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AMMONIA PRODUCTION BY INTESTINAL BACTERIA: THE EFFECTS OF LACTOSE, LACTULOSE AND GLUCOSE

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AMMONIA generated by bacteria in the colon and absorbed into the blood stream is thought to be an important factor in the genesis of hepatic coma (Schenker, Breen and Hoyumpa, 1974; Dawson, 1978). The origin of colonic ammonia is uncertain; urea has been suggested as the main source (Wolpert, Phillips and Summerskill, 1971) but Vince *et al.* (1976) have shown that considerable amounts of ammonia, in excess of those normally found in faeces, were generated by bacteria from non-urea sources in an in-vitro faecal incubation system. Addition of fermentable substrate—lactulose, glucose, mannitol or sorbitol—to this system caused a decrease in the ammonia concentration (Vince, Killingley and Wrong, 1978). This decrease was independent of reduction in pH, which reduces ammonia formation by inhibiting deaminating enzymes (Gale and Epps, 1942; Vince *et al.*, 1973). Decreased ammonia production and the lowering of pH, may contribute to the beneficial effect of lactulose therapy in the treatment of hepatic encephalopathy.

Studies on ammonia production from non-urea sources, e.g., peptides and amino acids, by intestinal bacteria have been confined mainly to the gram-negative aerobes and clostridia (Phear and Ruebner, 1956; O'Grady, 1966; Vince *et al.*, 1973). However, the numerical dominance of the non-sporing anaerobes in faeces suggests that they might be responsible for the formation of a substantial proportion of the total ammonia generated from the non-urea sources *in vivo*. Equally, it is possible that most of the decrease in ammonia concentration found in faecal incubation systems supplemented with fermentable substrates results from the metabolic activity of anaerobic organisms.

The purposes of this study were twofold: firstly, to determine which groups of intestinal bacteria are most active in forming ammonia from peptides and amino acids, and secondly, with the use of pure cultures, particularly gram-negative non-sporing anaerobes, to examine the mechanisms by which fer-

mentable substrates reduce the amount of ammonia formed in faecal incubation systems.

MATERIALS AND METHODS

Bacteria

These were isolated as described previously, mainly from the faeces of healthy adult subjects, but some isolates from the terminal ileum were also examined (Hamilton *et al.*, 1970; Vince *et al.*, 1976). Numerically dominant groups of bacteria were selected for testing, and placed in one of the following categories, account being taken of several criteria including, the ability to grow aerobically or anaerobically, gram-staining properties and spore formation: enterobacteria; streptococci; micrococci; *Bacillus* spp.; lactobacilli; yeasts; gram-negative anaerobic rods, e.g., bacteroides and fusobacteria; gram-positive non-sporing anaerobic rods, e.g., eubacteria and bifidobacteria; clostridia.

Enterobacteria and streptococci were identified further by the scheme of Cowan and Steel (1966) and clostridia and gram-negative anaerobes according to Holdeman, Cato and Moore (1977).

Ammonia production in a nutrient medium

The medium used for the experiments summarised in table I was Brain Heart Infusion Broth (Difco) supplemented with 1% Yeast Extract (Difco). A standard loopful of an overnight culture was used to inoculate 10 ml of medium. In addition, some bacteria were tested for ammonia production in Nutrient Broth (Oxoid CM67) supplemented with 1% Yeast Extract (Difco). Broths were incubated for 48 h at 37°C in an atmosphere containing 5% H₂, 10% CO₂ and 85% N₂. After 48 h, samples were taken for measurements of turbidity, dry weight, pH and ammonia.

We also examined the effect of lowered pH and raised levels of ammonia on ammonia production because these are factors that might occur as a result of bacterial metabolism in nutrient media and in the colon.

Effect of low pH. To test the effect of lowered pH, 1M HCl was added to broth to bring the pH to 5.0. To eliminate the possibility that reduction in ammonia formation at low pH was due entirely to reduced bacterial growth, a large inoculum, 0.5 ml (10¹⁰–10¹¹ cells/ml) of a washed suspension of 48-h cells in 0.15M NaCl added to 4.5 ml of broth, was used to give an initial bacterial concentration that was numerically equivalent to, or greater than, the maximal bacterial count normally reached after 48 h of growth.

Effect of increased ammonia concentration. To test the effect of increased ammonia concentration, ammonium chloride up to 68 mmol/litre was added to broth after autoclaving. The inocula used for these experiments were either a standard loopful of an overnight culture, or 0.5 ml of a washed saline suspension as used in the pH experiments. Results for the same organism tested with both types of inocula did not differ significantly.

Endogenous ammonia production from washed bacterial cells

The purpose of these experiments was to determine whether the endogenous metabolism of bacterial cells could act as a source for the release of ammonia.

Bacteria grown for 48 h in 200 ml of Brain Heart Infusion Broth (Difco) supplemented with 1% Yeast Extract (Difco) were centrifuged, washed three times in 200 ml of ammonia-free 0.15M saline and resuspended in 1–2 ml of saline to form a slurry containing approximately 100 mg (dry weight)/ml. Because so little ammonia was generated after 4 days' incubation, these washed cells were incubated for 2 weeks. To check bacterial viability, counts were made by inoculating serial tenfold dilutions of cell suspensions onto blood-agar plates at 0, 1, 4, 7 and 14 days. Samples for ammonia analyses were taken at the same times.

The effect of fermentable substrates on ammonia production

Ammonia formation from single amino acids. These experiments demonstrated the effect of fermentable substrates on the formation of enzymes involved in ammonia production. Cells were grown in the presence of either lactose or lactulose and then incubated with amino acids in the absence of lactose or lactulose.

Bacteria were grown for 24–48 h in 150 ml of Nutrient Broth (Oxoid CM 67) supplemented with 1% Yeast Extract (Difco), 0.05M alanine, 0.05M histidine, 0.05M methionine and 0.05M aspartic acid, 0.03M lactose or 0.03M lactulose (for sugar-containing broths), and 0.05M sodium phosphate buffer pH 7.2. Cells grown in the presence or absence of sugar were washed twice in 150 ml of ammonia-free 0.05M phosphate buffer. Cells grown in the absence of added sugar (control cells) were resuspended in 5 ml of 0.05M phosphate buffer, and cells from sugar-containing broths were resuspended in buffer to a turbidity equal to that of the suspension of control cells; 1-ml portions of these cell suspensions were taken for dry-weight determination and for ammonia estimations. After warming to 37°C, 1 ml of each cell suspension was added to 1 ml of 0.1M solutions of alanine, histidine, methionine or aspartate respectively and incubated at 37°C for 3 h—these amino acids were tested as representing the four main chemical groups of amino acids. The reaction was stopped by the addition of 0.2 ml of 1M HCl, and the suspension was centrifuged and analysed for ammonia content. To check for non-bacterial ammonia release, uninoculated amino-acid solutions were incubated with each assay. The organisms tested were mainly from groups known to be strong producers of ammonia. Enterobacteria were not tested with single amino acids because they have been studied extensively by other workers (Paigen and Williams, 1970).

Ammonia formation from a nutrient medium. The basal medium used was Brain Heart Infusion Broth (Difco) supplemented with 1% Yeast Extract (Difco). In each experiment 4.5-ml volumes of the following media were prepared: basal medium with no additions; basal medium containing 0.06M glucose, 0.03M lactose or 0.03M lactulose; basal medium containing 0.1M sodium phosphate buffer pH 7.2; basal medium containing 0.1M phosphate buffer and 0.06M glucose, 0.03M lactose or 0.03M lactulose.

A standard loopful of an overnight culture or 0.5 ml of a suspension of buffer-washed cells containing 10^{10} – 10^{11} cells/ml, was used as inoculum. Allowance was made for the dilution when test broths were studied with the 0.5-ml inoculum. In broths inoculated with 0.5 ml of washed cells (large inocula), the turbidities indicated that the bacterial counts at 48 h were often twice those obtained in broths that had been inoculated with a standard loopful of organisms. However, despite the increased counts, cultures in sugar-containing broths incorporating large inocula showed considerably less ammonia formation than cultures in sugar-containing broths inoculated with a standard loopful of organisms. For this reason, results are presented for both types of inocula. Because there was no difference between results obtained for inocula prepared in the presence or absence of sugar, all results refer to inocula prepared from starter cultures grown in the absence of sugar.

All cultures were incubated for 48 h at 37°C, and ammonia production, pH and turbidity were measured.

Analyses

Ammonia was measured with an EIL ammonia probe, by the method of Park and Fenton (1973) with 0.2M KCl as diluent. For calculation of ammonia formed in nutrient media, the concentration (2–4 mmol/litre) in an uninoculated broth was subtracted from that in an inoculated broth of the same type.

Dry weights were measured by drying 1 ml of either a saline or a buffer suspension of organisms and diluent blanks to constant weight at 100°C. The bacterial content was calculated after allowance for salt content. Standard curves of turbidity *versus* dry weight prepared for each of the bacterial groups tested were used to calculate the dry weight when only opacity was determined. Turbidity was measured with a Corning-Eel nephelometer. Within a particular bacterial group, for which ammonia production is not expressed in terms of dry weight, the

cultures compared had the same turbidity. However, in such cases results between groups have not been standardised.

A Radiometer pH meter 28 was used to measure the pH of all broths before and after incubation.

RESULTS

Ammonia production from nutrient medium

Gram-negative anaerobes, clostridia, enterobacteria and *Bacillus* spp. all produced considerable quantities of ammonia (table I). Streptococci, micrococci, and the gram-positive non-sporing anaerobes, e.g., bifidobacteria, produced moderate concentrations of ammonia whereas the gram-positive aerobic rods, mostly lactobacilli, produced very little ammonia. The two yeast isolates tested formed 0.0 and 0.1 mmol ammonia/litre respectively.

The most active group of ammonia-producing bacteria were the clostridia, most of which were *Clostridium perfringens*, followed closely by the gram-negative anaerobes. All the isolates of gram-negative anaerobes tested produced ammonia, although 20% produced 10 mmol/litre or less.

Some bacteria were identified to species level, and the ammonia produced by them is shown in table II. Within the *Bacteroides* group three species, *B. fragilis*, *B. ovatus* and *B. vulgatus* were isolated with sufficient frequency to allow more detailed analysis. Different isolates of *B. fragilis* and *B. vulgatus* showed a wide range of activity with only two of the 15 isolates producing < 20 mmol ammonia/litre and most strains producing 30 mmol/litre or more; conversely, of the eight *B. ovatus* isolates, only two produced 15 mmol/litre. The number of isolates and the species of *Bacteroides* and *Fusobacterium* listed in table II do not necessarily form a representative picture of all the gram-negative anaerobes isolated, because many organisms were either not kept or failed to survive until the end of the study period when the identifications were made. Identification of organisms in other groups showed that there was little difference between either *Escherichia coli* and

TABLE I
Ammonia formed by intestinal bacteria at a concentration of 15 mg/ml dry weight

Bacterial group	Number of isolates tested	Ammonia produced (mmol/litre)	
		mean ($\pm$ standard error)	range
Gram-negative anaerobic rods	82	31.4 ($\pm$ 1.5)	2.4-74.7
Clostridia	14	34.0 ($\pm$ 2.6)	8.8-54.8
Enterobacteria	61	21.6 ($\pm$ 1.2)	6.0-41.2
<i>Bacillus</i> spp.	3	23.5 ($\pm$ 1.9)	18.4-26.1
Streptococci	30	7.2 ($\pm$ 0.8)	0-17.6
Micrococci	17	7.2 ($\pm$ 1.3)	0-14.3
Gram-positive non-sporing anaerobic rods	65	7.2 ($\pm$ 1.2)	0-23.8
Lactobacilli	17	2.3 ($\pm$ 0.5)	0-5.4

TABLE II
Ammonia formed by individual bacterial species

Organism (number of isolates tested)	Ammonia produced (mmol/litre)	
	mean	range
<i>Bacteroides fragilis</i> (9)	33.4	10.1-59.7
<i>Bacteroides ovatus</i> (8)	11.1	2.5-18.0
<i>Bacteroides vulgatus</i> (6)	36.7	21.1-67.7
<i>Bacteroides diastonis</i> (2)	21.8	8.1-35.5
<i>Bacteroides melaninogenicus</i> ss <i>melaninogenicus</i> (2)	17.6	16.5-18.7
<i>Bacteroides capillosus</i> (2)	3.8	3.3-4.4
<i>Bacteroides multiacidus</i> (1)	64.3	...
<i>Bacteroides ruminicola</i> (1)	6.5	...
<i>Fusobacterium prausnitzii</i> (3)	18.2	2.9-33.5
<i>Escherichia coli</i> (15)	19.4	13.6-36.3
<i>Proteus mirabilis</i> (15)	23.1	14.1-41.1
<i>Streptococcus faecium</i> (8)	8.7	5.5-12.3
<i>Streptococcus faecalis</i> (17)	9.7	3.3-19.5

Proteus mirabilis or between *Streptococcus faecalis* and *Streptococcus faecium* in the amount of ammonia formed.

Individual strains of bacteria gave reasonably consistent results for ammonia production when tested on different occasions. Bacteria tested in duplicate on the same day usually gave almost identical results, but differences did occur when they were tested on different days in different batches of media. The average coefficients of variation for different organisms or groups of organisms were: *C. perfringens* 15%, gram-positive non-sporing anaerobic rods 12%, enterobacteria 10%, gram-negative anaerobic rods 8%, streptococci 6% and for one lactobacillus, 4%.

In addition to testing in brain-heart infusion broth many organisms, particularly the gram-negative aerobes and anaerobes, were tested in nutrient broth in which ammonia production was generally reduced. The mean concentrations for ammonia production by 32 isolates of gram-negative anaerobes were 25.0 and 22.4 mmol/litre in brain-heart infusion broth and nutrient broth respectively, and the corresponding values for 32 gram-negative aerobes were 29.6 and 19.8 mmol/litre.

Effect of pH. Less ammonia was generated by cells of all bacterial groups when the initial pH of the medium was 5.0, compared with cultures in which the initial pH was 7.0 (table III). Gram-negative aerobes were shown by Vince *et al.* (1973) to produce less ammonia at low pH.

Effect of ambient ammonia concentration on ammonia formation. There was no correlation between the initial ammonia concentration of the medium and the amount of ammonia formed by any of the bacterial groups tested

TABLE III
Ammonia formed by bacterial cells at pH 7.0 and pH 5.0

Bacterial group (number of isolates tested)	Mean concentration of ammonia produced (mmol/litre) ($\pm$ standard/error)	
	pH 7.0	pH 5.0
Gram-negative anaerobic rods (13)	17.0 ($\pm$ 1.0)	10.0 $\pm$ 0.7*
Clostridia (7)	22.7 ($\pm$ 5.3)	16.4 $\pm$ 4.3
Gram-positive non-sporing anaerobic rods (9)	10.1 ($\pm$ 3.3)	7.1 $\pm$ 2.0†
Streptococci (10)	8.4 ($\pm$ 0.9)	5.5 $\pm$ 0.7†

* $p < 0.001$.

† $p \leq 0.05$.

FIGURE.—Ammonia produced in relation to the initial ammonia concentration of the medium by: a) 53 isolates of enterobacteria; b) 37 isolates of gram-positive non-sporing anaerobes; c) 35 isolates of gram-negative anaerobes; d) 45 isolates of streptococci, tested at various concentrations of ammonia. ○=Inoculum was a standard loopful of organisms; ●=inoculum was 0.5 ml of a cell suspension containing 10^{10} – 10^{11} cells/ml.

(figure). Most isolates were tested at ammonia concentrations of 2–30 mmol/litre and a few were tested at 68 mmol/litre which is more than four times the mean concentration of ammonia in faeces.

Ammonia production due to endogenous bacterial metabolism

Washed cells obtained from pure cultures produced low concentrations of ammonia even after prolonged incubation. After 14 days a mean ammonia concentration of 1.8 mmol/litre was formed by six isolates of enterobacteria, and less than 1.0 mmol/litre was formed by each of three isolates of gram-negative anaerobic rods, two clostridial isolates, and two isolates of gram-positive non-sporing anaerobic rods. Less than 0.1 mmol/litre was formed by each of two streptococcal isolates, one micrococcus and two isolates of lactobacilli. Considerably more ammonia, 7.3 mmol/litre, was generated by a mixture of all those organisms when present in approximately the proportions found in faeces.

Viable counts of all suspensions fell during incubation. Those of pure cultures fell from 10^{11} /ml on day 0 to 10^4 /ml by day 4 and the count of the mixed cell suspension fell from 2.0×10^{11} /ml on day 0 to 1.0×10^4 /ml on day 7.

The effect of carbohydrate fermentation on ammonia production

Studies with single amino acids. Washed suspensions of gram-negative anaerobes, *C. perfringens* and *S. faecium* all showed varying degrees of reduc-

TABLE IV

Ammonia produced from alanine by suspensions of washed cells after growth with and without lactose or lactulose

Organism	Isolate serial no.	Ammonia production (μ mol/mg dry weight per hour) during growth in medium containing		
		no sugar	lactose	lactulose
<i>Bacteroides vulgatus</i>	1*	45.9	15.3 (66.7)‡	...
<i>Bacteroides vulgatus</i>	1†	53.3	...	19.4 (63.6)
<i>Bacteroides ovatus</i>	2	31.5	2.9 (90.8)	...
<i>Bacteroides multiaacidus</i>	3	16.1	...	11.7 (27.3)
<i>Bacteroides fragilis</i>	4	29.3	22.0 (24.9)	...
<i>Bacteroides fragilis</i>	4	30.8	...	13.9 (54.9)
<i>Clostridium perfringens</i>	1	91.1	72.6 (20.3)	...
<i>Streptococcus faecium</i>	1	11.0	5.1 (53.6)	5.1 (53.6)
<i>Streptococcus faecium</i>	2	8.1	1.5 (81.5)	...

* Mean of 3 assays.

† Mean of 2 assays.

‡ Figures in parentheses indicate percentage decrease in ammonia production by washed cells after growth in lactose or lactulose.

TABLE V

Ammonia produced from histidine by suspensions of washed cells after growth with and without lactose or lactulose

Organism	Isolate serial no.	Ammonia production ($\mu\text{mol}/\text{mg}$ dry weight per hour) during growth in medium containing		
		no sugar	lactose	lactulose
<i>Bacteroides vulgatus</i>	1*	46.2	11.0 (76.2)‡	...
<i>Bacteroides vulgatus</i>	1†	42.7	...	11.7 (72.6)
<i>Bacteroides ovatus</i>	2†	47.3	26.0 (45.0)	...
<i>Bacteroides multiacidus</i>	3	10.3	<0.1 (>99.0)	...
<i>Bacteroides fragilis</i>	4†	40.7	4.73 (88.4)	...
<i>Clostridium perfringens</i>	1	50.2	48.4 (3.6)	...
<i>Streptococcus faecium</i>	1	5.9	6.6 (0)	...

* Mean of 3 assays.

† Mean of 2 assays.

‡ Figures in parentheses indicate percentage decrease in ammonia production by washed cells after growth in lactose or lactulose.

TABLE VI

Ammonia produced from methionine by suspensions of washed cells after growth with and without lactose or lactulose

Organism	Isolate serial no.	Ammonia production ($\mu\text{mol}/\text{mg}$ dry weight per hour) during growth in medium containing		
		no sugar	lactose	lactulose
<i>Bacteroides vulgatus</i>	1	13.9	8.1 (41.7)*	...
<i>Bacteroides vulgatus</i>	1	13.9	...	7.3 (47.5)
<i>Bacteroides ovatus</i>	2	63.1	59.4 (5.9)	...
<i>Bacteroides multiacidus</i>	3	20.5	18.3 (10.7)	...
<i>Bacteroides multiacidus</i>	3	32.3	...	30.1 (6.8)
<i>Bacteroides fragilis</i>	4	22.0	9.5 (56.8)	...
<i>Clostridium perfringens</i>	1	86.5	62.9 (27.3)	...
<i>Streptococcus faecium</i>	1	8.8	3.7 (58.0)	9.5 (0)
<i>Streptococcus faecium</i>	2	8.8	5.9 (33.0)	...

* Figures in parentheses indicate percentage decrease in ammonia production by washed cells after growth in lactose or lactulose.

tion in ammonia formation from alanine, histidine and methionine, after growth in media containing either lactose or lactulose (tables IV–VI). In contrast, more ammonia was formed from aspartate by gram-negative anaerobes and *C. perfringens*, but not by *S. faecium*, after growth with lactose or lactulose (table VII).

Studies with a nutrient medium. Experiments were performed to differentiate between reduction in ammonia formation due to lactose, lactulose or glucose or their fermentation products *per se*, i.e., substrate inhibition, and inhibition due to fall in pH. The levels of ammonia produced in buffered medium in the presence and absence of sugar was compared with ammonia levels produced in unbuffered sugar broths. Inhibition due to the sugars themselves was taken to be the difference between ammonia levels in buffered medium lacking sugar and in buffered medium with sugar. Inhibition due to low pH was taken as the difference between ammonia levels in buffered and in unbuffered sugar-containing media.

Fermentation of lactose, lactulose or glucose significantly lowered the final ammonia concentration in cultures of gram-negative anaerobic and aerobic bacteria (tables VIII and IX). The decrease in ammonia formation was greater in cultures in which a large inoculum was used than in cultures inoculated with a standard loopful of organisms, despite the fact that the turbidity at 48 h was usually greater in the former.

Fermentation of sugars did not have nearly such a pronounced effect on ammonia production by gram-positive bacteria as it had on the metabolism of gram-negative bacteria. Indeed ammonia production by five gram-positive non-sporing anaerobes was not lowered in the presence of either lactose or lactulose. Three clostridial isolates showed a significant decrease of 26% in the presence of lactulose, whereas the decrease was only 14% for four clostridial

TABLE VII

Ammonia produced from aspartate by suspensions of washed cells after growth with and without lactose or lactulose

Organism	Isolate serial no.	Ammonia production ($\mu\text{mol}/\text{mg}$ dry weight per hour) during growth in medium containing		
		no sugar	lactose	lactulose
<i>Bacteroides vulgatus</i>	1	275.0	437.8 (+ 59.2)*	453.2 (+ 64.8)
<i>Bacteroides multiaacidus</i>	3	49.9	206.8 (+ 314.4)	...
<i>Bacteroides fragilis</i>	4	36.7	68.9 (+ 87.7)	...
<i>Clostridium perfringens</i>	1	50.2	67.8 (+ 35.1)	...
<i>Streptococcus faecium</i>	1	19.1	14.7 (– 23.0)	9.5 (– 50.3)
<i>Streptococcus faecium</i>	2	5.9	5.9 (0)	...

* Figures in parentheses indicate percentage change in ammonia production by washed cells during growth in lactose or lactulose; + denotes stimulation, – denotes inhibition.

TABLE VIII

Ammonia production by gram-negative anaerobes from brain-heart infusion broth with and without lactose or lactulose

Substrate (number of strains examined)		Ammonia produced (mmol/litre) ± standard error (mean and range)	Final pH	Percentage inhibition of ammonia formation due to substrate
Lactose*: (7)	control†	15.2 ± 2.6 (5.3–25.3)	6.2 ± 0.16	...
	buffered	11.9 ± 1.7§ (5.5–17.7)	6.6 ± 0.11	21.7
	unbuffered	8.9 ± 0.8 (5.0–12.7)	5.7 ± 0.16	...
Lactose†: (17)	control	27.1 ± 3.3 (5.4–44.3)	6.4 ± 0.03	...
	buffered	17.6 ± 2.9 (4.1–49.5)	6.3 ± 0.04	35.1
	unbuffered	12.7 ± 1.6 (3.9–30.7)	5.6 ± 0.08	...
Lactulose†: (10)	control	23.7 ± 3.3 (8.3–36.2)	6.5 ± 0.02	...
	buffered	14.6 ± 3.0 (4.3–34.1)	6.4 ± 0.05	38.4
	unbuffered	13.8 ± 2.4 (3.9–27.9)	5.8 ± 0.08	...

* Inoculum = standard loopful of organisms.

† Inoculum = 0.5 ml of a suspension containing 10^{10} – 10^{11} cells/ml

‡ Control = buffered medium without substrate.

§ $p = 0.05$.

|| $p = 0.00001$.

isolates grown with lactose. Conversely, seven streptococci produced 30% less ammonia in the presence of lactose, but showed virtually no decrease when grown with lactulose.

In addition to inhibition due to the substrates themselves, cells of all groups except streptococci showed further decreases of ammonia production due to lowering of pH.

DISCUSSION

Bacteria produce ammonia from proteins and their derivatives by several processes such as proteolysis, peptide degradation, deamination and deamidation, each of which will vary with the type of bacterium and with its nutritional status and growth phase. Brain-heart infusion broth contains mainly peptides and amino acids, so our results refer mainly to the breakdown of these substances.

The most active groups of ammonia producers were gram-negative anaerobic and aerobic rods, clostridia, and *Bacillus* spp. Within the colon, gram-negative anaerobes greatly outnumber clostridia and enterobacteria which in turn outnumber *Bacillus* spp., suggesting that anaerobes such as bacteroides may be the major contributors to intestinal ammonia formed from proteins

TABLE IX

Ammonia production by gram-negative aerobes from brain-heart infusion broth with or without lactose or glucose

Substrate (number of strains examined)	Ammonia produced (mmol/litre) ± standard error (mean and range)	Final pH	Percentage inhibition of ammonia formation due to substrate
Lactose*: control† (14)	23.3 ± 0.9 (16.7–30.1)	6.7 ± 0.03	...
buffered	17.6 ± 0.7§ (12.9–21.5)	6.6 ± 0.03	24.5
unbuffered	15.0 ± 0.3 (12.7–16.7)	5.8 ± 0.05	...
Lactose†: control (13)	29.3 ± 1.2 (20.4–37.6)	6.4 ± 0.03	...
buffered	18.7 ± 0.8§ (15.7–26.5)	6.4 ± 0.02	36.2
unbuffered	16.3 ± 0.7 (11.9–21.0)	5.5 ± 0.04	...
Glucose†: control (5)	23.1 ± 1.2 (20.2–26.3)	6.8 ± 0.02	...
buffered	16.7 ± 1.5§ (12.4–19.2)	6.7 ± 0.04	27.7
unbuffered	13.7 ± 0.7 (12.7–16.3)	5.5 ± 0.12	...

* Inoculum = standard loopful organisms

† Inoculum = 0.5 ml of a suspension containing 10^{10} – 10^{11} cells/ml

‡ Control = buffered medium without substrate

§ $p < 0.005$

and their derivatives, rather than the gram-negative aerobes as has been previously assumed. *B. vulgatus* and *B. fragilis* consistently produced substantial quantities of ammonia. These bacteria are among the organisms isolated most frequently from the faecal flora (Moore and Holdeman, 1974) and may therefore make a substantial contribution to the ammonia concentration in the colon. The finding that eight isolates of *B. ovatus* all produced ammonia at less than 20 mmol/litre suggests that there may be species of *Bacteroides* that characteristically produce only moderate or low concentrations of ammonia. All the numerically dominant gram-negative anaerobes, and all the clostridia, mostly *C. perfringens*, that we tested formed ammonia. A larger survey would probably reveal some strains of intestinal bacteria, e.g., *Fusobacterium russii*, *Bacteroides eggerthii*, *Clostridium ramosum* and *Clostridium innocuum*, that may not form ammonia (Holdeman, Cato and Moore, 1977).

The relative contributions made by endogenous secretions, undigested food residues and bacterial cells to colonic ammonia concentrations is not clear. We have shown that small amounts of ammonia are produced due to endogenous bacterial metabolism and, because it has been estimated that over 50% of the total nitrogen in human faeces is contained in bacteria (Matill and Hawk, 1911), bacterial cells could provide a proportion of the non-urea ammonia formed in the colon.

Low pH is known to decrease ammonia production by pure cultures of enterobacteria and by mixtures of faecal organisms (Vince *et al.*, 1973; Vince *et al.*, 1978). The results presented in this study show that clostridia and gram-negative anaerobes also form less ammonia at low pH. This means that the three groups of intestinal bacteria that are most active in producing ammonia all show a marked decrease in activity as the pH falls. This is a particularly useful observation from the therapeutic point of view because lactulose, the synthetic disaccharide given to patients with hepatic encephalopathy to reduce the formation and absorption of ammonia, causes a marked decrease in caecal, and to a lesser extent faecal, pH (Agostini *et al.*, 1972; Bown *et al.*, 1974). These and earlier results suggest that intestinal bacteria are metabolically tolerant over a fairly wide pH range, i.e., 5.5–7.0, but show a marked decrease in activity, as judged by ammonia release, when the pH falls to 5.0 or less (Vince *et al.*, 1973; Vince *et al.*, 1978). However this value is lower than the normal colonic pH of 5.5–7.6 (Bown *et al.*, 1974) so that, *in vivo*, pH would not usually be a major factor limiting ammonia release.

It also seems unlikely that the ambient ammonia concentration is a factor limiting ammonia production in the colon. The mean concentration of faecal ammonia in normal subjects was found to be 11.2 mmol/litre with a 95% range of 2.9–44 mmol/litre (Wilson *et al.*, 1968). We found that these concentrations did not inhibit ammonia production by any of the major groups of intestinal bacteria. Furthermore, strains of enterobacteria tested in media containing 68 mmol ammonia/litre, which is several times greater than the mean concentration in the colon, produced amounts of ammonia similar to those formed in media containing 2 or 3 mmol/litre. Thus, even in uraemic subjects for whom there is evidence that the amounts of urea entering the colon and the amounts of ammonia formed (mean 17.9 mmol/litre, 95% range 4.3–75 mmol/litre) are both significantly greater than in normal subjects (Wilson *et al.*, 1968), the ambient ammonia concentration would probably not be a factor limiting ammonia release.

Growth of bacteria in the presence of either lactose or lactulose significantly lowered the concentration of ammonia formed subsequently from individual amino acids. A similar phenomenon was demonstrated originally by Stephenson and Gale in 1937 and by Epps and Gale in 1942. They showed with growing *E. coli* cells that the formation of alanine and glutamic deaminases and aspartase, as measured by ammonia formation, was inhibited by glucose fermentation, and that the inhibition was independent of fall in pH. This ability of glucose, 'the glucose effect', to inhibit the synthesis of inducible and constitutive enzymes has been termed catabolite repression (Magasanik, 1961). The effect is not restricted to glucose, but may extend to other substances that can act as readily available sources of intermediary metabolites and energy.

Since the original description of 'the glucose effect' there have been numerous reports of carbohydrate fermentation inhibiting the amino-acid deaminases of aerobic bacteria (Paigen and Williams, 1970), but information was lacking on the effect of carbohydrate fermentation on the metabolism of anaerobic intestinal organisms. This study has shown that an effect similar to 'the glucose effect' also occurs when gram-negative non-sporing anaerobes, e.g., *B. vulgatus* and *B. fragilis* are grown in the presence of sugars such as lactose and lactulose.

In addition to repressing the synthesis of enzymes, fermentation of glucose and other sugars may also inhibit the activity of preformed enzymes (Paigen and Williams, 1970). Such inhibition, known as catabolite inhibition, probably accounts for a substantial part of the reduction in ammonia formation that we observed in sugar-containing media when a large inoculum of 10^{10} – 10^{11} bacteria/ml was used. This count approaches the maximum number of organisms that can be accommodated in 1 ml of medium and allowed for only limited further growth during incubation, suggesting that most of the reduction in ammonia formation was due to a decrease in the activity of preformed enzymes.

Inhibition of enzymes responsible for release of ammonia, e.g., amino-acid deaminases, offers a simple mechanism for decreasing ammonia production by intestinal bacteria. These results help to explain the effect that lactulose and other fermentable substrates have in changing the balance of ammonia meta-

bolism from net production to net utilisation in a faecal incubation system (Vince *et al.*, 1978), for if bacterial uptake of ammonia continues normally and production falls, the net concentration of ammonia in the system will decrease accordingly.

Catabolite repression and catabolite inhibition, or other similar mechanisms, may be partially responsible for the beneficial effects of lactulose therapy in portosystemic encephalopathy (Bircher, *et al.*, 1966). In this syndrome, the symptoms of cerebral intoxication are attributed to nitrogenous metabolites, especially ammonia, formed by intestinal bacteria. We have found that fermentation of lactulose lowers the amount of ammonia formed by intestinal bacteria, and if a similar effect occurs *in vivo* one of the results of lactulose therapy will be to decrease the concentration of colonic ammonia available to exert a toxic effect on the host.

The effect of carbohydrate fermentation on the metabolism of amino acids was not restricted to inhibition; we also observed a stimulatory effect when some bacteria were incubated with aspartic acid. Another stimulatory effect for the production of carbon dioxide by decarboxylation of aspartic acid by *Clostridium welchii* was described by Meister, Sober and Tice (1951). The combined effects of stimulation and inhibition of ammonia production from amino acids may explain partially why the reduction in ammonia formation observed in brain-heart infusion broth was sometimes considerably less than that seen with washed cells in the presence of single amino acids. However, the net effect of carbohydrate fermentation, both in a nutrient medium and in the faecal-incubation system, was to decrease the final ammonia concentration (Vince *et al.*, 1978).

It is unlikely that the effects of lactulose and other fermentable substrates on the formation and activity of bacterial enzymes are restricted to enzymes involved in ammonia production; indeed the paper of Stephenson and Gale (1937) describing the glucose effect contains a reference to the inhibition of dephosphorylation of adenosinetriphosphate by Bacteriaceae grown in glucose. Further research on the effects of poorly absorbed carbohydrates on the metabolism of colonic bacteria will undoubtedly produce more evidence that the metabolic activities of intestinal bacteria are influenced by the nature of the diet consumed.

SUMMARY

Ammonia production by eight groups of intestinal bacteria was measured, and the effect on ammonia production of lowered pH and ambient ammonia concentration was determined. Endogenous ammonia production from bacterial protoplasm was also examined.

To examine the mechanisms by which fermentable substrates reduce ammonia formation in a faecal incubation system, the effect of lactose, lactulose or glucose on ammonia release by pure cultures of intestinal bacteria was studied.

The largest amounts of ammonia were generated by gram-negative anaer-

obes, clostridia, enterobacteria, and *Bacillus* spp. Gram-positive non-sporing anaerobes, streptococci and micrococci formed modest amounts, and lactobacilli and yeasts formed very little ammonia. All groups of bacteria formed less ammonia at pH 5.0 than at pH 7.0 and production of ammonia was not inhibited when 30 mmol ammonia/litre was included in the medium. Small amounts of ammonia were formed due to endogenous metabolism of bacterial cells.

Washed cell suspensions of four isolates of *Bacteroides*, one clostridial isolate and two streptococcal isolates formed less ammonia from alanine, methionine or histidine after growth in the presence of either lactose or lactulose. In contrast, the *Bacteroides* isolates formed more ammonia from aspartate than from either lactose or lactulose. Also, cultures of gram-negative anaerobes and enterobacteria, and to a lesser extent clostridia and streptococci, formed significantly less ammonia in nutrient broth when lactose, lactulose or glucose was included in the medium. This decrease in ammonia formation was not due to a fall in pH of the medium. Ammonia production by gram-positive non-sporing anaerobes was not affected by carbohydrate fermentation.

These results suggest that gram-negative anaerobic bacteria make a major contribution to ammonia generated from peptides and amino acids *in vivo*, and that ammonia may be formed from bacterial cells in the colon. Fermentation of lactose and lactulose may repress the formation and inhibit the activity of enzymes responsible for ammonia release. In the human colon these substrate effects may decrease the amount of ammonia available to exert a toxic effect on the host, and thus contribute to the beneficial effects of lactulose when it is used in the treatment of portosystemic encephalopathy.

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