

Ammonia production by intestinal bacteria

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SUMMARY Bacterial growth and the production of ammonia from urea and by deamination of peptone has been examined at various pHs in both conventional static bacterial cultures and in a continuous cultivation system.

Growth occurred on primary testing of 93 out of 100 strains of aerobic Gram-negative bacteria at pH 5, and 48 out of 50 strains of *Esch. coli* at pH 4.6.

Hydrolysis of urea by *Proteus mirabilis* decreased steadily from pH 7.2 to pH 5.3; below pH 5.3 little hydrolysis occurred. Ammonia production from peptones by *Esch. coli* decreased from pH 7.2 to pH 4.6. Considerable variation was noted in the ability of different strains to produce ammonia. Experiments with cultures containing both *Esch. coli* and *Pr. mirabilis* showed that more ammonia was produced at low pH than was produced by cultures of single organisms.

At low pH reduction in the count of organisms was not found to be an essential prerequisite for reduction of ammonia formation.

Several workers have found lactulose to be an effective alternative to neomycin in the treatment of hepatic encephalopathy (Fung and Khoo, 1968; Bircher, Scollo-Lavizzari, Hoffman, and Haemmarli, 1969; Elkington, Floch, and Conn, 1969; Ma, McLeod, and Blackburn, 1969; Zeegen, Drinkwater, Fenton, Vince, and Dawson, 1970). Lactulose is a synthetic disaccharide which is not split by intestinal disaccharidases but passes unaltered to the terminal ileum and colon. There it is split by bacterial action into a number of substances, including lactic and acetic acids, with a consequent lowering of the colonic pH.

Ammonia intoxication is an important factor in the genesis of hepatic coma and the beneficial effect of lowering colonic pH could be due to various factors, acting singly or in combination, causing a fall of portal blood ammonia. One theory is that at a low pH ammonia, which is mainly absorbed by non-ionic diffusion, is almost completely ionized and so not absorbed (Castell and Moore, 1971). This seems unlikely to be the dominant mechanism because there is not a commensurate rise of faecal ammonia (Zeegen *et al.*, 1970). Alternatively, increased growth of acidophilic organisms such as lactobacilli and bifidobacteria could depress the growth of putrefactive, ammonia-producing organisms such as *Escherichia coli* and *Bacteroides* spp.

However, counts of ammonia-producing organisms do not necessarily fall during lactulose administration (Vince, Zeegen, Drinkwater, O'Grady, and Dawson, in preparation). Another possibility is that a low pH alters bacterial metabolism, so that less ammonia is produced.

Ammonia in the large gut is thought to be derived from either hydrolysis of urea by bacterial, and possibly mucosal, ureases (Wolpert, Phillips, and Summerskill, 1970) or from deamination of proteins and other nitrogenous substrates. Many viable intestinal organisms, eg, bacteroides, bifidobacteria, clostridia, *Proteus* spp, and *Klebsiella* spp possess urease activity. Others, notably *Esch. coli* (the dominant Gram-negative aerobic bacilli in the intestines of most subjects) do not, so that ammonia released by these organisms will be by deamination of substances other than urea. Some organisms will produce ammonia by both mechanisms. O'Grady (1966) showed that the most active of the intestinal organisms in the production of ammonia were Gram-negative aerobic bacilli, eg, *Esch. coli*, *Klebsiella* spp, *Proteus* spp, and *Pseudomonas* spp.

The purpose of the present investigation was to determine whether production of ammonia by intestinal bacteria was inhibited or reduced at the pHs observed in the caecum during the administration of lactulose, when a pH-sensitive radiotelemetry pill was used to record the pH. The pH range found was 3.7-6.2 although most readings were less than 5

(Bown, Sladen, Rousseau, Gibson, Clark, and Dawson, 1972). Bacterial growth and production of ammonia from urea and by deamination of peptone has been examined at various pHs in both conventional static bacterial cultures and a continuous cultivation system. Ammonia production by mixtures of organisms has also been studied, as it is recognized that the behaviour of one organism may be modified by the presence of another in the complex ecosystem of the gut.

Materials and Methods

ORGANISMS

Organisms studied were isolated from the colonic effluent of patients who had undergone colonic exclusion and from the small and large bowel and faeces of patients investigated by this department.

CULTURE MEDIUM

Oxoid nutrient broth (CM67) was used. It was adjusted to the required pH by the addition of varying amounts of 0.1 M citric acid and 0.1 M sodium citrate.

SCREENING OF ORGANISMS FOR ABILITY TO GROW AT LOW pH

One hundred organisms were tested: 58 *Esch. coli*, 16 *Klebsiella aerogenes*, 14 *Proteus mirabilis*, and 12 *Pseudomonas* spp. Aliquots, each of 0.1 ml, of overnight cultures of the organisms in nutrient broth were used to inoculate broths at the stated pHs. Plate counts were made initially and after 24 hours' incubation (static) at 37°C to determine whether growth had occurred. Continuous growth records of some of these static cultures were also obtained photometrically, using a 12-channel modification (Mackintosh, Watson, and O'Grady, 1973) of the simple turbidity monitoring system described by Watson, Gauci, Blache, and O'Grady (1969).

CULTURE SYSTEMS FOR THE EXAMINATION OF UREA HYDROLYSIS AND PEPTONE DEAMINATION
Strains of *Esch. coli*, which does not produce urease, and *Proteus mirabilis*, which produces urease, used in these studies were isolated from excluded colonic segments of patients with hepatic encephalopathy.

Static culture

Four hundred ml of broth was inoculated with 0.1 ml of a two-hour culture of the organism and incubated at 37°C. Samples were withdrawn at regular intervals, centrifuged, and ammonia and urea in the supernatant measured either immediately or following storage at -20°C for not more than 24 hours. This had previously been shown not to

affect the ammonia content of the samples. Viabi[®] bacterial counts were performed on all samples as described previously (Hamilton, Dyer, Dawson, O'Grady, Vince, Fenton, and Mollin, 1970). Urea was added, where required, immediately before inoculation to a final concentration of 20 or 30 mg/100 ml. Control samples for urea and ammonia estimations were taken immediately before, and immediately after, inoculation.

Continuous culture

The system consisted of a tube containing approximately 35 ml of broth, maintained at 37°C, and closed with a rubber bung fitted with an inlet connected to a flask containing fresh broth, and an outlet for disposal. A constant volume of fluid was maintained in the tube by connecting the tubes taking fresh medium in and discharge out through the same peristaltic pump (Watson-Marlow Ltd, Falmouth). The tube was inoculated with 0.1 ml of a two-hour culture of the organism(s) under test and left to incubate for a further one and a quarter hours before the pump was switched on. The flow rate (around 1 ml per min, depending on the organism) was adjusted to maintain a constant number of organisms (10^7 - 10^8) in the tube. Where required urea was added to the tube when the pump was switched on.

AMMONIA AND UREA MEASUREMENT

Ammonia was estimated as described by Fenton and Williams (1968). Urea was measured by the diacetyl monoxime technique on the AutoAnalyzer.

Results

THE EFFECT OF pH ON THE GROWTH AND AMMONIA PRODUCTION OF ORGANISMS

A hundred strains were tested for their ability to grow at reduced pH. None had been exposed to low pH in any previous *in vitro* tests. The results are given in table I.

Fifty-four of the 58 strains of *Esch. coli* examined grew readily on primary testing at pH 5. Of 50

Organism	Number of Strains Showing a Significant Increase in Count after 24 Hours at pH			
	7.2	5.0	4.6	4.0
<i>Escherichia coli</i>	58/58	54/58	48/50	0/50
<i>Klebsiella aerogenes</i>	16/16	15/16	—	—
<i>Proteus mirabilis</i>	14/14	13/14	—	—
<i>Pseudomonas</i> spp	12/12	11/12	—	—

Table I Growth of enterobacteria in nutrient broth at different pHs

Ammonia production by intestinal bacteria

Esch. coli tested at pH 4.6 and pH 4, 48 grew at pH 4.6 but none grew at pH 4. None of these organisms could hydrolyse urea.

Forty-two strains of other organisms, ie, *Klebsiella aerogenes*, *Proteus mirabilis*, and *Pseudomonas* spp, were tested for growth at pH 5. One strain of each of the three species failed to grow at pH 5 within 24 hours. All 42 strains split urea *in vitro*.

Although, as indicated, most of the organisms tested grew at reduced pH, the total viable count reached after 24 hours' incubation at pH 5 was in many cases half, or even less, than that reached at neutral pH. The effect of reduction in pH on the growth of organisms is more readily seen when a continuous record of the growth curve is examined. The growth curves at various pHs of one strain of *Esch. coli* and one strain of *Proteus mirabilis* are given in figure 1. From these it can be seen that there is a restricted pH range over which maximal growth occurs for any given bacterium and a much wider pH range over which growth still occurs, but at a much slower rate. At low pH, the lag phase before

active growth commences is still considerably longer than at neutral pH, and the climax, or total, population achieved is proportionately smaller.

Two organisms (those shown in fig 1a, 1b) were selected for a more detailed study of ammonia production at low pH. However, it is important to realize that although this study is restricted to single strains there is considerable variation in the amount of ammonia produced by different strains of *Esch. coli* (which do not produce urease), both at neutral and reduced pH (table II). In contrast, three strains of *Pr. mirabilis*, which produce urease, produced fairly similar amounts of ammonia from cultures containing urea (table II).

AMMONIA PRODUCTION FROM UREA

Static system, single organism culture

The amount of ammonia formed from urea by a pure culture of *Pr. mirabilis* decreased steadily with decreasing pH from 7.2 to 5.3. At pH 5.3 the amount of ammonia formed within 24 hours was about half

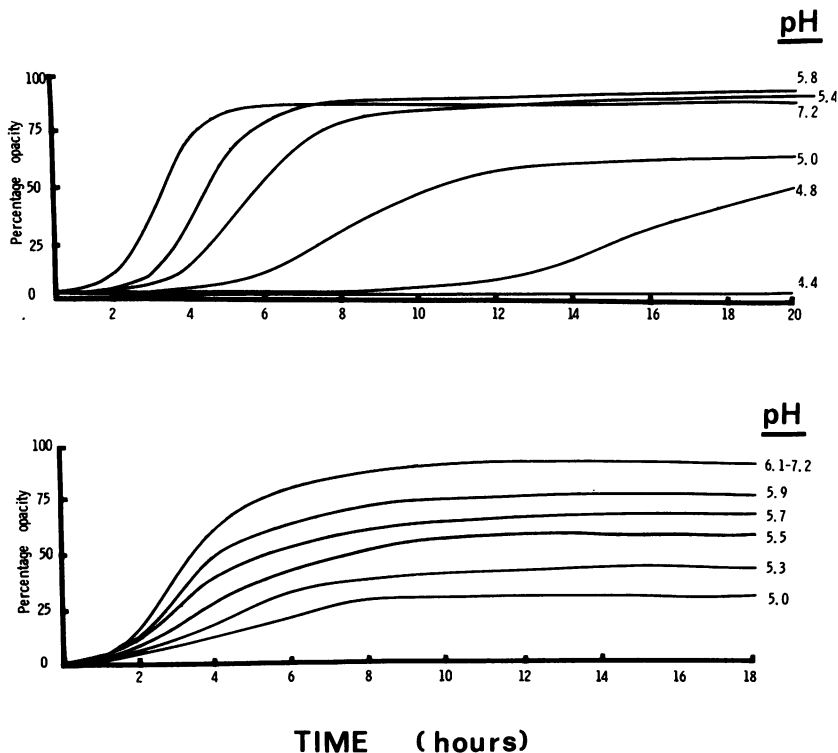


Fig. 1 Growth of *Esch. coli* (upper graph) and *Proteus mirabilis* (lower graph) in nutrient broth at different pHs, as shown by increase in turbidity of the medium.

Organism	Medium	Number of Strains Tested	pH	Ammonia ($\mu\text{g}/100\text{ ml}$)	
				Mean	Range
<i>E. coli</i>	Nutrient broth	6	4.6	2638	1200- 4040
		6	5.0	3820	900- 6280
		6	7.2	12 173	8560-16 160
		3	7.2	30 826	29 200-33 200
<i>Proteus mirabilis</i>	Nutrient broth 30 mg% urea	10	7.2	1100	920- 1300
Blank determination	Nutrient broth				

Table II Production of ammonia by different strains of *Esch. coli*, *Proteus mirabilis*, and medium alone after 24 hours' incubation at 37°C

that formed at pH 7.2 (fig 2). Below pH 5.2 growth occurred (fig 1) but a minimal amount of urea was split.

Once organisms had entered the maximal growth phase at the various pHs the ammonia content of the medium increased steadily with increasing organism count, so that the curves for ammonia production and growth at different pHs followed similar patterns at pH 5.3 and above (compare figs 1 and 2).

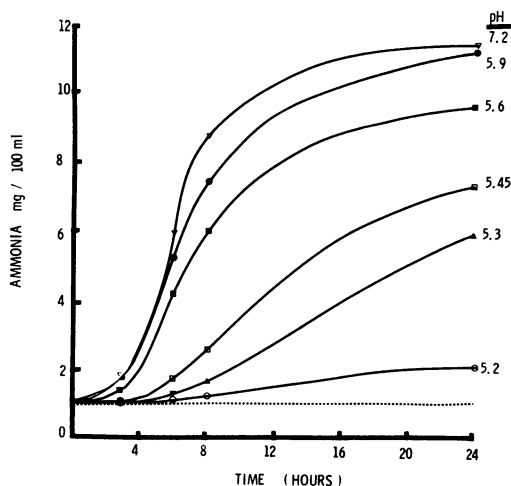


Fig. 2 Ammonia production from nutrient broth containing 20 mg/100 ml urea by *Proteus mirabilis* at different pHs, in static culture. The ammonium content of the medium is indicated by the broken line.

Continuous system, single organism culture

The findings were similar to those obtained in the static system. Urea hydrolysis by *Pr. mirabilis* was not detected at pH 5.3 or less, the cut-off point being sharply defined (fig 3), most of the ammonia produced below this pH in the static system presumably being derived from deamination and not from urea hydrolysis. As the pH was increased from 5.4 to

6.0 urea hydrolysis increased, but above pH 6.0 no further decrease in urea concentration occurred, even when the pH was increased to 7.6.

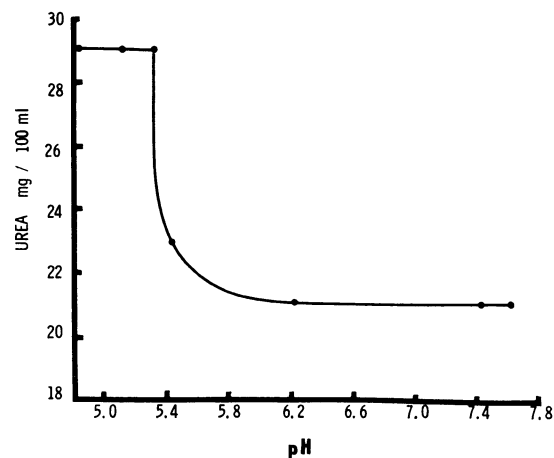


Fig. 3 Hydrolysis of urea by *Proteus mirabilis* at different pHs during continuous flow culture of the organism in nutrient broth containing 29 mg/100 ml urea.

AMMONIA PRODUCTION BY DEAMINATION

Static system, single organism culture

The ammonia produced by a pure culture of *Esch. coli* from a solution containing peptones and other protein derivatives decreased steadily with decreasing pH (fig 4). As with the release of ammonia from urea, there was virtually no difference in the amount of ammonia produced over a 24-hour period as the pH was lowered from 7.2 to 6.0. Further reduction in pH reduced ammonia formation, but not as dramatically as when ammonia was formed from urea by *Pr. mirabilis*. More ammonia was produced at low pH by *Esch. coli* than by *Pr. mirabilis* even when *Pr. mirabilis* had urea as a substrate. At pH 5.0 the amount of ammonia formed in 24 hours was still more than half the amount formed at pH 7.2 and

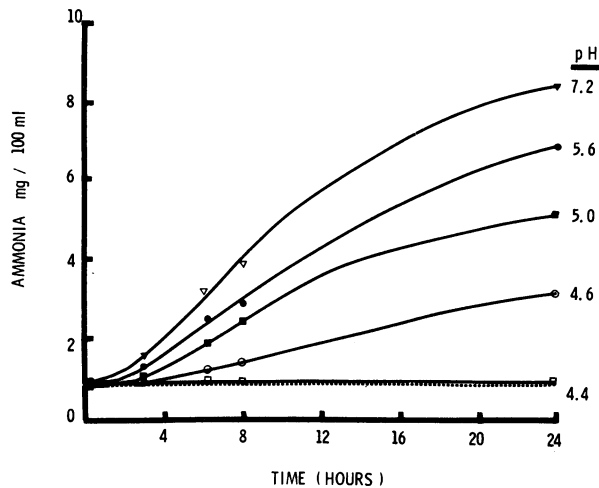


Fig. 4 Ammonia production from nutrient broth by *Esch. coli* at different pHs in static culture. The ammonium content of the medium alone is indicated by the broken line.

even at pH 4.6 small amounts of ammonia were still produced. It was notable that although *Esch. coli* grew equally well at pH 5.4 as at pH 7.2, less ammonia was produced at the lower pH. At pH 4.4 and below no growth of organisms occurred (fig 1) and no ammonia was produced.

Continuous system, single organism culture

The lowest pH at which ammonia production by *Esch. coli* was tested in this system was 5.2. In agreement with the static culture results a substantial amount of ammonia was formed.

AMMONIA PRODUCTION BY DEAMINATION AND FROM UREA BY MIXTURES OF ORGANISMS
The ability of mixtures of *Esch. coli* and *Pr. mirabilis* to produce ammonia at different pHs from solutions with and without urea was tested over a 24-hour period. At higher pH, ie, 5.6 and above, the picture was similar to that obtained using single cultures of organisms in that ammonia production increased steadily with increasing pH, both from solutions containing urea and from those without it (fig 5). However, in contrast with results obtained from cultures of single organisms, ammonia production from systems with and without urea did not decline, but continued steadily down to pH 4.7. The difference in the amount of ammonia produced from the parallel systems to which urea was or was not added indicates that a small quantity of ammonia was still produced from urea at a pH as low as 4.7 in a culture containing both *Esch. coli* and *Pr. mirabilis*, although none had been produced from urea by a pure culture of the same strain of *Pr. mirabilis* at such a low pH.

Discussion

During the administration of lactulose, the pH of the colon, especially the caecum, drops considerably (Bown *et al*, 1972). This drop in pH may initially depress counts of some ammonia-producing organisms and so cause a temporary reduction in the amount of ammonia available for absorption. However, acidophilic strains of genera not initially adapted for growth at low pH soon emerge, which explains why counts of organisms such as *Esch. coli*, which metabolize lactulose weakly if at all (Hoffman, Mossel, Korus, and Kamer, 1964), are frequently unaffected by lactulose administration.

Whilst at higher pHs reduction in viable count was related to a reduction in the amount of ammonia formed (both from urea and by deamination) the relationship did not hold at low pH, where, although growth of organisms continued, ammonia production was considerably curtailed. These findings confirm that a reduction in the count of ammonia-producing organisms is not an essential prerequisite for a reduction of ammonia formation at low pH.

Considerable variation was observed in the ability of intestinal organisms to produce ammonia, the variation being more pronounced for ammonia production from peptones by *Esch. coli* than for urea hydrolysis by *Pr. mirabilis*. Such variation amongst intestinal organisms, combined with the presence or absence of urea-splitting organisms in the gut, might explain the different responses of patients with hepatic encephalopathy to various forms of therapy.

Reduction in ammonia formation following

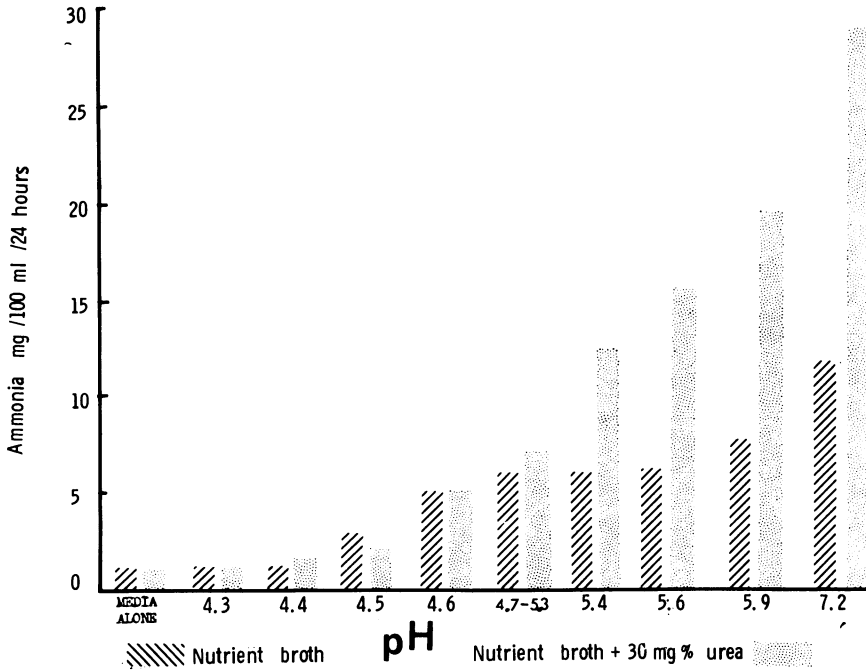


Fig. 5 Production of ammonia from nutrient broth with and without the addition of urea (30 mg/100 ml), by a mixed culture of *Esch. coli* and *Proteus mirabilis* after 24 hours' incubation.

lactulose therapy must depend to some extent on what constitutes the major source of ammonia in the gut. Decreasing the pH had a much greater effect on urea hydrolysis than on ammonia production from peptones. This suggests that if urea is ordinarily a major source of ammonia in the intestine, patients colonized by large numbers of urea-splitting organisms should show a more marked reduction in colonic ammonia during lactulose administration than patients lacking such organisms.

Growth conditions in the colon presumably occupy a position intermediate between the extremes of continuous and static culture, which was why both static and continuous culture systems were used in this study. Similar results were obtained from the static and continuous systems, indicating that activity of the organisms was similar in both growth systems. The experiments with mixed cultures indicate that ammonia production is enhanced rather than depressed when more than one organism is present. Cooperative efforts between intestinal organisms have been reported previously (Gustafsson, Midtvedt and Norman, 1968) and there must be numerous opportunities for group activity in the production of ammonia by deamination. A

molecule partly degraded by one organism might easily be further degraded by another.

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