

## STOMACH

<sup>13</sup>C urea breath test (UBT) in the diagnosis of *Helicobacter pylori*: why does it work better with acid test meals?

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**Background:** Acid test meals may improve the accuracy of the <sup>13</sup>C urea breath test (UBT). This has been attributed to changes in gastric emptying rather than to the effects of gastric pH on *Helicobacter pylori* urease.

**Aims:** To determine whether enhancement of <sup>13</sup>CO<sub>2</sub> excretion in the UBT in *H pylori* infected volunteers by acidification of a test meal is due to a delay in gastric emptying.

**Methods:** Urease activity in vitro was measured in intact bacteria and in bacterial homogenates. Urease activity in vivo was assessed by means of the UBT. Eleven *H pylori* infected subjects underwent UBTs with neutral Ensure (pH 7.0), acidified Ensure (pH 3.0), and apple juice (pH 3.0). Gastric emptying was assessed by <sup>13</sup>C sodium acetate breath test.

**Results:** From pH 7 to pH 3, the in vitro urease activity of intact bacteria increased sixfold. In contrast, urease activity of bacterial homogenates was inactivated by low pH. In vivo, urease activity, as measured by the UBT 20 minutes after meal ingestion, was higher with apple juice ( $\delta^{13}\text{CO}_2=21.1$ ;  $p=0.03$ ) and acidified Ensure ( $\delta^{13}\text{CO}_2=25.5$ ;  $p=0.01$ ) than with neutral Ensure ( $\delta^{13}\text{CO}_2=12.5$ ). Gastric emptying was faster with apple juice ( $T_{\text{max}}=36.7$  (8) minutes) but not with acidified Ensure ( $T_{\text{max}}=63.3$  (5) minutes;  $p=0.06$ ) than with neutral Ensure ( $T_{\text{max}}=65.0$  (3) minutes;  $p=0.04$ ).

**Conclusions:** The higher UBT found with acidified compared with neutral test meals was independent of the emptying rates of the test meals but may have been due to medium acidity dependent activation of intra-bacterial urease in intact *H pylori*.

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The <sup>13</sup>C urea breath test (UBT) is widely used for determination of *H pylori* infection.<sup>1</sup> On average, this test is quite accurate<sup>2</sup> but false negative results are obtained in conditions which are frequently encountered in *H pylori* infected subjects, such as atrophic gastritis<sup>3</sup> and acid suppression by acid pump inhibitors.<sup>4-8</sup> It was argued that the accuracy of the UBT may be improved by prolonging the contact of the test meal with *H pylori* urease.<sup>9-10</sup> As citric acid slows gastric emptying and improves the accuracy of the UBT, it was concluded that citric acid had a favourable effect because of its slowing effect on gastric emptying.<sup>9-11</sup> Acidification of test meals has become a standard recommendation.<sup>1</sup>

In this study we examined an alternative mechanism for the effect of acidification by citric acid. The activity of intra-bacterial urease, which represents the major compartment of *H pylori* urease,<sup>12</sup> depends on the medium pH, as medium acidity activates urea entry via activation of UreI, the urea channel in the inner membrane of *H pylori*. This increased urea entry in acidic medium results in increased urease activity,<sup>13,14</sup> and hence increased acidity of the bacterial environment is predicted to increase the UBT.

## MATERIALS AND METHODS

### Assessment of *H pylori* urease activity at different pH values in vitro

#### Growth of *H pylori*

*H pylori* strain ATCC43504 was grown on blood agar plates (BBL TSA 5% sheep blood; Becton-Dickenson, Franklin Lakes, New Jersey, USA) in a microaerobic atmosphere (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) at 37.0° C for 24 hours. Cells from one plate were harvested in 300 µl of 1 mM phosphate buffer, pH 7.0, from which aliquots were taken for the urease assay described below.

### Urease assay in vitro

The method chosen was release of radioactive <sup>14</sup>CO<sub>2</sub> from <sup>14</sup>C urea to resemble the UBT, as previously described.<sup>15</sup> The incubation medium contained 5 mM labelled <sup>14</sup>C urea with a specific activity of 10 µCi/µmol. In the experiments we used 100 mM sodium phosphate buffer at pH 3.0, 5.0, and 7.0 containing NaCl 138 mM, KCl 5 mM, CaCl<sub>2</sub> 1 mM, MgCl<sub>2</sub> 2 mM, glucose 10 mM, and glutamine 1 mM. pH was adjusted with HCl. pH did not change over the 30 minute measurement period carried out at 37°C and urease activity was linear with time. Incubation was carried out in a tube sealed with a rubber stopper that contained a well with a filter paper soaked in 0.5 N KOH. <sup>14</sup>CO<sub>2</sub> generated was trapped in the well following injection of 5 N H<sub>2</sub>SO<sub>4</sub> into the medium. Counting was done in an LKB scintillation counter. Protein determination was performed by the Lowry method.<sup>16</sup> Urease activity was measured as a function of varying medium pH, both in a bacterial homogenate generated by French press treatment and in intact bacteria. The former gives total urease activity, the latter activation of intra-bacterial urease.

### Assessment of *H pylori* urease activity at different pH values in vivo

#### Study population

Eleven *H pylori* positive subjects (eight men, three women; age range 26-36 years) participated in this crossover randomised study. Eleven *H pylori* negative subjects served as controls (six men, five women; age range 23-36 years). All were healthy with no history of gastrointestinal disease or other illness, and

**Abbreviations:** UBT, <sup>13</sup>C urea breath test; DOB, delta over baseline; T<sub>max</sub>, time of maximal gastric emptying rate; T<sub>1/2</sub>, half emptying time.

no current gastrointestinal symptoms. The volunteers were not taking any medications other than oral contraceptives. Subjects who had taken antibiotics, proton pump inhibitors, H<sub>2</sub> receptor antagonists, and bismuth containing preparation in the four weeks before enrolment were excluded from the study. The study was conducted according to the Declaration of Helsinki and the rules of Good Clinical Practice.

### Diagnosis of *H pylori* infection

The presence or absence of *H pylori* infection was diagnosed at enrolment by an enzyme linked immunosorbent assay (ELISA; Roche, Switzerland) and a standard UBT. Fasting blood samples were collected for determination of specific IgG anti-*H pylori* antibodies. The standard <sup>13</sup>C UBT was performed using 100 mg of <sup>13</sup>C urea and orange juice as a test meal, as previously described.<sup>2</sup> Baseline and 30 minute breath samples were analysed by isotope ratio mass spectrometer (PDZ Europa, Crewe, UK). The diagnostic cut off value for the standard <sup>13</sup>C-UBT was  $\delta^{13}\text{CO}_2 = 5.0\%$  delta over baseline (DOB).<sup>17</sup>

### Study protocol

Each volunteer underwent three different UBTs in a crossover randomised design. After an overnight fast, the following test meals were administered: standard Ensure (237 ml, 250 kcal, 9 g protein, 40 g carbohydrate, 6 g fat; pH 6.8), Ensure acidified with 4 g of citric acid (237 ml, 250 kcal, 40 g carbohydrate, 9 g protein, 6 g fat; pH 3.0), and apple juice (200 ml, 45 kcal, 11 g carbohydrate, 0 g protein, 0 g fat; pH 3.0). A commercial apple juice was used. Its composition, as stated by the manufacturer, was: citric acid (4 g), ascorbic acid (2 g), malic acid (2 g), and small amounts of isocitric acid and *cis*-acotinic acid with a resulting pH of 3.

Breath samples were collected into plastic breath collectors (Alimenterics, Inc., New York, USA) at baseline and at 10 minute intervals over 60 minutes after administration of the test meal with <sup>13</sup>C urea. <sup>13</sup>C labelled urea (99%) was administered in a solution of distilled water (5 ml) immediately after ingestion of a test meal. <sup>13</sup>C/<sup>12</sup>C ratio was measured by the Laser Assisted Ratio Analyzer (LARA System; Alimenterics Inc.).<sup>17</sup> Values are expressed as  $\delta$  excess over baseline per ml. The diagnostic cut off value for our laboratory is  $\delta^{13}\text{CO}_2 = 5.0\%$  DOB.<sup>17</sup>

### Gastric emptying studies

Gastric emptying of test meals was assessed by <sup>13</sup>C sodium acetate breath test in 11 *H pylori* positive volunteers. Breath samples for gastric emptying studies were obtained at baseline and at 10 minute intervals over 120 minutes after administration of a test meal with 150 mg of <sup>13</sup>C labelled sodium acetate.<sup>18</sup> <sup>13</sup>CO<sub>2</sub> excretion was measured, as above, by the Laser Assisted Ratio Analyzer System (Alimenterics Inc.). The results were expressed as percentage of <sup>13</sup>C dose recovered (<sup>13</sup>C-PDR). <sup>13</sup>C-PDR was calculated according to the formula of Schoeller and colleagues.<sup>19</sup>

$$^{13}\text{C-PDR} = \frac{((^{13}\delta_t + ^{13}\delta_{t+1})/2) \times (t_{+1} - t) \times R_{\text{PDR}} \times 10^{-3} \times \text{CO}_2 / (D/M_t) \times (P \times n/100))}{100\%}$$

where  $^{13}\delta = ((R_t/R_{\text{PDR}}) - 1) \times 10^3 - ((R_0/R_{\text{PDR}}) - 1) \times 10^3$  ( $R_t = ^{13}\text{C}:^{12}\text{C}$  in the sample at time  $t$ ;  $R_0 = ^{13}\text{C}:^{12}\text{C}$  in the sample at baseline;  $R_{\text{PDR}} = ^{13}\text{C}:^{12}\text{C}$  in international standard PeeDeeDelemonite = 0.0112372);  $\text{CO}_2$  is  $\text{CO}_2$  production rate;  $D$  is the dose of substrate administered;  $M_t$  is molecular weight of the substrate;  $P$  is <sup>13</sup>C atom % excess; and  $n$  is the number of labelled carbon positions.<sup>19</sup> Total  $\text{CO}_2$  production rate was assumed to be 5 mmol/m<sup>2</sup> of body surface/min.

Body surface area was calculated according to the formula of Haycock and colleagues.<sup>20</sup>

$$\text{Body surface area (m}^2\text{)} = H^{0.396} \times W^{0.5378} \times 0.02465$$

where  $H$  is height (m) and  $W$  is weight (kg).

### Statistical methods

DOB values obtained in UBT studies were analysed using Sigma Stat 2.0 software (SPSS Inc., Chicago, Illinois, USA). The Komolgorov-Smirnov test was applied to test the normality of the distribution of the data. As the data were not normally distributed, the overall effect of different test meals on <sup>13</sup>CO<sub>2</sub> excretion was assessed by the Friedman repeated measures analysis of variance on ranks. To isolate the group or groups that differed from the others, we used Dunnett's method as the all pairwise comparison procedure. Pairwise comparisons were made using the Wilcoxon signed rank test. Data are presented as group median values with interpercentile range (10–90 centiles). Values of  $p < 0.05$  were considered significant.

The data obtained by the <sup>13</sup>C acetate breath test were fitted by a mathematic formula using the least squares procedure.<sup>18</sup> The formula is given by:

$$y = a^b e^{-ct}$$

where  $y$  is the percentage of <sup>13</sup>CO<sub>2</sub> excretion per hour;  $t$  is time in hours; and  $a$ ,  $b$ ,  $c$  are constants.

Cumulative <sup>13</sup>C recovery data were fitted to the formula:

$$y = m (1 - e^{-kt})^b$$

which is the inverse of the scintigraphic radioactivity decay curve.<sup>21</sup> From these constants, the time of maximal gastric emptying rate  $T_{\text{max}} = b/c$  and the half emptying time  $T_{1/2} = -1/k \ln(1 - 2^{-1/b})$ , were calculated. Non-linear regression analysis of these formulas was performed using a commercial software program (NLREG 5.0, Brentwood, Tennessee, USA).

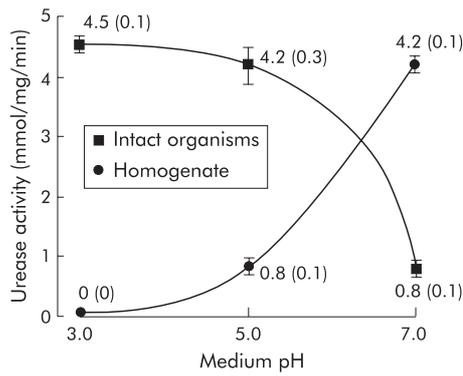
## RESULTS

### In vitro urease activity at different medium pH values

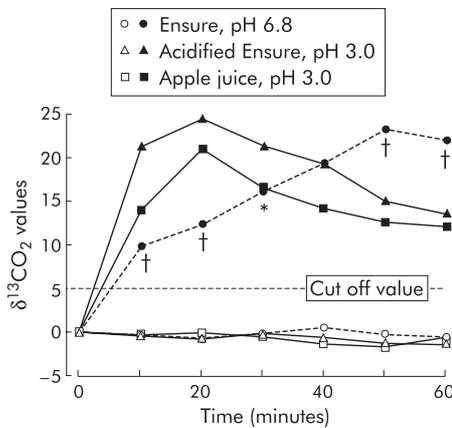
The results of the urease assays at pH values of 7.0, 5.0, and 3.0 are shown in fig 1. Urease activity of intact bacteria is low at pH 7.0, rises steeply at pH 5.0, and remains essentially the same at pH 3.0. The opposite is true for urease activity assayed in bacterial homogenates where activity is maximal at pH 7.0, declines steeply at pH 5.0, and is absent at pH 3.0. These data are in accordance with previously published results showing that the enzyme has a pH optimum close to neutrality and is irreversibly inactivated below pH 4.5 in bacterial homogenates.<sup>14</sup> In contrast, urease activity of the intact bacteria increases with acidification of the medium due to increased urea permeability via UreI, allowing a large increase in urea access to intra-bacterial urease.<sup>14</sup>

### <sup>13</sup>C UBT studies

All 11 *H pylori* negative and 11 *H pylori* positive subjects completed the study. Group median  $\delta$  values are shown in fig 2. Median <sup>13</sup>CO<sub>2</sub> excretion rates at 10, 20, and 30 minutes were lower with standard Ensure at a pH of 6.8 than with acidified Ensure and apple juice, both initially at pH 3 ( $p < 0.05$ ) (fig 2). There were no significant differences in <sup>13</sup>CO<sub>2</sub> excretion between the three <sup>13</sup>C UBT protocols at 40 minutes (fig 2). The amount of <sup>13</sup>CO<sub>2</sub> excretion was higher with Ensure than with both acidic test meals only during the 50 minute and 60 minute periods of the study ( $p < 0.05$ ) (fig 2). Hence the major effect of acidification occurred between 0 and 30 minutes, presumably because of buffering of the administered acidity, blunting the stimulation of urea entry, with buffering by the citric acid in the acidified Ensure being more effective than buffering in apple juice.



**Figure 1** In vitro urease activity in intact (n=3) and homogenised *Helicobacter pylori* (n=3). In the intact organism, urease activity increased sixfold as the medium pH declined whereas urease activity of the bacterial homogenate decreased with a fall in medium pH. Values are mean (SEM).



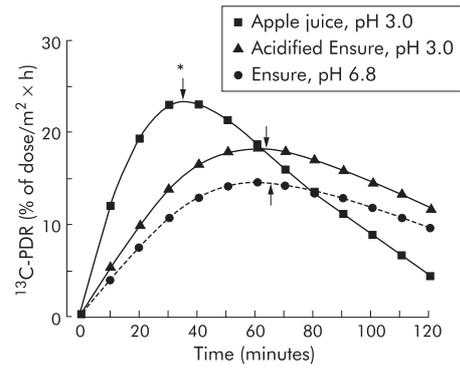
**Figure 2** In vivo urease activity with test meals (see methods) in *Helicobacter pylori* positive and negative subjects. Median  $\delta^{13}\text{CO}_2$  values of *H pylori* positive subjects are shown in table 1. \*p<0.05 versus acidified Ensure pH 3.0; †p<0.05 versus apple juice and acidified Ensure pH 3.0. Filled symbols depict values in *H pylori* positive subjects; open symbols in *H pylori* negative subjects.

All *H pylori* negative subjects had  $^{13}\text{CO}_2$  excretion values below the cut off value of 5.0% DOB at all collection times (fig 2).

**Gastric emptying studies**

The results are shown in fig 3. Gastric emptying was faster with apple juice ( $T_{\text{max}}=36.7$  (8) minutes) but not with acidified Ensure ( $T_{\text{max}}=63.3$  (5) minutes; p=0.06) than with neutral Ensure ( $T_{\text{max}}=65.0$  (3) minutes; p=0.04).

Similarly, the  $T_{1/2}$  of gastric emptying was lower with apple juice (71.7 (9) minutes; p<0.05) than with both Ensure meals



**Figure 3** Gastric emptying (see methods) of the three types of test meals. Data are medians of 11 *Helicobacter pylori* positive subjects. Arrows indicate the time of  $T_{\text{max}}$ . \*p=0.03 and 0.04 versus Ensure pH 6.8 and acidified Ensure pH 3.0, respectively.  $^{13}\text{C}$ -PDR, percentage of  $^{13}\text{C}$  dose recovered.

(acidified Ensure 103.4 (7) minutes; neutral Ensure 105.6 (6) minutes) (fig 3). Acidification of the Ensure meal did not slow gastric emptying, contrary to the hypothesis explaining the effect of citric acid on  $\text{CO}_2$  excretion<sup>9</sup>.

**DISCUSSION**

We have confirmed previous observations in *H pylori* infected subjects<sup>10 11 22</sup> that had shown that acidification of a test meal by citric acid enhanced  $^{13}\text{CO}_2$  excretion in a UBT. Apple juice which contains a variety of organic acids<sup>23</sup> also increases  $^{13}\text{CO}_2$  excretion.

It has been known since the classical studies by Hunt and Knox,<sup>24</sup> and recently been described by Shiotani and colleagues,<sup>25</sup> that organic acids, in particular citric acid, slow gastric emptying in a dose dependent manner. This has led to the hypothesis that citric acid increases  $^{13}\text{CO}_2$  excretion because it slows gastric emptying and thus prolongs contact of the test meal with *H pylori* and its urease.<sup>9 10 22</sup>

To test the gastric emptying hypothesis, we performed gastric emptying tests in *H pylori* positive volunteers who had undergone UBTs. Ensure meals were emptied more slowly than apple juice which had a pH identical to the acidified Ensure meal. The emptying rates of the neutral and Ensure test meal acidified with citric acid were identical. The difference in gastric emptying between Ensure test meals and apple juice is explained by the different caloric density of the test meals.<sup>26</sup> Ensure as a meal with higher caloric density empties less rapidly than apple juice. Thus when using a test meal of comparable caloric content but different pH, the rate of gastric emptying is determined by its caloric density and not by its pH. The observations reported here show that slowed gastric emptying does not explain enhancement of the UBT induced by acidification of the test meal.

Our in vitro results suggest that the pH at the gastric surface activates *H pylori* urease by activating urea entry via UreI, and

**Table 1** Median  $\delta^{13}\text{CO}_2$  values (10–90 centiles) of *Helicobacter pylori* positive subjects

	Ensure (pH=6.8)	Acidified Ensure (pH=3.0)	Apple juice (pH=3.0)
Collection time			
10 min	9.9 (3.6–14.0)†	21.4 (10.9–36.4)	14.0 (6.5–34.3)
20 min	12.5 (3.3–28.4)†	24.5 (12.9–41.5)	21.1 (13.3–42.6)
30 min	16.8 (6.1–35.2)*	21.4 (17.6–49.5)	16.7 (11.9–45.6)
40 min	19.5 (10.1–39.5)	19.3 (15.6–48.1)	14.3 (10.4–37.2)
50 min	23.4 (12.7–45.1)†	15.1 (12.1–30.1)	12.7 (7.2–29.9)
60 min	22.1 (16.3–49.7)†	13.7 (10.1–20.4)	12.3 (5.6–24.9)

\*p<0.05 versus acidified Ensure pH 3.0; †p<0.05 versus apple juice and acidified Ensure pH 3.0.

that this effect is the main determinant of the acidity dependent  $^{13}\text{CO}_2$  excretion rate in vivo.

In vitro, external acidity leads to stimulation of urease activity in intact bacteria (fig 1) due to facilitation of urea access across the bacterial inner membrane to intra-bacterial urease via the proton gated urea channel UreI.<sup>13,14</sup> Thus when acid in the form of apple juice or acidified test meals is given to *H pylori* infected volunteers, urease is stimulated due to acidification of the bacterial environment, and increased  $^{13}\text{CO}_2$  excretion in vivo occurs rapidly (fig 2) compared with neutral pH meals. As similar  $^{13}\text{CO}_2$  excretion was observed with an Ensure test meal containing only citric acid as an acidifier and apple juice, a test meal which had an identical pH but contained a large variety of organic acids, especially malic acid,<sup>23</sup> we suggest, in contrast with previous authors,<sup>9</sup> that it is the pH and not the type of organic acid that determines the magnitude of the  $^{13}\text{CO}_2$  response.

It has previously been observed that administration of neutral Ensure led to an increase in intragastric pH to ~4.0 and to increased plasma gastrin levels.<sup>27</sup> One hour after this neutral Ensure meal, intragastric pH started to fall. Hence maximal stimulation of intra-bacterial urease activity would be delayed by one hour compared with acidified Ensure.<sup>27</sup> This explains the rapid increase in  $^{13}\text{CO}_2$  excretion with acidified Ensure compared with neutral Ensure, as observed here.

Prior studies have shown a dose-response effect with citric acid but no dose-response effect with ascorbic acid solutions in spite of their similar pH values.<sup>9</sup> In addition, endogenous stimulation of acid secretion by pentagastrin appeared not to have an effect on urease activity.<sup>9</sup> It has therefore been argued that acid does not play a direct role in  $^{13}\text{CO}_2$  excretion in UBTs.<sup>28</sup> The lack of effect of ascorbic acid compared with citric acid can be explained by the lower buffering capacity of the monocarboxylic ascorbate compared with the tricarboxylic citric acid. In addition, ascorbic acid is a potent inhibitor of a variety of bacterial ureases, including *H pylori* urease.<sup>29</sup> Hence the dose dependent effect of citric acid can be explained by its increased buffering capacity and its ability to maintain a low pH in the bacterial environment<sup>30</sup> and perhaps also by its ability to cross the diffusional resistance of the gastric mucus at higher concentrations.<sup>31,32</sup> Pentagastrin injection increases gastric acid secretion but does not necessarily reduce pH at the site of bacterial colonisation. Juxtamucosal pH has been claimed to be maintained at a relatively neutral pH during pentagastrin stimulated acid secretion pH 1, unless acid is applied intraluminally.<sup>33</sup> Administration of pentagastrin also leads to increased mucus gel thickness.<sup>34</sup>

The UBT may give false negative data in *H pylori* infected subjects with atrophic gastritis<sup>3</sup> and, in particular, during treatment with proton pump inhibitors.<sup>4,8</sup> When these subjects undergo UBTs with meals containing citric acid, the diagnostic accuracy of the test is improved.<sup>10,11,22</sup> It has been argued that a false negative UBT in subjects on proton pump inhibitor therapy is due to a decreased bacterial load.<sup>28</sup> However, more likely, as proton pump inhibitor therapy increases intragastric pH, intra-bacterial urease activity will decrease.<sup>35</sup> Intragastric titration from pH 3 to 5 and to 7 led to a decrease in urease activity in vivo, as assessed by the  $^{13}\text{C}$ -UBT.<sup>36</sup>

Acidification increased *H pylori* load in gastric aspirates from chronic proton pump inhibitor treated subjects.<sup>37</sup> Chronic proton pump inhibitor treatment may therefore result in a decreased bacterial load. However, immediate improvement in the rapid urease test at pH 7.0 found with acidification cannot be due to an immediate change in the bacterial load.<sup>37-39</sup> Thus the explanation for the decrease in UBT during proton pump inhibitor treatment is more likely due to the pH increase induced by proton pump inhibitors and a decrease in intra-bacterial urease activity because of inactivation of urea entry into the organism. After chronic therapy, a decrease in the number of bacteria may also contribute to the decrease in

UBT. Acidification of the test meal is therefore desirable for accurate UBT results, particularly in subjects on proton pump inhibitor therapy.

In conclusion, we suggest that activation of the cytoplasmic urease by urea entry into the cytoplasm of *H pylori* rather than gastric emptying may explain the increased  $^{13}\text{CO}_2$  excretion when an acid test meal is given.

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