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Faecal Microflora and Urease Activity during the First Six Months of Infancy

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ABSTRACT

Gastrointestinal degradation of urea might, according to a new hypothesis, have consequences for the regulation of acid-base balance as well as control of breathing during infancy. Thirteen infants were investigated from their first few days of life to the age of 6 months by collecting faecal samples at the age of 3 days, 2, 3, and 6 months, respectively. The faecal microflora was determined after aerobic and anaerobic cultivation and the faecal urease activity was assessed after 36 h aerobic and anaerobic preincubation. The infants were mostly breast fed and had a faecal microflora containing anaerobic bacteria such as Bifidobacteria, Bacterioides and Lactobacilli but also aerobics such as Escherichia coli, Enterococci and sometimes Klebsiella.The faecal pH increased from approximately 5.30 to 5.90, the pH after anaerobic preincubation being on an average 0.2 pH units lower than after aerobic preincubation. Simultaneously the nitric oxide production of the faecal specimens increased approximately 10-fold and the urease activity decreased by a factor of 3 to 5. We also found an inhibitory action of nitrate, nitrite (in μmolar concentration) and nitric oxide (in parts per million concentration) on the faecal urease activity. Hence, the present results warrant further research in order to determine more precisely the action of different concentrations of various nitrous oxides on individual bacterial species, and furthermore, to assay the faecal urease activity in victims of sudden infant death syndrome as well as in infants dead due to other causes.

INTRODUCTION

Some 10 years ago Atkinson & Camien (1) suggested that normal acid-base balance in mammals was greatly dependent on an acidification mechanism neutralising the endogenous production of bicarbonate (for review, see ref. 21). A vivid discussion of this hypothesis has taken place but failed to confirm its importance as regards maintenance of acid-base metabolism in general. However, it has been confirmed that blood-borne urea is also distributed to the gut contents where it is metabolised to ammonium ions by bacterial urease after which they are absorbed and transported to the liver where new urea synthesis takes place again, forming an entero-hepatic circuit of urea (14,20). Human breast milk contains large amounts of non-protein nitrogen (8, 10, 17) in the form of urea and ammonium ions. Subsequent bacterial conversion of the enteric content of urea to ammonium ions and bicarbonate would implicate an important and large hepatic production of urea and hydrogen ions. It has been hypothesized (21) that failure of the bacterial transformation of urea to ammonium ions could result in an alkalinization of such magnitude as to cause a ventilatory insufficiency and even Sudden Infant Death Syndrome (SIDS).

The aim of the present investigation was therefore to elucidate the development of the faecal microflora and its ability to convert urea to ammonium ions in normal infants up to the age of 6 months.

METHODS

During a period of approximately 10 months faecal specimens from 13 normal infants were collected on four separate occasions, at the age of 3 days, 2 months, 3 months and 6 months. The kind of feed received by the infants was recorded. The investigation was approved of by the Ethics Committee of the Medical Faculty of Uppsala University. During these investigations faeces after spontaneous defecation were carefully collected and subsequently treated under aseptic precautions. A minor portion of the faecal specimen from each patient was subject to subsequent bacteriological aerobic and anaerobic culture to reveal the presence of any microorganisms (15). The urease

activity of each bacterial species was monitored by a pH indicator (phenol red) after incubation of the specific bacterial species for 2 h in the presence of urea (0.3 mol/L) and a commercial bacteriological substrate (6) at pH 6.8-6.9. Analogously, another faecal portion from each patient was reserved for additional biochemical examinations as follows.

Preanalytical procedure

On each occasion four faecal specimens, each of approximately 40 mg, were harvested from each individual and suspended in 4 mL of sterile, physiological NaCl solution in sterile test tubes. Two of the suspensions were preincubated anaerobically and the other two aerobically for 36 h at 33°C, followed by centrifugation to separate faecal material from a clear supernatant that was either colourless or yellowish in colour. Before centrifugation the gas in the aerobic tubes was transferred into a syringe for subsequent immediate analysis of nitric oxide (NO). The anaerobic preincubation was carried out in an anaerobic tight chamber containing a gas mixture of 90% CO_2 and 10% H_2 circulated over a palladium catalyst (GasPak Plus', Becton Dickinson Microbiology Systems, Cockeysville, Md USA) while aerobic preincubation was done in ambient air in sealed test tubes.

After having been tested for pH the 4 supernatants (2 from the anaerobic conditions and 2 from aerobic conditions), were then subdivided into 400 µL-samples (in duplicate), each of which was mixed with 3 mL of sterile physiological NaCl solution forming an incubation mixture. Hence, 8 incubation mixtures (4 from the anaerobic conditions and another 4 from aerobic conditions) were supplemented with urea to a final concentration of 1.95±0.12 mmol/L, and another 8 incubation mixtures to a final urea concentration of 3.40±0.22 mmol/L for subsequent incubations. Concomitantly, 4 other incubation mixtures were saved (2 from anaerobic conditions and 2 from aerobic conditions) for further incubation without any additional urea in order to determine the endogenous faecal content of urea. Finally, sterile, physiological NaCl solutions (3.4 mL) containing 1.95±0.12 and 3.40±0.22 mmol/L of urea, respectively, each in duplicate, were also incubated (blanks). Hence, each patient generated a total of 24 tubes to be incubated for 30h at 33°C.

Biochemical analyses

The NO concentration of the gaseous atmosphere of the aerobic tubes was determined in a Chemiluminescence NO-NO_2-NO_x analyser (Model 42, Thermo Environmental Instruments Inc, USA) and expressed in parts per billion (ppb) or parts per million (ppm). NO in the anaerobic tubes was not determined as measurements of the gas in the anaerobic chamber (without preincubation tubes) showed some content of NO.

All incubation mixtures were analysed regarding their contents of urea and ammonium ions in an Hitachi 911 analytical system. Urea was measured employing auxiliary enzymes to convert urea by urease to CO_2 and ammonium ions. The ammonium ions thus formed were reacted in a reductive amination of α-ketoglutarate catalysed by glutamate dehydrogenase with concomitant oxidation of NADH which was followed spectrophotometrically at 340nm. Hence, strictly stoichiometric relationships existed between the amounts of NADH converted and ammonium ions formed (as a function of the actual urea concentration) in the urease reaction. Ammonium ions in the samples were assayed similarly but without urease. The urea and ammonium ion concentrations were expressed in mmoles and μmoles per gram (wet weight) faeces, respectively, after blank corrections. All chemicals were of *pro analysi* grade from Boehringer-Mannheim, Germany.

Ad hoc analyses

Using the same technique as above the urease activity of 2 pooled faecal samples from each 3 infants was investigated after addition of known concentrations of NO (0.8, 2.5, 10, 100, 200, 500 and 1000 ppm), sodium nitrate and nitrite (0.1 and 1 μmol/L, respectively). Similarly, different amounts of urease (EC 3.5.1.5, jack bean urease type IV, Sigma Chemical Co., St Louis MO, USA) was added to sterile normal saline containing 1.95±0.12 and 3.40±0.22 mmol/L urea, respectively.

Statistics:

Results of biochemical analyses were presented as means ± SD, and range, while results of the quantitative bacteriological cultures were given as box plots displaying the 10th, 25th, 50th (median), 75th, and 90th percentiles.

RESULTS

Most of the infants were breast-fed during their first few months of life, after which they were gradually weaned (Table 1). Their faecal microflora was mainly of the anaerobic kind with large numbers of Bifidobacteria and Bacteroides, but also, from their first week of life, aerobic bacteria such as Streptococci, Enterococci, Escherichia coli, and Klebsiella. One of the infants (No. 7) had a respiratory infection and had finished an oral cephadroxil treatment 3-4 days before the last faecal specimen was collected. At this stage the faecal anaerobic micoflora was almost eliminated and among the aerobics only Enterococcus faecalis, unidentified gram negative rods and Candida remained.

The faecal pH was 5.6±0.8 after aerobic preincubation and 5.3±0.6 after anaerobic preincubation, respectively, during the first week of life. At 6 months of age it had increased to 5.9±0.6 and 5.7±0.7, respectively. Thus, pH in the anaerobic preincubation mixtures continued to be approximately 0.2 pH units lower than the aerobically preincubated samples throughout the whole study period (Table 2, Fig 9).

Gender (M/F)	3 days	2 months	3 months	6 months
F	BM	BM	BM	BM
M	BM	BM	BM	BM
M	BM	BM	BM	BM
F	BM	BM	BM	BM
M	BM	BM	–	–
M	BM	BM	BM	BM
F	BM	BM	BM	BM+BF+SS*
M	BM	BM	BM	BM+SS
M	BM	–	–	–
M	BM	BM	BM+BF	BF+SS
F	BM	BM	BM	BM+SS
F	BM	BM	BM	BM+SS
M	BM	BM	BM	BM+SS

BM=breast milk; BF=baby food; SS=soft solids; – =not accessible

*= taken 3-4 days after oral antibiotic therapy

Table 1. Gender of infants and type of feeding at various sampling times.

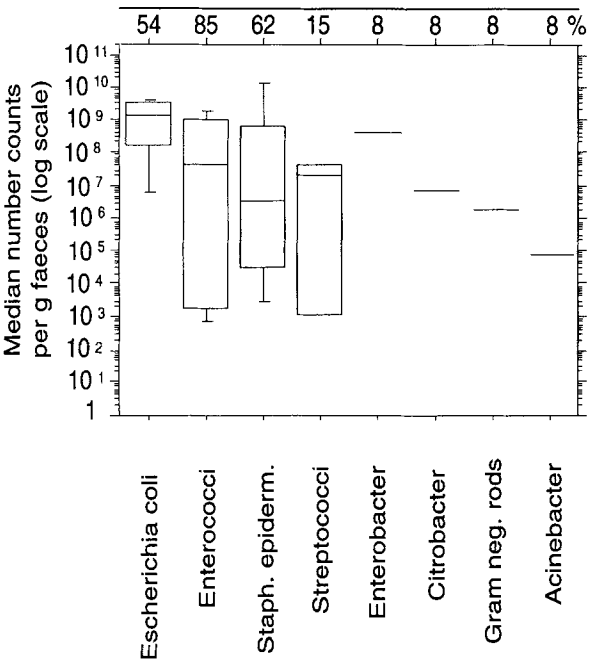

Fig 1
Aerobic faecal microflora at 3 days of age (per g faeces wet weight) presented as the median, 10th, 25th, 75th, and 90th percentiles (log scale). The prevalence (%) of each species is indicated at the top.

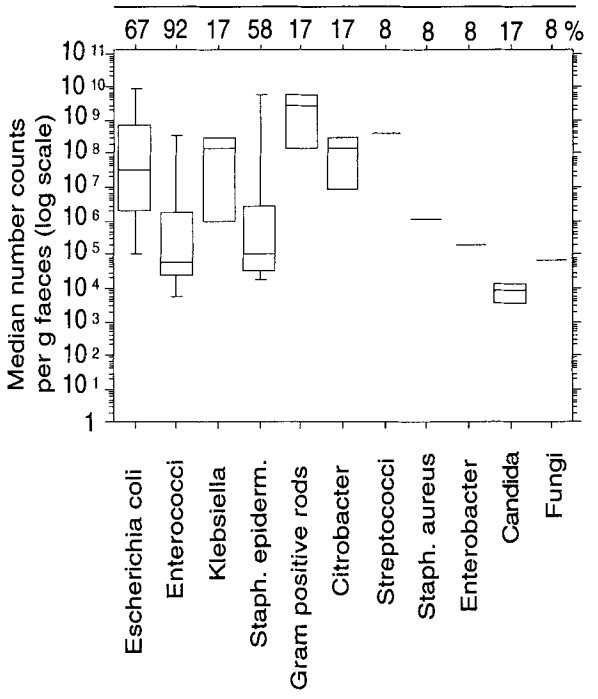

Fig 2
Aerobic faecal microflora at 2 months of age. Presentation as in Fig 1.

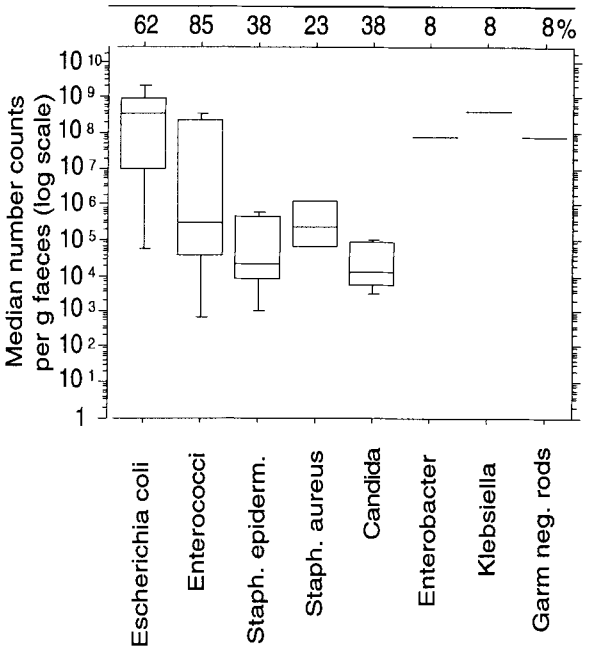

Fig 3
Aerobic faecal microflora at 3 months of age. Presentation as in Fig 1.

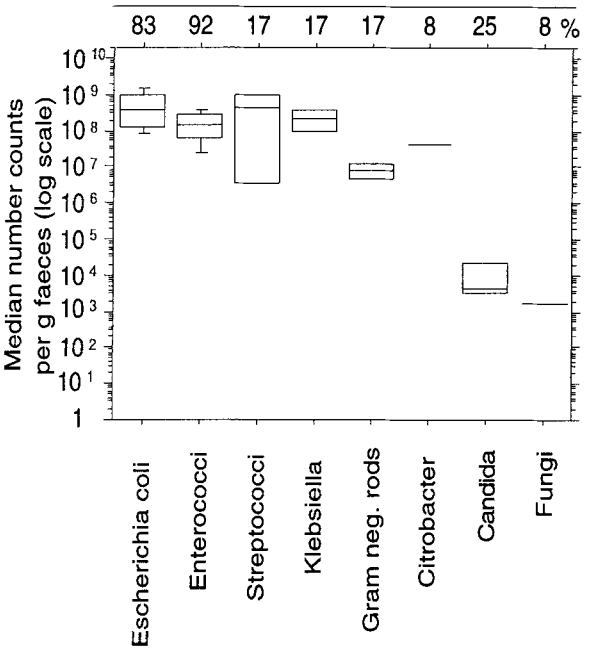

Fig 4
Aerobic faecal microflora at 6 months of age. Presentation as in Fig 1.

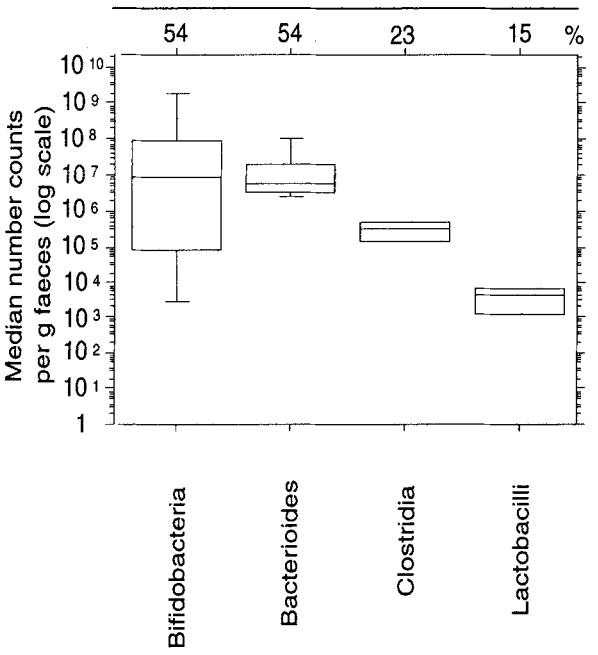

Fig 5
Anaerobic faecal microflora at 3 days of age. Presentation as in Fig 1.

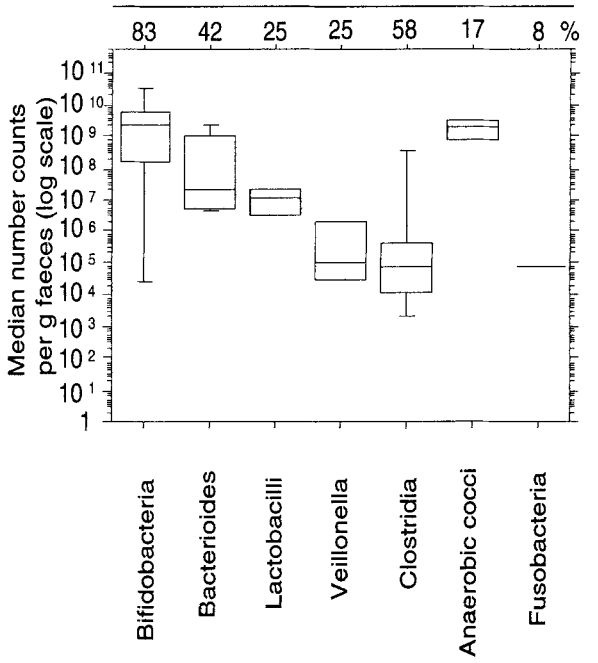

Fig 6
Anaerobic faecal microflora at 2 months of age. Presentation as in Fig 1.

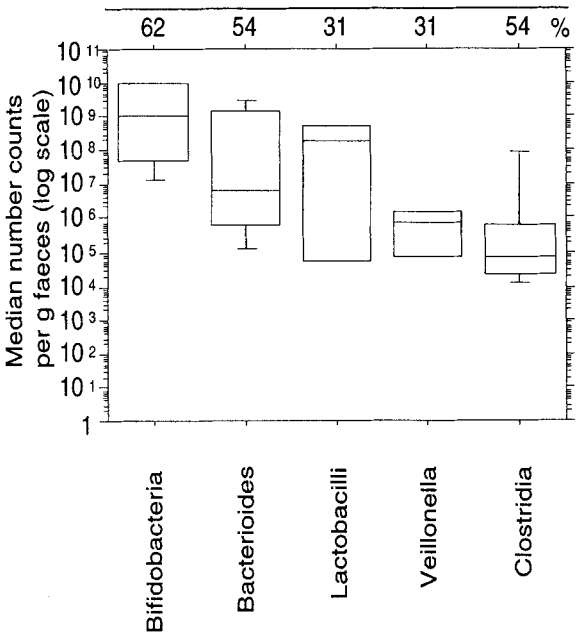

Fig 7
Anaerobic faecal microflora at 3 months of age. Presentation as in Fig 1.

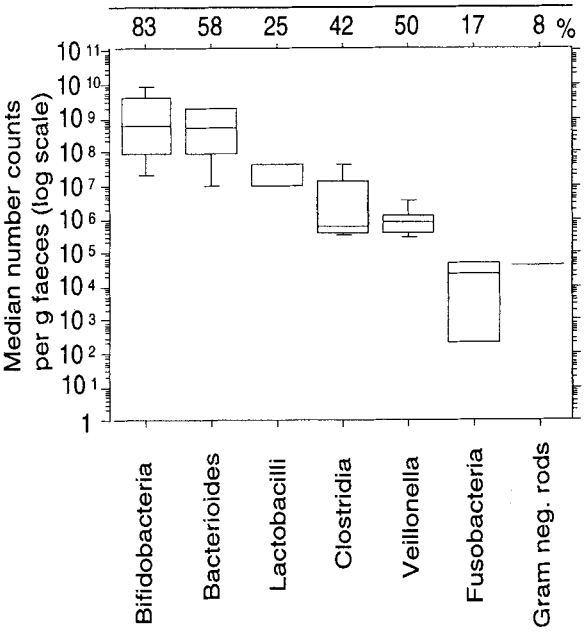

Fig 8
Anaerobic faecal microflora at 6 months of age. Presentation as in Fig 1.

The faecal urease activity expressed as the ammonium ion formation per gram faeces (wet weight) after 30h incubation (Table 3, Fig 10) slowly decreased throughout the 6 months' study period.

Age	Aerobic pH	Anaerobic pH	Nitric oxide concentration
3 days	5.55±0.76 (4.20-6.40)	5.34±0.58 (4.25-6.00)	52±35 (15-120)
2 months	5.52±0.61 (4.45-6.40)	5.32±0.63 (4.30-6.12)	151±62 (51-234)
3 months	5.80±0.52 (4.80-6.30)	5.52±0.54 (4.50-6.20)	149±86 (36-372)
6 months	5.92±0.58 (4.40-6.50)	5.70±0.67 (4.50-6.50)	575±419 (36-1440)

Table 2. Faecal concentrations of nitric oxide (ppb) at different infant ages under aerobic preincubation conditions. Actual pH:s of preincubation suspensions are given (range given in parenthesis).

	Aerobic preincubation		Anaerobic preincubation	
Incubation urea concentration	1.95±0.12	3.40±0.22	1.95±0.12	3.40±0.22
3 days	56±68 (2-212)	67±74 (6-298)	40±34 (3-128)	60±34 (5-111)
2 months	40±60 (7-221)	57±79 (12-289)	36±46 (12-179)	47±56 (16-221)
3 months	36±67 (3-255)	42±72 (5-276)	22±26 (3-94)	32±35 (3-136)
6 months	15±14 (0-43)	20+17 (3-51)	11±8 (2-27)	12±9 (1-29)

Table 3. Faecal ammonium ion production expressed as ammonium ion formed (mmol/ g faeces wet weight after 30 h incubation) (range given in parenthesis) at different infant ages using a low and a high substrate concentration, and under 2 different preincubation conditions.

The phenol red method detected bacterial ammonium ion only in approximately 50% of the specimens containing aerobic species, and only in 1% of the specimens containing anaerobic bacteria, whereas all investigated faecal specimens exhibited urease activity throughout the study period on biochemical analysis.

The faecal urea content in the present material was generally 5-15 µmol/g faeces wet weight. On a few occasions, especially during the first week of life, the faecal urea content was somewhat higher, but in the present material the maximum was approximately 40 µmol/g faeces wet weight.

Age	µmol/g faeces (wet weight)	mmol/L water (assuming 90% water content, ref 7, 16)
3 days	39±57 (0-170)	0.8±1.2 (0-3.7)
2 months	4±12 (0-43)	0.1±0.3 (0-0.9)
3 months	5±17 (0-60)	0.1±0.4 (0-1.3)
6 months	6±14 (0-43)	0.1-0.3 (0-0.9)

Table 4. Faecal urea content ± SD (range given in parenthesis) at different infant ages .

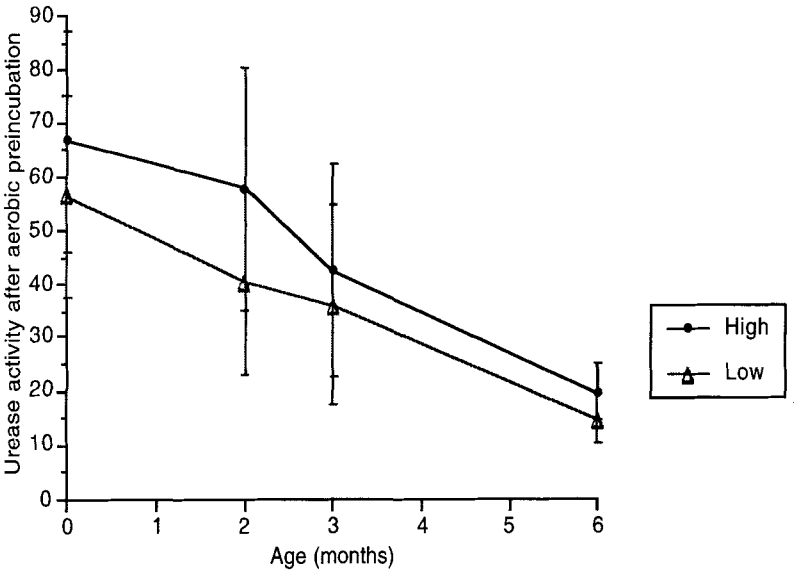

Fig 9.
Faecal urease activity expressed as ammonium ions formed in mmol/per g faeces wet weight after 30 h incubation. The assay was carried out at a high (upper curve) and a low (lower curve) urea concentration. Data shown also in Table 3.

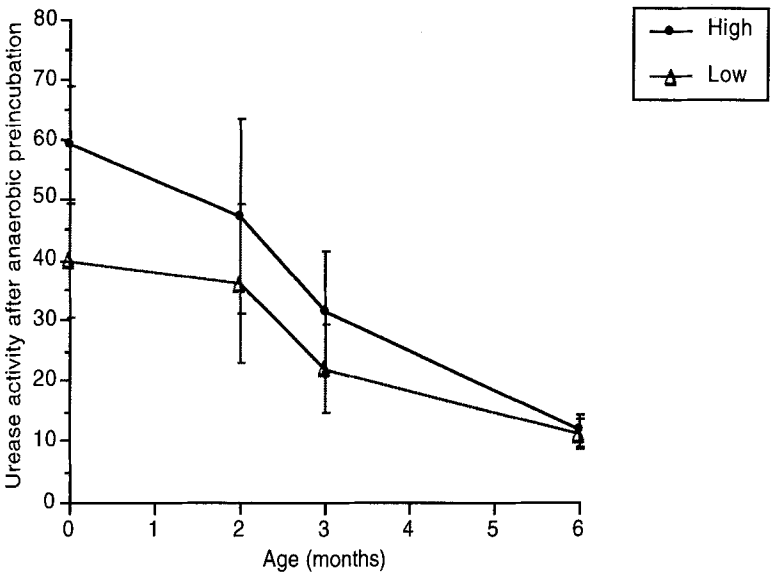

Fig 10
Faecal urease activity expressed as ammonium ions formed in mmol/per g faeces wet weight after 30 h incubation. The assay was carried out at a high (upper curve) and a low (lower curve) urea concentration. Data also shown in Table 3.

The NO concentration in the aerobically preincubated test tubes increased from 52±35 to 575±419 ppb (Table 2, Fig 12) during the observation period, i.e. the gaseous concentration increased approximately 10-fold during the first 6 months of life.

There was no urease activity when more than 10 ppm of NO was equilibrated with any of the preincubation mixtures with varying urease activity between 1 and 10 ppm. Similarly, the bacteriological urease activity was reduced by 10-25% and 75-90% when the concentration of nitrate or nitrite in the preincubation mixture was 0.1 or 1 µmol/L, respectively. In contrast, addition of NO to sterile urea solutions with jack bean urease resulted in unchanged, full urease activity, NO having no effect on the activity of this enzyme.

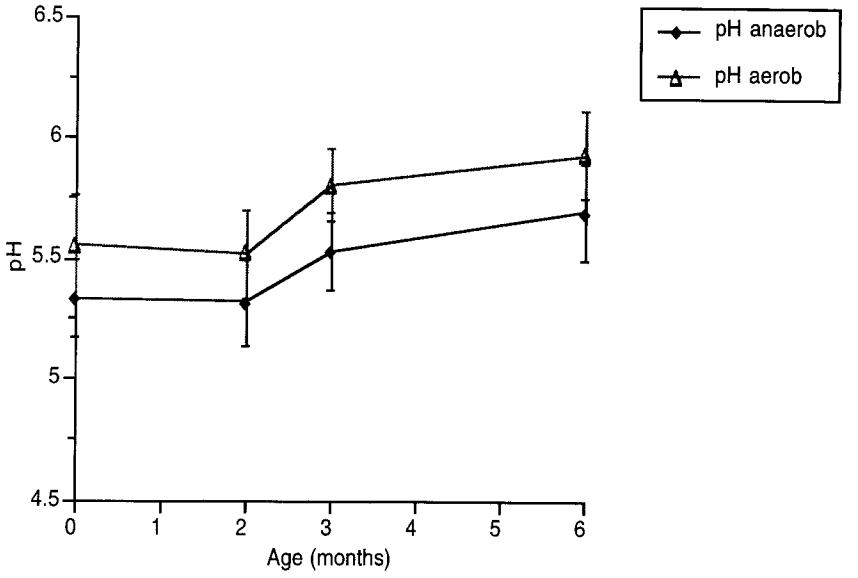

Fig 11

pH in faecal suspension after aerobic and anaerobic preincubation.

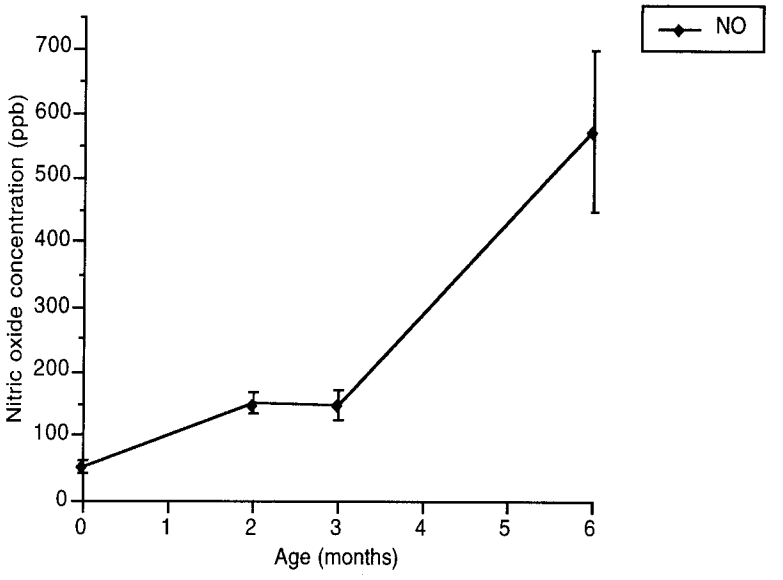

Fig 12

Nitric oxide concentration (in parts per billion) in test tubes after aerobic preincubation.

DISCUSSION

Human breast milk contains large amounts of non-protein nitrogen (8, 10, 17) in the form of urea (3-6 mmol/L) and ammonium ions (100-500 µmol/L). Metabolic conversion of the enteric content of urea to ammonium ions is considered to be achieved by the action of bacterial urease (19) resulting in a normal faecal content of urea considered close to zero (7). The present results seem to confirm this view, and furthermore, our measurements indicate that the normal faecal urea content during infancy is of the order 5-10 µmol/g faeces (wet weight). As the majority of the infants were breast fed, thus ingesting 2-6 mmoles urea (equivalent to 400-1200 µmoles ammonium ions) daily, it is obvious that the gastro-intestinal urea content was eliminated with great efficiency. It should, however, be noted that although the faecal urease activity was generally high, it varied during the first few days of life when, also the unmetabolized faecal urea was a more frequent finding than later.

The present investigation has also documented a faecal bacterial content similar to what has been reported in recent years from different parts of the western world where aerobic enteric bacteria currently play an increasing role eliminating an earlier difference between breast-fed infants and those being bottle-fed (2, 5 , 15). It may be noted that almost all newborns, inspite of being breast-fed from the very first few days of life, have large numbers of Escherichia coli and enterococci in their stools. Although the phenol red method detected urease activity in some aerobic bacterial species, it seems that this method for detection of anaerobic ammonium ion production is not sensitive enough, the reason probably being that these bacteria simultaneously produce acids which keep the pH of the bacterial suspensions at a pH of approximately 5, at some distance from the pH range (approx. 6.6-8.0) (9) where the phenol red indicator has its colour transition zone. The average faecal pH gradually increased with age and seemed, in accordance with other investigators (5), to increase in conjunction with weaning, when aerobic bacteria were abundant, and simultaneously with an increasing bacterial production of NO.

As an ad hoc analysis we also investigated the effect of NO, sodium nitrite and sodium nitrate on the urea degrading activity in faecal samples from 6 infants in the present series. These asseys showed an inhibitory effect on the faecal urease activity by NO (total inhibition at 10 ppm) as well as nitrate and nitrite (75-90% inhibition at 1μmol/L in both cases). It has previously been described that NO, in μmolar concentration, may inhibit the reductive enzymes of some anaerobic bacteria (4, 11) blocking the nitrate reductase enzyme. These results are of special interest as several of the appliances and means of entertainment used in modern society create increasing concentrations of nitrous oxides both in the earth and in the atmosphere (13, 18) but fortunately not in milk (12). In addition, our finding of an unchanged activity of jack bean urease after addition of NO seemed to confirm that the NO effect is exerted already in the bacterial production of urease.

The faecal urease activity, expressed as the production of ammonium ions per gram faeces wet weight during 30 h, decreased throughout the first 6 months of life both after aerobic and anaerobic preincubation. However, as the faecal mass is increasing by age (16) this does not mean that the ability of the infants to degrade the enteric urea content decreased. The urea content of the breast milk is in the range 3-6 mmol/L, while that of the infant formula, made from cow's milk, contains approximately half of this concentration. Thus, with a daily oral intake of 3-6 mmoles urea, a mean estimated enteral passage time of 25h (7), and a daily faecal mass of approximately 100 g (at 3 months of age), the urea degrading capacity recorded in the present series is in excess of what is needed for total degradation of all dietary urea to ammonium ions and carbon dioxide under normal conditions. The consistently very low faecal urea content is a proof of this kind of reasoning. It should, however, be noted that use of antibiotics can destroy the faecal microflora and simultaneously the urea degrading capacity of the GI tract (3).

The urease reaction at pH 7 is generally described as:

$H_2N–CO–NH_2 + 2H_2O \leftrightarrow NH_4^+ + NH_3 + HCO_3^-$

However, when in an acid solution the result of the reaction would be NH_3, NH_4^+ and CO_2, as any bicarbonate produced should be protonized to carbonic acid which subsequently is dissociated to CO_2 and water. Hence, we also noted visible gas bubbles in many of the preincubation tubes. As pH in these tubes was in the range 4.2-6.5 the action of faecal urease cannot be expected to balance the acidifying action of the urea cycle to any appreciable extent (1).

The urea content in faeces has been described as close to zero (7). When analysed more extensively we found that this concentration generally was 5-15 μmol/g faeces wet weight. On a few occasions, especially during the first week of life, the faecal urea content was somewhat higher, but in the present material less than 50 μmol/g faeces in healthy infants. In comparison, sigmoid faeces from SIDS victims at the postmortem pilot investigation contained on an average 100 (range 50-1700) μmol/g faeces (22). Assuming the present results could be confirmed, values above 100 μmol/g faeces indicate a possible risk of development of respiratory failure.

Conclusions: In newborn infants the GI tract is quickly colonized not only by anaerobic but also aerobic bacteria. Also in breast-fed infants aerobic bacteria are common from the first few days of life to the age of 6 months. During the same period of time faecal pH increases and the NO production is 10-fold. The faecal urease activity during the first few days of life is variable, leaving significant amounts of undegraded urea in faeces of some infants. Later the faecal urease activity gradually decreases but is sufficient to keep the faecal urea content very low (<50 μg/g faeces wet weight).

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