Suicidal destruction of Helicobacter pylori: Metabolic consequence of intracellular accumulation of ammonia

W D Neithercut, M A Greig, M Hossack, K E L McColl

Abstract

The role of pH, citrate buffer, and urea were investigated in the suicidal destruction of *Helicobacter pylori*, with particular reference to the organism's urea and ammonia metabolism. The median five minute survival of *H pylori* in the presence of 50 mmol/l urea in 0·2 M citrate buffer at pH 6·0 was only 14%, compared with 53% in the same solution at pH 7·0. The median amount of ammonium released into the incubating solution over five minutes was lower at pH 6·0 (9 μmol) than at pH 7·0 (18 μmol) despite similar uptake of urea. The median five minute survival of *H pylori* in 0·2 M citrate buffer, pH 6·0, decreased from 89% to 14% when the urea concentration was increased from 1 mmol/l to 50 mmol/l. Likewise, the recovery in the incubating solution of ammonia resulting from the hydrolysis of urea fell from 27% to 3% when the initial urea concentration was increased from 1 mmol/l to 50 mmol/l. Survival of *H pylori* in the presence of 30 mmol/l urea at pH 6·0 was compared in 0·2 M citrate, acetate, and phosphate buffers. The median five minute survival was less in the citrate buffer, at 29%, than in either the acetate buffer 80% or the phosphate buffer 100%. The percentage recovery of ammonia was similar in the three buffers.

These findings indicate that the suicidal destruction of the bacterium may be explained by intracellular accumulation of ammonia due to production in excess of the rate of excretion.

University Department of Pathological Biochemistry, Western Infirmary, Glasgow
W D Neithercut

University Department of Bacteriology and Immunology
M A Greig

University Department of Medicine and Therapeutics
M Hossack
K E L McColl

Correspondence to:
Dr W D Neithercut, Department of Chemical Pathology, Arrowe Park Hospital, Arrowe Park Road, Upton, Wirral, Merseyside L49 5PE.

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Colonisation of the gastric antral mucosa by *Helicobacter pylori* is associated with the development of duodenal ulcers.[1][2] Eradication of the organism from the gastric mucosa reduces the rate of relapse from 84% to 21% a year.[3–5]

Despite its ability to colonise the gastric mucosa, the organism does not survive well under conditions of high acidity.[6][7] The presence of physiological concentrations of urea, however, enhances the survival of the organism at low pH.[6–8] It has been suggested that the production of ammonia by the organism's high urease activity may create an "alkaline microenvironment" and thereby promote its survival in acidic conditions.[9]

In contrast to the protective effect of urea at acidic pH, we recently observed that the organism rapidly dies in the presence of urea in citrate buffer, pH 6·0.[10] The speed of death under these conditions suggests suicidal destruction mediated by the organism's urease activity. To investigate the biochemical basis of the suicidal process we compared the organism's urea metabolism in environments causing its rapid death with those in which it survives.

Methods

As our earlier studies had indicated that pH, urea concentration, and type of buffer may have contributed to the suicidal process, the effect of each of these on the organism's urea metabolism and survival was examined.

1 *Effect of pH* The survival of the organism, its urea consumption, and ammonia release in 0·2 M citrate buffer (pH 6·0) containing 50 mmol/l urea were compared with those in the same solution at pH 7·0. Similar studies were also conducted with unbuffered isomolar saline at pH 6·0 containing 50 mmol/l urea and 0·2 M citrate buffer (pH 6·0) without urea.

2 *Effect of urea concentration* The effect of increasing initial urea concentration on urea consumption, ammonia release, and survival of the organism in 0·2 M citrate buffer (pH 6·0) was investigated. The following urea concentrations were examined: 1 mmol/l; 5 mmol/l; 10 mmol/l; 20 mmol/l; 30 mmol/l; and 50 mmol/l.

3 *Effect of buffer ammonium ion concentration* The effect of the addition of 10 mmol/l ammonium chloride to 0·2 M citrate buffer (pH 6·0) on the survival of the organism was investigated.

4 *Effect of type of buffer* The survival of the organism in the presence of 30 mmol/l urea in 0·2 M citrate buffer (pH 6·0) was compared with survival in 0·2 M acetate buffer (pH 6·0) containing 30 mmol/l urea, and with 0·2 M phosphate buffer (pH 6·0) also containing 30 mmol/l urea. Urea consumption and percentage ammonia release were also measured.

To start each experiment 1 ml of a 72 hour broth culture suspension (BHI broth, 0·25% yeast extract, and 10% horse serum) of the NCTC 11637 strain of *H pylori* was added to 9 ml of buffer solution containing urea, or to buffer without urea, and to isomolar saline

Table 1 Comparison of survival and ammonium production at pH 6·0 and pH 7·0

Buffer	Urea hydrolysed μmol/5 min Median and (range)	NH₄+ produced μmol/5 min Median and (range)	% Survival Median and (range)
0·2 M citrate pH 6·0 50 mmol/l urea n = 8	154 (126–162)	9* (7–10)	14* (0–22)
0·2 M citrate pH 7·0 50 mmol/l urea n = 8	162 (73–188)	18 (8–21)	53 (31–112)
Isomolar saline pH 6·0 50 mmol/l urea n = 8	200 (182–225)	10 (7–12)	65 (29–113)
0·2 M citrate no urea pH 6·0 n = 8			60 (33–114)

*p < 0·01.

used as controls. The suspensions were incubated at 37°C for five minutes. Samples were collected at the start and end of the five minute incubation for bacterial culture, urea, and ammonium analyses. Each experiment was repeated to obtain a minimum of seven replicates for statistical analysis.

The method of Miles and Misra was used to establish survival of the organism in the timed samples.[11] Viable colony counts were obtained on blood agar plates after six days of incubation at 37°C in a microaerophilic atmosphere (BBL CampyPak gas-generating system). Survival of the organism was expressed as the percentage of starting inoculum calculated from the dilution of the viable colony count in the initial broth suspension.

The 1 ml sample collected for urea and ammonium analysis was filtered (Gelman Sciences, Acrodisc 0·2 μm) to remove the organism, snap frozen, and stored at −20°C until analysis. The urea concentration was measured by the o-phthalaldehyde method using a perspective analyser (American Monitor, West Sussex). The ammonium concentration was measured following dilution in 0·2 M phosphate buffer (pH 7·4) by an enzymatic method (Sigma Chemical Company, Dorset), adapted for the Cobas Bio centrifugal analyser (Roche, Welwyn Garden City).

The percentage recovery of ammonia was calculated, assuming complete hydrolysis of urea removed from the incubate by the bacterium. The five minute urea concentration in the buffer was subtracted from the initial urea concentration and the result multiplied by two to give the expected final ammonia concentration. The measured concentration of ammonia was then expressed as a percentage of the expected final ammonia concentration calculated from the fall in urea concentration.

The stock buffer solutions used were 0·2 M sodium citrate buffers (pH 6·0) and (pH 7·0), 0·2 M sodium acetate buffer (pH 6·0), and 0·2 M sodium phosphate buffer (pH 6·0). Isomolar sodium chloride solution was adjusted to pH 6·0 before use. To obtain a range of urea concentrations stock solutions with urea concentrations of 0·1 M, 0·5 M, 1 M, 2 M,

3 M and 5 M were made. All the stock solutions were stored at −20°C until use. To set the initial urea concentration in the buffer 100 μl of the appropriate stock urea solution was added to 8·9 ml of the required buffer to give urea concentrations of 1 mmol/l, 5 mmol/l, 10 mmol/l, 20 mmol/l, 30 mmol/l, or 50 mmol/l in the final suspension. Buffers were equilibrated at 37°C and their pH checked before each experiment.

All reagents used were analar grade. The statistical method used to analyse the data was the Mann-Whitney U test.

Results

EFFECT OF pH

The median five minute survival of H pylori, when incubated at 37°C in 0·2 M sodium citrate buffer (pH 6·0) with an initial urea concentration of 50 mmol/l was only 14% (range 0–22%) compared with 53% (range 31–112%) when incubated in the same solution but at pH 7·0 (p < 0·01) (fig 1). The survival of the organism in citrate buffer at pH 6·0 with 50 mmol/l urea was also reduced in comparison with survival in isomolar saline containing 50 mmol/l urea (median 65%, range 29–113%) (p < 0·01) and with survival in 0·2 M sodium citrate buffer (pH 6·0) without any added urea (median 60%, range 33–114%) (p < 0·01) (fig 1).

Urea utilisation was similar in pH 6·0 buffer and in pH 7·0 buffer, being 154 μmol/five minutes (126–162 μmol) and 162 μmol/five minutes (73–188 μmol), respectively (table 1). The median five minute amount of ammonium found in the incubate at pH 7·0 was 18 μmol, however (range 8–21 μmol), which was significantly greater than at pH 6·0 (9 μmol) (range 7–10 μmol) (p < 0·01) (table 1).

EFFECT OF UREA CONCENTRATION

The median five minute survival of the organism in 0·2 M citrate buffer (pH 6·0) progressively fell with increasing initial urea concentrations from 89% (range 19–124%) at 1 mmol/l to 39% (range 13–87%) at 10 mmol/l,
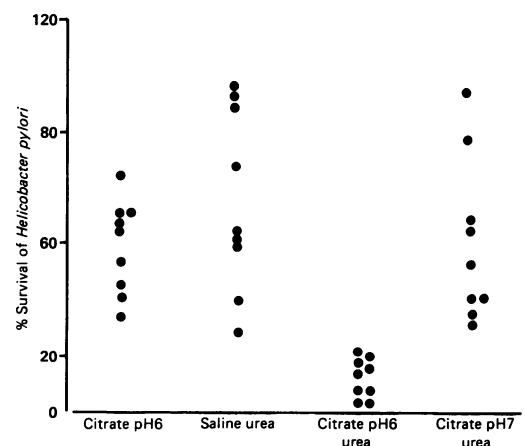

Figure 1 Percentage survival of H pylori NCTC 11637 at the end of a five minute incubation (n = 9), using 0·2 M citrate buffer (pH 6·0), isomolar saline containing 50 mmol/l urea, 0·2 M citrate buffer (pH 6·0) containing 50 mmol/l urea, and 0·2 M citrate buffer (pH 7·0) containing 50 mmol/l urea.

Figure 2 Amount of urea used by H pylori during the five minute incubation in 0·2 M citrate buffer (pH 6·0) containing initial urea concentrations from 1 to 50 mmol/l. Urea concentrations of 1 to 20 mmol/l (n = 7), 30 mmol/l (n = 17), 50 mmol/l (n = 9).
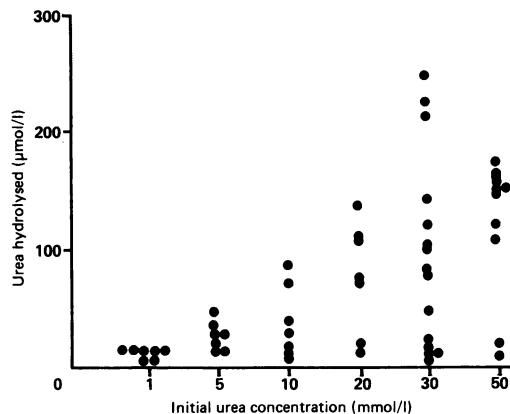
29% (range 10–64%) at 30 mmol/l and to 9% (range 0–22%) at 50 mmol/l. Although the median percentage survival of the organism fell as the initial urea concentration increased, the amount of urea used by the organism during the five minute incubation increased from 9 μmol (range 7–9 μmol) at an initial urea concentration of 1 mmol/l to 32 μmol (range 14–92 μmol) at 10 mmol/l (p < 0·05), 98 μmol (range 15–281 μmol) at 30 mmol/l (p < 0·01), and 146 μmol (range 11–171 μmol) at an initial urea concentration of 50 mmol/l (p < 0·01) (fig 2). The percentage recovery of ammonia from the hydrolysis of urea fell as the initial urea concentration increased (fig 3). With an initial urea concentration of 1 mmol/l, the median five minute recovery of ammonia was 27% (range 7–94%) compared with 18% (range 15–80%) at 10 mmol/l, 16% (range 4–315%) at 30 mmol/l, and only 3% (2–22%) at 50 mmol/l urea (p < 0·01 compared with 1 mmol/l urea).

EFFECT OF BUFFER AMMONIUM ION CONCENTRATION

The survival of the organism in 0·2 M citrate buffer (pH 6·0) in the presence of 10 mmol/l ammonium chloride was investigated. The median ammonium concentration of the 12 replicate experiments when measured at the start was 10·7 mmol/l (range 9·7–13·1 mmol/l) and at the end it was 10·9 mmol/l (range 10·6–11·3 mmol/l). The five minute survival of the organism in buffer with ammonium chloride (median 64%, range 20–137%) was similar to its survival in 0·2 M sodium citrate buffer (pH 6·0), without any additions (median 60%, range 33–148%).

EFFECT OF TYPE OF BUFFER

The median five minute survival in buffer

Figure 3 Recovery of ammonia, expressed as a percentage of the amount of ammonia expected to have been formed as a result of hydrolysis of urea by H pylori in 0·2 M citrate buffer (pH 6·0) with initial urea concentrations from 1 to 50 mmol/l. Urea concentrations of 1 to 20 mmol/l (n = 7), 30 mmol/l (n = 17), and 50 mmol/l (n = 9).
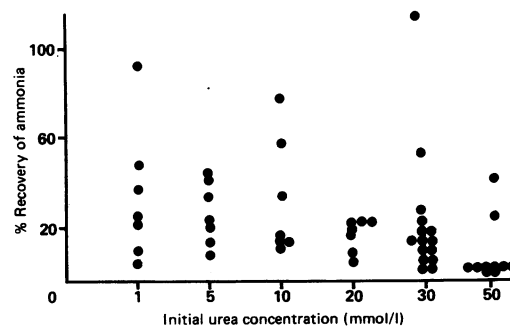
pH 6·0 in the presence of 30 mmol/l urea when citrate buffer was used was 29% (range 10–64%) compared with 80% (range 26–160%) when acetate buffer was used (p < 0·01) (table 2). The amount of urea used by the organism in the acetate buffer (median 215 μmol, range 18–272 μmol) was not significantly different from that in the citrate buffer (median 97 μmol, range 5–282 μmol). The median five minute ammonium release was similar in the acetate buffer (33 μmol, range 7–132 μmol) to that in the citrate buffer (19 μmol, range 7–41 μmol). The percentage of ammonia recovered from the hydrolysis of urea in the acetate buffer (12·1%) was also similar to that (8·6%) in the citrate buffer. In the absence of urea the five minute survival in citrate buffer (pH 6·0) was still reduced (68%, range 33–163%) when compared with survival in the acetate buffer at pH 6·0 (95%, range 36–211%) (p < 0·05).

The median five minute survival of *H pylori* in the presence of 30 mmol/l urea in phosphate buffer at pH 6·0 (100%, range 72–128%) was also greater than that in citrate buffer (42%, range 27–55%) (p < 0·05) (table 3). In the absence of urea survival was similar in citrate (83%, range 75–103%) and phosphate buffers (90%, range 82–126%). A similar amount of urea was consumed in the phosphate buffer (83 μmol, range 0–296 μmol) and in the citrate buffer (120 μmol, range 19–264 μmol). The median percentage recovery of ammonia from urea hydrolysed was again similar for both buffers, 17% for citrate and 15% for phosphate (table 3).

Discussion

It has been proposed that the ammonia produced by the urease activity of *H pylori* generates an alkaline microenvironment to allow the organism to survive in the acid gastric environment.[9] It was therefore of interest to discover that incubating the organism in citrate buffer at pH 6·0 in the presence of a high urea concentration resulted in its rapid suicidal destruction. We have previously observed this with all strains of the bacterium tested.[10]

The importance of the hydrogen ion concentration of the buffer in the suicidal process was shown by improved survival in citrate buffer solution (pH 7·0) with 50 mmol/l urea compared with that in the same solution at pH 6·0. Although the organism consumed similar amounts of urea at pH 6·0 and pH 7·0, less ammonia was released into the incubate at pH 6·0. This reduced excretion of ammonia at pH 6·0 may explain the organism's impaired survival at this pH in the presence of urea.

The survival of *H pylori* decreased with increasing initial urea concentration in the pH 6·0 citrate buffer, indicating that the suicidal destruction was linked to urease activity. This observation was consistent with our previous finding that the addition of the urease inhibitor hydroxyurea enhanced the survival of the organism in citrate buffer at pH 6·0.[10] The percentage recovery of ammonia following hydrolysis of urea fell with increasing initial urea concentration, indicating that the

Table 2   Comparison of survival and ammonium production in citrate buffer and acetate buffer

Buffer	Urea hydrolysed µmol/5 min Median and (range)	NH₄+ produced µmol/5 min Median and (range)	% Recovery of NH₄+ Median and (range)	% Survival Median and (range)
0·2 M citrate buffer pH 6·0 with 30 mmol/l urea n = 18	97 (5–282)	19 (7–41)	8·6% (2·6–61)	29* (15–64)
0·2 M acetate buffer pH 6·0 with 30 mmol/l urea n = 18	215 (18–272)	33 (7–132)	12·1% (3·5–90)	95 (36–211)

*p < 0·01.

organism may have been unable to excrete the ammonia generated by urea hydrolysis as rapidly as it was produced. This failure to excrete ammonia may explain its rapid death.

Our studies have shown that citrate was essential for the suicidal process to occur. *H pylori* survived in phosphate and acetate buffers at pH 6·0 with concentrations of urea which caused suicidal destruction in citrate buffer. The percentage recovery of ammonium from the urea consumed during incubation in these buffers, however, was similar to that in citrate buffer. The contribution of citrate to the suicidal process, therefore, cannot be explained by changes in urease activity or ammonia excretion.

Localisation of the organism's urease activity by means of urease linked precipitation of silver followed by visualisation of the precipitate by electron microsopy had suggested that urease activity was associated with the periplasmic membrane of the organism.[12] In our experiments less ammonia was recovered from the incubate than calculated from urea consumed and ammonium ions added to the incubation medium were not taken up by the organism. This indicates that the urease activity of *H pylori* is either located within the cell or that it is closely associated with the cell membrane, taking up extracellular urea and releasing ammonia within the cell.

Ammonium produced by urease activity may be used in the formation of glutamate and glutamine by glutamate dehydrogenase and glutamine synthetase, respectively. Both of these reactions consume high energy intermediates, but glutamate dehydrogenase also uses α ketoglutarate in the formation of glutamate. Although citrate is used in the formation of α ketoglutarate, when present in high concentrations it may act as a competitive inhibitor of isocitrate dehydrogenase.[13] The activity of this enzyme is required for the formation of α ketoglutarate from citrate. The simultaneous inhibition of synthesis and stimulation of utilisation of α ketoglutarate could result in the complete depletion of this compound in the bacterium. This would result in intracellular accumulation of ammonia and impairment of energy production.

H pylori may need an active transport process to excrete excess ammonia produced by urease activity. Impairment of energy production would also impede this and would therefore tend to increase intracellular accumulation of ammonia. Through these mechanisms citrate, urease activity, and pH could combine to produce a rapid catastrophic accumulation of ammonia within the organism resulting in its death.

H pylori is difficult to eradicate with conventional treatment. Even triple treatment with tri-potassium dicitratobismuthate, metronidazole, plus either amoxycillin or tetracycline for two to four weeks fails to eradicate the infection in 10% of patients.[1] The development of antibiotic resistance is also proving a problem.[14] Our in vitro observations may indicate a potential new approach to killing the organism in vivo. We have previously shown that infusion of solutions containing urea using a nasogastric tube can stimulate ammonia production by the organism[15] thereby raising the possibility that the in vitro conditions required for eradication of the organism may be reproduced in the human stomach.

In conclusion, *H pylori* rapidly dies in citrate buffer (pH 6·0) containing urea. This may be explained by the metabolic consequences of rapid intracellular accumulation of ammonia.

Table 3   Comparison of survival and ammonium production in citrate buffer and phosphate buffer

Buffer	Urea hydrolysed µmol/5 min Median and (range)	NH₄+ produced µmol/5 min Median and (range)	% Recovery of NH₄+ Median and (range)	% Survival Median and (range)
0·2 M citrate buffer pH 6·0 with 30 mmol/l urea n = 7	120 (19–264)	39 (32–41)	17% (7·4–21)	42* (27–55)
0·2 M phosphate buffer pH 6·0 with 30 mmol/l urea n = 7	83 (0–296)	30 (5–36)	15% (4·3–19%)	100 (72–127)

*p < 0·05.

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