the treatment of *H. pylori* infections.

Introduction

Colonisation of the gastric antral mucosa by *Helicobacter pylori* is strongly associated with active and chronic gastritis as well as with peptic ulcer diseases [1,2]. Despite its ability to colonise the gastric mucosa, the organism does not survive *in vitro* conditions of high acidity [3], and growth is restricted to a pH range of 6.5-7.5 [4,5]. The pH for optimum growth of *H. pylori* is reported to be c. 7.0 [6]. Recently, and as an exception to what others have found, Kangatharalingam and Amy [7] reported growth of *H. pylori* at a pH value as low as 4.5 in a modified Brucella broth medium without serum. *In vitro*, urea increased survival at low pH [3] but Greig *et al.* [8] observed a self-destruction of bacteria in the presence of urea at pH 6. The effect was prevented by addition of hydroxyurea and was not seen at pH 7, showing that the bactericidal effect was dependent on pH and urease activity.

All clinical isolates of *H. pylori* show urease activity, which is believed to facilitate colonisation [9,10] by generating ammonia to buffer gastric acidity [11]. Administration of the urease inhibitor, flurofamide, to rats infected with *H. felis* did not affect the growth of *H. felis*, showing that there is no requirement for urease activity after the bacteria have reached their unique niche beneath the mucus layer of the gastric mucosa, where pH may be close to neutrality [12,13].

Antimicrobial agents generally display good activity against *H. pylori in vitro* [14]. However, the bactericidal activity of single antibiotics against *H. pylori in vivo* is very poor [15-17] and they do not succeed in eradicating the organism. Apart from
antibiotics combined with acid inhibitors, the most effective treatment regimen for the eradication of *H. pylori* [18,19], given good compliance by patients [20], is triple therapy with bismuth subcitrate and two antimicrobial agents (tetracycline or amoxicillin and metronidazole).

A possible explanation for the success of combined therapy may reside in the synergy between metronidazole and amoxicillin or tetracycline that has been described *in vitro* [21]. Bismuth subcitrate also interacts synergically *in vitro* with several antimicrobial agents, including metronidazole and β-lactams [22]. The antimicrobial activity of several antibiotics *in vitro* is also affected by pH, with increased MICs under acid conditions [23–25]. The synergic effect obtained *in vivo* between amoxicillin and the proton pump inhibitor, omeprazole [26], is probably due to the increase in pH in the vicinity of *H. pylori*, improving the conditions for antibiotic activity. More recently, optimal short-term treatment of *H. pylori* infections with omeprazole in combination with two antibiotics has been recommended [27,28]. *In vitro*, the culture conditions, affecting growth rates [29], as well as whether the bacteria are planktonic or adherent to cells [30], are other important determinants of antibiotic susceptibility.

The aim of the work reported here was to investigate how pH affects the growth and survival of *H. pylori* and its susceptibility to different antibiotics *in vitro*, in order to provide a possible explanation for variations in antibiotic efficacy *in vivo*.

Materials and methods

**Bacterial strains**

The strains of *H. pylori* used in this study are listed in Table 1. Stock cultures were stored at −70°C in Brucella Broth (Difco) with heat-inactivated (56°C, 30 min) fetal calf serum (FCS) 10%, pH 7.0, supplemented with glycerol 20%. The type strains of *H. pylori* selected for this study were NTCC 11637 and CCUG 15818. Strains AH 5 and AH 28 from our collection were clinical isolates from duodenal ulcer patients. Strains N6, wild-type, and N6KmV, its isogenic urease-deficient mutant [31], were kindly provided by A. Labigne, Institute Pasteur, Paris.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genotype/phenotype</th>
</tr>
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<tbody>
<tr>
<td>NTCC 11637</td>
<td>MtzR*</td>
</tr>
<tr>
<td>CCUG 15818</td>
<td></td>
</tr>
<tr>
<td>N6</td>
<td>MtzR</td>
</tr>
<tr>
<td>N6 KmV</td>
<td>MtzR, KmR, ureB1</td>
</tr>
<tr>
<td>AH5</td>
<td></td>
</tr>
<tr>
<td>AH28</td>
<td></td>
</tr>
</tbody>
</table>

*MtzR*, metronidazole resistant.

*Kanamycin-resistant transformant. The ureB gene is disrupted by insertion of a mini-Tn3-Km transposon.

**Media and buffers**

Solid medium for *H. pylori* was Columbia blood agar comprising Columbia Agar Base II (Oxoid) 42.5 g/L, Bactoagar (Oxoid) 15 g/L, horse blood 7% and IsoVitale X (BBL Microbiology System) 1%, pH 7.3 ± 0.2. Growth medium was, if not stated otherwise, Brucella Broth (Difco) supplemented with FCS 10%, pH 7.0. Brucella broth pH 5.0 was adjusted to that pH with HCl.

Brucella broth, pH 5.9 and 7.9, was prepared in 66 mM sodium-phosphate buffer. Citrate-phosphate buffer (pH 3.0, pH 5.0, pH 7.0) and glycine-NaOH (pH 9.0) were used at 100 mM for studying the survival of bacteria. Phosphate-buffered saline (PBS) (pH 7.2) was used for washing and dilution of bacteria.

**Chemicals**

Urea and antibiotics were purchased from Sigma. Flurofamide was synthesised by Synthelec AB (Lund, Sweden) and omeprazole was from Astra Hässle AB (Mölndal, Sweden). Other salts and solvents were from commercial sources and were of the highest purity available.

**Growth conditions**

Bacteria stored at −70°C were thawed and subcultured twice on Columbia blood agar for 2–3 days at 37°C in an automatic CO2/O2 incubator (Forma Scientific), under micro-aerophilic conditions (N2 85%, CO2 10%, O2 5%) before being used in an experiment or as an inoculum for broth cultures. Viable counts, expressed as cfu/ml, were determined on blood agar after washing and dilutions in PBS, and colonies were counted after incubation for 3 days under the conditions described above.

**Determination of growth and pH**

Growth of bacteria in liquid media was determined by reading the optical density in a spectrophotometer (Shimadzu UV-120-01) at 560 nm or in a microtitration plate reader (Molecular Devices) at the same wavelength. pH was monitored with a glass pH electrode (Radiometer, Copenhagen).

**Determination of surviving bacteria in buffer**

*H. pylori* strains grown on Columbia blood agar plates were harvested and washed in PBS by centrifugation at 4000 rpm for 10 min at room temperature and resuspended in tubes with 1.0 ml of sterile buffer at different pH values. Cell density was adjusted to 10^7–10^8 cfu/ml. Viable counts were determined by a modified Miles and Misra technique [32] by dilution in PBS and spreading on agar plates at time zero, and after
different incubation times under microaerophilic conditions at 37°C. The results were expressed as mean and SEM. The survival of bacteria under different conditions was compared by the paired $t$ test.

**Determination of MBC and fractional inhibitory concentration (FIC) index**

Minimal bactericidal concentrations (MBCs) for amoxycillin, tetracycline, erythromycin, clarithromycin and metronidazole dissolved in dimethylsulphoxide (DMSO) were determined by serial dilutions in Brucella broth plus FCS 10% at pH 5.9, 7.2 and 7.9, at a cell density of $10^6$ cfu/ml in 96-well microtitration plates. After incubation for 72 h at 37°C under microaerophilic conditions, 10 μl from each well were applied with a replicator to large (120 mm × 120 mm × 17 mm) Columbia blood agar plates. The plates were read after incubation for 72 h. MBC was defined as the lowest concentration of the compound giving >99.9% killing. The combined activity of omeprazole and amoxycillin was quantified by the simplified broth dilution checkerboard technique, in Brucella broth plus FCS 10% at pH 7 against the type strains NTCC 11637 and CCUG 15818, as described by Krogstad and Moellering [33]. With this method synergy is defined as a fractional inhibitory concentration (FIC) index ≤0.5; additivity as an FIC index of 0.5–1.0 and antagonism as an FIC index of 1.0–2.0.

**Results**

Survival of non-growing bacteria at different pH values and the effect of urea

The decrease in viability of *H. pylori* strains N6 and N6KmV in buffers with and without urea is shown in Fig. 1. When urea was added to buffer at a final concentration of 6.25 mM at pH 3 and pH 9, the cell viability for the wild-type strain N6 was markedly

![Fig. 1. Log$_{10}$ decrease in survival of bacteria after 1 h in buffers at different pH values at 37°C under micro-aerophilic conditions. a, *H. pylori* N6 (wild-type); b, *H. pylori* N6 KmV (urease-deficient). □, no additions; ■, 40 μM flurofamide; ⌞, 6.25 mM urea; ⌝, 40 μM flurofamide + 6.25 mM urea. Each bar represents the mean and SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.](image)
affected during the incubation period of 1 h. At pH 3, survival was significantly increased (p < 0.001), probably depending on the ammonium ions formed by the urease activity, which results in an increase in pH adjacent to the bacteria. This was reflected by an increase in buffer pH to 3.65 (data not shown). Addition of flurofamide, an inhibitor of urease, reduced the protective effect exerted by the urease reaction (p < 0.01). In contrast, at pH 9, urea further significantly reduced viability (p < 0.05) and all bacteria were killed. However, if flurofamide was added simultaneously to a final concentration of 40 μM, the effects of urea at both low and high pH values were reduced. At pH 5 and 7, the numbers of viable bacteria

Fig. 2. Growth of *H. pylori* in Brucella broth plus FCS 10% at 37°C under micro-aerophilic conditions at pH 5 and 7. a, Strain N6 (wild-type); b, N6 KmV (urease-deficient). □, Brucella broth; ■, Brucella broth + 6.25 mM urea.
were unaffected by addition of urea or flurofamide. The urease-deficient mutant was not significantly affected by urea or flurofamide under any of the test conditions, and no alterations in pH values were observed.

Effect of urea on growth of *H. pylori* at different pH values

*H. pylori* strains N6 and N6KmV were grown in microtitration plates in Brucella broth with FCS 10% at pH 5 and pH 7 in the presence or absence of 6.25 mM urea. The absorbance at 560 nm and the pH were read at the start and after incubation for 72 h at 37°C under micro-aerophilic conditions. The results in Fig. 2 show that neither the wild-type strain nor the urease-deficient mutant grew at pH 5, and that growth was obtained for the wild-type strain at pH 5 only in the presence of urea. During incubation, the medium pH increased to 7.1 (data not shown). However, at a starting pH of 7, there was a reduction in growth of the wild-type strain when urea was added, and pH increase to 7.9 compared to 7.5 for the urease-deficient mutant (data not shown). Growth of the urease-deficient mutant at pH 5 and at pH 7 was not affected by addition of urea.

Growth and antibiotic susceptibility at different pH values

Four strains of *H. pylori* were grown in microtitration plates in Brucella broth plus FCS 10% at different pH values at 37°C under micro-aerophilic conditions. The increase in absorbance was determined after incubation for 72 h. At pH 5.9, growth was slower than at pH 7.2 (Table 2). At pH 7.9 the bacteria were again affected, and growth was similar to that obtained at pH 5.9.

At pH 5.9, the bactericidal activities of amoxycillin, clarithromycin and erythromycin were poor, but at pH 7.2 and 7.9, the bacterial susceptibility was increased, resulting in much lower MBCs (Table 2). The susceptibility to amoxycillin increased 10-20-fold, and even more for the macrolides. The lowest MBCs were achieved at pH 7.9. The MBCs for tetracycline and metronidazole were not appreciably affected by pH. Only strain AH5 was sensitive to metronidazole.

Growth of *H. pylori* in flasks with and without shaking

*H. pylori* strains NTCC 11637 and AH 28 were incubated in 125-ml flasks with 20 ml of Brucella broth plus FCS 10% at pH 7.0, and the effect of shaking on growth was studied by incubating the flasks with and without shaking at 150 rpm, at 37°C under micro-aerophilic conditions. Growth was followed by determination of viable counts (cfu/ml). Fig. 3 shows the results from three different experiments. There was an increase of >1 log_{10} cfu/ml during the first 12 h of incubation with shaking. Mean Δlog_{10} for strains NTCC 11637 and AH 28 was 1.528 SEM 0.1043 and 1.246 SEM 0.1429, respectively. The numbers of growing bacteria were then rapidly reduced, especially for strain AH 28. The growth pattern in stationary cultures was different and probably reflects a steady state. The increase in viable counts was poor, if any, and no reduction in viability was observed during the incubation period of 72 h studied. The difference in growth measured after incubation for 12 h between shaken and non-shaken cultures was significant (NCTT 11637, p < 0.01 and AH 28, p < 0.01). The fact that growth was much better in shaking flasks suggests that a good transfer of the gas mixture into the broth medium improves growth of *H. pylori*.

Killing activity of amoxycillin, clarithromycin and metronidazole in different bacterial growth conditions

The difference in growth of *H. pylori* with or without shaking (Fig. 3) was used to study whether the killing

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>pH value</th>
<th>ΔA_{660}</th>
<th>Amox</th>
<th>Cla</th>
<th>Ery</th>
<th>Tet</th>
<th>Met</th>
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<tr>
<td>N6</td>
<td>5.9</td>
<td>0.23</td>
<td>0.2</td>
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<td>ND</td>
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<tr>
<td>AH5</td>
<td>5.9</td>
<td>0.04</td>
<td>&gt;0.4</td>
<td>&gt;0.4</td>
<td>&gt;4</td>
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<tr>
<td>NTCC11637</td>
<td>5.9</td>
<td>0.10</td>
<td>&gt;0.4</td>
<td>&gt;0.4</td>
<td>&gt;4</td>
<td>2</td>
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<tr>
<td>N6</td>
<td>7.2</td>
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<td>0.025</td>
<td>0.025</td>
<td>0.12</td>
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<tr>
<td>N6KmV</td>
<td>7.2</td>
<td>0.60</td>
<td>0.012</td>
<td>0.012</td>
<td>0.25</td>
<td>2</td>
<td>ND</td>
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<td>AH5</td>
<td>7.2</td>
<td>0.14</td>
<td>0.05</td>
<td>0.025</td>
<td>0.12</td>
<td>2</td>
<td>1</td>
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<td>NTCC11637</td>
<td>7.2</td>
<td>0.32</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td>4</td>
<td>ND</td>
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<tr>
<td>N6</td>
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<td>0.012</td>
<td>0.0031</td>
<td>0.031</td>
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<td>ND</td>
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<td>0.16</td>
<td>0.0062</td>
<td>0.0031</td>
<td>0.031</td>
<td>0.25</td>
<td>ND</td>
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<tr>
<td>AH5</td>
<td>7.9</td>
<td>0.03</td>
<td>0.05</td>
<td>0.0016</td>
<td>0.016</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NTCC11637</td>
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<td>0.05</td>
<td>0.0031</td>
<td>0.062</td>
<td>1</td>
<td>ND</td>
</tr>
</tbody>
</table>

Amox, amoxycillin; Cla, clarithromycin; Ery, erythromycin; Tet, tetracycline; Met, metronidazole. ND, not done (strains are MtzR).
rate for amoxycillin, clarithromycin and metronidazole was growth dependent at pH 7. The results obtained for *H. pylori* strain AH 28 (Fig. 4) show that the bactericidal activity exerted by amoxycillin was slow in comparison with that of clarithromycin and metronidazole. The effect of amoxycillin and clarithromycin on slow or non-growing bacteria in the stationary culture was reduced compared with the effect on fast-growing bacteria in the shake-culture, indicating that the effect of amoxycillin and clarithromycin was growth rate dependent. For metronidazole, the highest killing rate was obtained in flasks without shaking, where the reduction in viable bacteria was almost 4 log$_{10}$ CFU/ml during incubation for 12 h. The results from this and other experiments with two different strains (NTCC 11374 and AH 28) show that the differences in killing rate between fast- and slow-growing bacteria are significant: amoxycillin (*n* = 4) *p* < 0.05, clarithromycin (*n* = 3) *p* < 0.05, and metronidazole (*n* = 3) *p* < 0.001.

**Interaction between omeprazole and amoxycillin**

The results obtained in checkerboard titration for the type strain of *H. pylori* NTCC 11637 and strain CCUG 15818 (FIC index = 0.625) indicated that the combined activity of the proton pump inhibitor, omeprazole (MIC = 64 µg/ml), and amoxycillin (MIC = 0.1 µg/ml) was additive rather than synergic.

**Discussion**

Although *H. pylori* is a fastidious organism requiring special culture conditions in vitro, i.e., micro-aerophilic or CO$_2$ 5–10% environments with an optimum temperature of 35°–37°C at pH 6.5–7.5 [34, 35], it adapts to a unique niche beneath the mucus layer of the gastric mucosa. Studies of the pH at this location in experimental animals indicate a pH of c. 7.0 [12, 13], and, therefore, protection against acid may not be required after adhesion of bacteria to epithelial cells.
Fig. 4. Growth and killing rates for different antibiotics in shaken (●) and stationary (○) cultures of *H. pylori* strain AH 28 grown in Brucella broth plus FCS 10% at pH 7.0 (−) and with antibiotics (−−−−) − a, amoxycillin 0.25 μg/ml; c, clarithromycin 0.25 μg/ml; m, metronidazole 4 μg/ml. The results of a single experiment are shown.

However, the passage through the acid lumen requires protective bacterial urease activity [9,10]. These results clearly show that, in vitro, *H. pylori* is sensitive to acid both in buffer and in broth, and that supplementation with urea increased survival at pH 3. We suggest that survival at this low pH is accomplished by a sufficient increase in the pH immediately surrounding the bacteria by the action of urease on urea, resulting in ammonium ion production. This was also reflected by an increase in medium pH to 3.65. These results are in agreement with those previously described by Marshall *et al.* [3]. Comparison of the effects of the potent urease inhibitor, flurofamide, and wild-type strain on a urease-deficient mutant suggests that the protection is mediated by urease.

In contrast to the finding at pH 3, supplementation with urea at both pH 7 and pH 9 adversely affected growth and cell viability. A suicidal effect reported by Greig *et al.* [8] was also suggested to be due to the urease activity. The killing mechanism at high pH in the presence of urea may, according to Neithercut *et al.* [36], depend on an imbalance in ammonium metabolism, resulting in a lack of α-ketoglutarate, which is a necessary intermediate in the biosynthesis of the glutamate family of amino acids.

Wild-type *H. pylori* did not grow in Brucella broth at pH 5 except in the presence of 6.25 mM urea, resulting in an increase in pH to 7 after incubation for 72 h. The urease-deficient mutant did not respond to urea supplementation. To increase the buffer capacity of Brucella broth and keep pH at a relatively stable level, the broth was prepared in 66 mM sodium-phosphate buffer, and in this medium the lowest pH at which *H. pylori* grew was 5.9. The optimum pH for growth of *H. pylori* in vitro has been reported to be c. 7.0 [5,6], which fits with the data in the present study. However, Kangatharalingam and Amy [7] recently reported growth of *H. pylori* at pH as low as 4.5, and urea neither stimulated growth further nor led to an increase in pH. Whether these contradictory results depend on the modified Brucella medium used by the group or other factors was not discussed by the authors.

*H. pylori* does not grow aerobically [37] and is categorised as an obligate micro-aerophile [7]. Shahmat *et al.* [38] compared a stationary growth system with an agitated system, and growth in shaken culture was much less than that in the stationary system. However, the present results are in agreement with those reported by Morgan *et al.* [6], showing only slight growth in flasks incubated without shaking. These data indicate that a good dispersion of the gas mixture throughout the liquid medium is essential for optimal growth of *H. pylori*.

MBCs for amoxycillin, erythromycin and clarithromycin were much higher at low pH. The reason for these differences in antibacterial activity depend either on pH effects directly on the antimicrobial agents, on
lower expression of bacterial targets or on lower binding. The effects of pH on the antibacterial activity of a range of antibiotics against H. pylori and other bacteria have been studied previously [14, 39] and, in accordance with what was reported, the present results show that susceptibility to tetracycline and metronidazole was not pH-dependent and not affected by the poor growth obtained at pH 5.9 and 7.9. On the contrary, slow-growing bacteria were more susceptible to metronidazole than fast-growing ones. However, this could be due to more anaerobic conditions prevailing in the flask without shaking, thus improving the anaerobic killing mechanism of metronidazole. The results of the present study further show that, at a higher growth rate at neutral pH, the bactericidal activity of amoxycillin and clarithromycin was enhanced, possibly due to an increase in target expression. However, the susceptibilities for amoxycillin, erythromycin and clarithromycin seem to be more pH-dependent than growth-dependent, as the poor growth at pH 7.9 compared to 7.2 did not increase MBCs. Growth patterns of strain N6 (the wild-type strain), and the isogenic urease-deficient mutant (N6KmV) seem to be different. At pH 5.9, growth of the wild-type strain was better than that of the mutant. However, at pH 7.9 the opposite was true, which further strengthens the difference in response to pH for the wild-type and the mutant strains.

However, these results clearly show that both the pH of growth medium and the growth rate of H. pylori affect antibiotic susceptibility in vitro, and we suggest that these factors may also play an important role in antibacterial activity in vivo.

The data provide further support for the hypothesis that, in vivo, omeprazole exerts a synergic effect with amoxycillin and other antibiotics [26, 40], probably by improving growth condition for H. pylori and thus improving the conditions for antibacterial action in vivo. Furthermore, the interaction obtained between omeprazole and amoxycillin in vitro was additive rather than synergic, a fact that would refute the suggestion that synergy in vivo is exerted via a direct action on the bacterium. The poor antibiotic effect exerted in vivo by a single drug may be attributed to the fact that important factors for growth of H. pylori or for antibacterial efficacy vary substantially between different parts of the gastric and duodenal mucosa. These results suggest that pH, growth rate and possibly the partial pressure of oxygen, affecting the killing mechanism of antibiotics, are such important factors. This interpretation provides support for the findings that omeprazole, in combination with antibiotics, enhances the efficacy of eradication of H. pylori.

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References

EFFECT OF pH AND UREA ON SURVIVAL OF H. PYLORI


