Generation of ammonia from non-urea sources in a faecal incubation system

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Summary

1. A 25% faecal suspension in sodium chloride solution, incubated anaerobically at 37°C for 48 h, showed excellent survival of all the main groups of faecal bacteria.

2. All faecal incubation systems studied generated large amounts of ammonia, particularly those in which bacterial counts fell during incubation. As normal faeces contain negligible amounts of urea this ammonia must have been generated from sources other than urea.

3. Ammonia was also generated by faeces delivered by sodium chloride enema, and by ileostomy fluid, indicating that the phenomenon is not confined to distal colonic contents.

4. Ammonia generation by incubated faeces was inhibited by prior autoclaving of the sample, but not by sterilization with gamma-irradiation.

5. Generation of ammonia by incubated stool was accompanied by release of large amounts of organic anion and a fall in pH.

6. These observations are interpreted as evidence that ammonia generated within the colon in situ is not derived exclusively from urea, but also from bacterial deamination of amino acids, peptides and proteins. Simultaneously bacterial activity generates large amounts of organic acid. The presence of living bacteria is not essential for ammonia generation, provided that bacterial enzymes are present.

7. Bacterial generation of organic solute in faeces which have left the body is sufficiently rapid to cast serious doubts on the validity of faecal centrifugation, or other time-consuming techniques involving lengthy handling of faeces, as methods of obtaining extracellular faecal fluid for measurements of organic constituents or ammonia.

Key words: amino acids, ammonia, bacteria, colon, faeces, urea.

Introduction

Ammonia is known to be generated in large amounts in the colon. The main source is usually thought to be bacterial hydrolysis of urea, but substantial amounts might also be formed by bacterial metabolism of other nitrogenous substances in dietary residues, desquamated epithelial cells and endogenous secretions.

Gamble (1915) showed that faeces continue to generate ammonia after they have left the body. It is now known that normal faeces contain negligible amounts of urea (Wrong, Metcalfe-Gibson, Morrison, Ng & Howard, 1965; Owens & Padovan, 1975); Gamble's original observation was therefore evidence of colonic production of ammonia from sources other than urea. To investigate this phenomenon further we have studied the generation of ammonia in a faecal incubation system.

Incubation of faeces or faecal homogenates in vitro is a simple way of studying the metabolic activity of faecal organisms. It has been used to investigate amino acid metabolism (Asatoor, Lacey, London & Milne, 1962), the breakdown of cholesterol (Wood & Hatoff, 1970) and the production of ammonia in experimental uraemia (Bourke, Milne
& Stokes, 1966). These previous studies lacked bacteriological control. The ideal incubation system would be one in which bacterial activity does not differ substantially from that within the colon. Few bacteriological studies of the proximal colon have been made, but the flora of distal colonic contents is that of faeces, and the aim of the present work was therefore to develop a faecal incubation system in which the bacterial flora remains substantially unchanged during incubation.

In addition to freshly passed faeces we have incubated stool delivered by a sodium chloride enema, and material obtained from an ileostomy, to discover whether the contents of the more proximal colon behave like freshly passed faeces in their generation of ammonia. In other experiments ammonia production has been examined in faeces sterilized by autoclave or by gamma-irradiation; this was done because many of the bacteria in faeces are known to be dead, and the biochemical changes observed on incubation could be the result of the combined metabolic activity of living bacteria and enzymes liberated from dead organisms.

Methods

Incubation of faeces

Collection of faeces. Stools were obtained from six normal subjects and one subject with an ileostomy. Except for the latter subject, who used a clean ileostomy bag, all stool samples were passed directly into polythene bags which were weighed intact. Only faecal samples weighing over 50 g were used.

25% suspension of faeces. This was the principal type of faecal incubation system used. A stream of oxygen-free nitrogen, piped through butyl rubber tubing, was directed over the specimen within 5 min of collection, and all subsequent manipulations were performed under nitrogen. Samples of whole stool were taken for analysis at zero time, and the remaining faeces were homogenized for 7–10 min with three times their weight of sodium chloride solution (154 mmol/l; saline) in a Colworth 'stomacher' 3500. The homogenate was transferred to a disposable polypropylene beaker, dialysis capsules were added in order to obtain subsequent samples of diffusate for analysis (Wrong et al., 1965) and a polythene cover was fixed over the top of the beaker with tape. The nitrogen line was inserted through a small hole in the cover and anchored so that nitrogen was expelled over the surface of the mixture. The flow of nitrogen was adjusted to approx. 200 ml/min. The beaker was placed on an orbital bed (Luckham Rotatest R/100) and shaken at 30 rev./min in an incubator kept in a fume cupboard and adjusted so that the temperature remained between 36 and 37°C. The initial weight of the beaker, contents and cover was noted and checked periodically, and sterile water added at intervals to compensate for weight losses caused by evaporation of water (about 1 g/h).

33% suspension of faeces. In seven early studies a 33% suspension of faeces was used. The stool was homogenized in a Waring Blender with twice its weight of saline; it was then incubated under nitrogen in a glass beaker covered with a watch glass, and stirred continuously on a magnetic stirrer placed in an incubator at 37°C. Otherwise the procedure was that used with the 25% suspension. Later it was found that bacteria survived better in the 25% suspension, and more precise temperature control was achieved with the shaker. Significantly less ammonia was generated by the 25% suspension than by the 33% suspension. To check that this difference resulted from dilution rather than from the other changes in methodology, one 33% faecal suspension was incubated with the system described for the 25% suspension. Ammonia results fell within 1 SD of those obtained for the mean of the original five 33% suspensions. These previous studies lacked

Undiluted faeces. In order to repeat Gamble's (1915) original observations a 240 g sample of faeces from two subjects was mixed by thorough kneading through a polythene bag on ice, and a small portion taken for analysis. The remaining specimen was divided into six approximately equal samples (to assist subsequent sampling) in separate polythene bags, which were flushed with nitrogen, sealed by heat and then incubated at 37°C.

Colonic contents from more proximal intestine

(a) 550 g of faecal material was obtained by use of a saline enema (1000 ml) in a normal subject. No further dilution was made and the total result of the enema was homogenized and incubated in the same way as the samples of 25% faecal suspension.

(b) A 21-year-old subject who had had a total colectomy and ileostomy for ulcerative colitis 18 months earlier provided a fresh 92 g sample of
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ileostomy fluid, which was homogenized and incubated without further dilution in the same way as the 25% faecal incubations.

Sterilized stool

Autoclaving. A 150 g sample of freshly passed faeces was thoroughly mixed by kneading in a polythene bag, and divided into three roughly equal samples which were placed in glass beakers, covered with aluminium foil and autoclaved for 15 min at 15 lb/in². One sample was tested for sterility, total nitrogen and ammonia at zero time, immediately after autoclaving, and the other two samples were incubated at 37°C and similarly tested at 24 and 48 h respectively for sterility and ammonia. Specimens were not diluted with saline in this experiment or the subsequent two involving gamma-irradiation, as there was no need to maintain optimum conditions for bacterial survival.

Gamma-irradiation. Two incubation studies were performed on stool sterilized by gamma-irradiation at AERE (Harwell, Oxon., U.K.). In each experiment freshly passed stool from four subjects was mixed by kneading in a polythene bag and then divided into eight or ten separate samples and sealed in smaller polythene bags. All specimens were then placed on ice and sterilized by gamma-irradiation. One sample in each experiment was analysed for sterility, total nitrogen and ammonia at the start of incubation (zero time), and further samples were opened and analysed at intervals up to 48 h. Final aerobic and anaerobic cultures were sterile up to 14 days after gamma-irradiation.

Sampling

Base-line determinations were obtained in some studies by use of faecal dialysis in vivo (Wrong et al., 1965), the subject ensuring that the specimen of stool studied contained dialysis capsules by swallowing several 24–72 h earlier. Faecal homogenates were sampled for chemical analysis by (1) dialysis in vitro, in which dialysis capsules were added 6 h before a sample was taken, usually at the time of previous sampling, or (2) low-speed centrifugation at 3000 g and 0°C or (3) high-speed centrifugation at 40 000 g and 0°C, as recommended by Tarlow & Thom (1974). These methods gave very similar results; in twenty-seven observations with all three techniques the ammonia concentration of diffusate in vitro was 104·8 ± 1·6 (SEM)% and that of low-speed centrifugate was 99·5 ± 2·9% of the concentration found in high-speed centrifugate. We considered these differences to be so small that they could be disregarded for the purpose of the study, and for convenience we usually sampled by dialysis in vitro. High-speed centrifugation (Tarlow & Thom, 1974) was the method of sampling used in incubation studies in which stool had not been diluted (sterilized and ileostomy specimens).

Bacteriological studies

Media. Saline (NaCl solution, 154 mmol/l) was sterilized shortly before use and kept under nitrogen. Transport broth, based on that of Crowther (1971), contained peptone (Oxoid L37) 10 g, cysteine hydrochloride 0·5 g, glycerol 100 ml, and distilled water 900 ml. Blood agar base (Oxoid CM 271), with 10% blood (Oxoid SR 50) and 1% yeast extract (Difco), was used to isolate all groups of organisms. Azide agar (Oxoid CM 259 and 10% blood) was also used for the isolation of streptococci.

Sampling. Samples of 1 ml of faecal suspension were taken at 0, 24 and 48 h and added to 9 ml of sterile transport broth. Serial tenfold dilutions were prepared under a stream of nitrogen, and 0·1 or 0·001 ml aliquots were spread over the surface of the plates with a glass spreader. In five 25% suspension studies which were submitted to statistical analysis each dilution was plated and counted in quadruplicate. Total cell counts (living and dead cells) were made in duplicate with the aid of a Thoma counting chamber.

Incubation of plates. Plates for the isolation of anaerobes were incubated at 37°C for 5 days in hydrogen/carbon dioxide/nitrogen (1:2:17, by vol.). Blood-agar plates were incubated aerobically at 37°C for 24 h and azide plates for up to 3 days.

Identification and counting of organisms. Six groups of organisms were counted: (1) total cell count, (2) total viable anaerobes, (3) Gram-negative anaerobic non-sporing rods (e.g. bacteroides, fusobacteria), (4) Gram-positive anaerobic non-sporing rods (e.g. bifidobacteria, eubacteria, propionibacteria), (5) Gram-negative aerobic rods (e.g. Escherichia coli, Klebsiella aerogenes) and (6) streptococci (all catalase-negative, Gram-positive cocci). Colonies were
counted by means of a Cook electronic colony counter. For statistical analysis bacteriological data, after logarithmic transformation, were analysed as a two-factor (bacteria and times) factorial experiment in randomized blocks.

**Chemical methods**

Analytical methods were essentially unchanged from those used earlier (Wrong et al., 1965). Total water content was determined by drying 0.2–1.0 g samples of faeces or faecal fluid to constant weight at 98–102°C, osmolality by freezing-point depression with an Advanced Instruments Inc. osmometer, and pH and Eh by a Radiometer pH meter 26. Total nitrogen was determined by a micro-Kjeldahl procedure, organic anion by a micro-modification of the method of Van Slyke & Palmer (1920) and ammonia by the microdiffusion method of Conway (1957). The last method measures volatile bases, and is not specific for ammonia but also includes substances such as dimethylamine and other low-molecular-weight amines which are known to be generated in the colon (Simenhoff, Asatoor, Milne & Zilva, 1963). Therefore duplicate analyses comparing Conway values with the results of the Nessler procedure were performed on many occasions, as previously described (Wrong et al., 1965). The results of these two estimations were always within 1 mmol/l, indicating that the concentration of volatile base other than ammonia in an incubated suspension of faeces must be less than this amount.

**Results**

**Bacteriological findings**

25% faecal suspension. Total cell counts, which include both living and dead cells, and counts of the major groups of viable organisms remained substantially unaltered over 48 h. The results from five incubations that were studied in greater detail are shown in Tables 1 and 2. No significant change occurred in either the total or viable cell counts over the 48 h period (P<0.05 in each group) although counts of five of the six groups were slightly lower at

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<tr>
<th>Table 1. Bacterial cell counts in faecal homogenate</th>
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<td>Results are expressed as mean log counts/ml of faecal homogenate.</td>
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<th>Log no. of cells/ml</th>
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<th>Gram-positive non-sporing anaerobes</th>
<th>Total viable anaerobes</th>
<th>Coliforms</th>
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TABLE 2. 25% and 33% faecal suspensions: greatest reductions observed in viable bacterial counts (log units)

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<th>Time of incubation (h)</th>
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48 h than at zero time. The greatest reduction observed in any group of organisms was a fall of 0.5 log unit in coliforms at 48 h in one study (Table 2).

33% faecal suspension. In this system there was a steady and progressive decrease in the counts of all groups of organisms over 48 h (Tables 1 and 2). When the 33% suspension was incubated with the system described for the 25% suspension no change occurred in counts over the first 24 h, but counts of all groups had fallen at 48 h. In some experiments counts of individual groups fell by as much as 3 log units (Table 2).

Undiluted faeces. Counts of streptococci fell by 1 log unit but no other significant change occurred during the first 24 h. Counts of coliforms and streptococci were reduced by 1–2 log units at 48 h (Table 1).

Saline enema. At 24 h counts of organisms were similar to initial values, with the exception of the Gram-negative non-sporing anaerobes, whose counts had decreased by 1 log unit. At 48 h there was a general decrease in the counts of anaerobic organisms (Table 1).

Ileostomy fluid. Initial counts were similar to those described for ileostomy fluid by Vince, O'Grady & Dawson (1973), showing lower total anaerobes than freshly passed faeces. In contrast to all the other incubations, bacterial counts increased over the first 24 h, and some of these increases were maintained at 48 h (Table 1).

Chemical results

At the pH of the faecal samples of these experiments (5.1–7.3) ionized ammonium constitutes the major part of total ammonia, but for convenience we have used the term ‘ammonia’ for the sum of ammonium and free un-ionized ammonia.

![Fig. 1. pH of faecal suspensions over 48 h of incubation. The 25% and 33% suspensions showed similar changes, and are not distinguished.](image-url)
were 33 mmol/l for the 25% and 89 mmol/l for the 33% suspensions. In order to compare results from different faecal suspensions of various water content, ammonia concentrations are expressed in Fig. 2 per 20 g of stool solids, this being the solid content of approximately 100 g of wet faeces, or the average normal amount of stool passed per day. In Fig. 3 we have attempted to make allowance for the various nitrogen contents of different stools by expressing ammonia production as a percentage of total faecal nitrogen. The profile of ammonia production was similar in the two incubation systems, but production was less in the 25% suspensions in which the bacterial population survived better, and after the first 12 h this difference in ammonia production was significant ($P = 0.01$ or less) whether expressed per 20 g of stool solids or as a proportion of total faecal nitrogen.

A rise in ammonia concentration should render a faecal incubation system more alkaline unless there is a corresponding reduction in the concentration of some other base or generation of an equivalent amount of acid. The progressive fall in pH of all these faecal incubations indicates that the accumulation of acid or the neutralization of base other than ammonia was occurring even more rapidly than the accumulation of ammonia. Analysis of organic anion concentrations in several experiments showed that the explanation lay in the generation of large amounts of organic acid. Thus in three experiments with the 33% suspension, the concentration of organic anion rose in 48 h by 76, 79 and 89 mmol/l, and ammonia concentrations simultaneously rose by 32, 34 and 38 mmol/l respectively. In two further studies with the 25% suspension organic anion concentration rose in 48 h by 92 and 122 mmol/l, and ammonia by 70 and 82 mmol/l respectively. The osmolality of faecal suspensions rose as predicted, but the only experiments in which simultaneous determinations of organic anion, ammonia and osmolality were made were the two last referred to, in which osmolality increased in 48 h by 144 and 190 mosmol/kg respectively. These increases in osmolality are less than the calculated contributions from ammonium and organic anions, a discrepancy which is probably due to the overestimation of organic anions by the Van Slyke & Palmer procedure (Collin & McCormack, 1974), and the fact that at the molarity of these solutions ammonium and acetate (the predominant organic anion in faeces) contribute only 95% of their calculated osmolar values (Hall & Sherrill, 1928). We have not been primarily concerned with organic acid production by these incubations, but it is clear from these results that the amounts generated are at least as great as those of ammonia.

Undiluted faeces. Fig. 4 shows the rapid rise in ammonia concentration found in high-speed centrifugate of incubated whole stool. Concentrations are again charted as the proportion of total faecal nitrogen in the form of ammonia, but in addition uncorrected molar concentrations are shown. The latter rose from 6.4 mmol/l initially to 236 mmol/l (252
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FIO. 4. Ammonia generation by undiluted faeces incubated anaerobically for 48 h.

mmol/kg corrected for solid content) at 48 h of incubation.

Colonic contents from more proximal bowel. Ammonia concentrations are shown in Fig. 5, expressed as proportions of total faecal nitrogen. Actual molar concentrations were 32 and 27 mmol/l at 48 h in the enema and ileostomy sample respectively. In both studies the yield of ammonia on incubation, expressed as a percentage of faecal nitrogen, was greater than in either the 25% or 33% suspensions, but in ileostomy fluid this difference could be accounted for by the ammonia derived from breakdown of urea, which was present initially in a concentration of 4.5 mmol/l and disappeared within the first few hours of incubation. The enema sample of stool

FIG. 5. Ammonia generation by incubated enema product (Δ) and ileostomy sample (■). When ammonia derived from urea hydrolysis was deducted from the figures for the ileostomy sample, the 48 h ammonia concentration was equivalent to 18% of total faecal nitrogen.

Fig. 6. Ammonia generation in two samples of faeces incubated after gamma-irradiation. The broken line shows the mean results of the 25% faecal suspension (from Fig. 3).

contained no urea, and generated significantly more ammonia from other sources than either the 25% (P < 0.001) or the 33% (P < 0.001) suspension systems. In this enema incubation, the pH fell from 7.21 at 0 h to 5.94 at 24 h to 5.76 at 48 h and the corresponding values for the ileostomy fluid were 8.15, 5.30 and 5.30.

Sterilized faeces. Stool sterilized by autoclaving did not increase its ammonia concentration on incubation, and is therefore not charted. Fig. 6 shows the ammonia generated by stool incubated after sterilization by gamma-irradiation. In both studies ammonia was generated rapidly within the first 24 h and then reached a plateau which showed no further increase from 24 to 48 h. The final 48 h value was not significantly different from that achieved in the living 25% faecal suspension system (broken line in Fig. 6).

Discussion

Faecal incubation system

An incubation system in vitro for studying the metabolic activity of colonic organisms has two pre-requisites. The representation of the major groups of organisms should be similar to that found in vivo, and it should not change substantially over the incubation period, since marked changes in the numbers of any group of organisms could affect the metabolism of others. With faeces as a starting material, the first requirement is automatically filled, and in the 25% system described here the second requirement was obtained.
The stability of the total counts in the 25% suspensions indicated that little bacterial growth occurred over 48 h. Bacteria tended to die off, especially during the second day, when either 33% faecal suspensions or undiluted faeces were used. These findings are similar to those of Crowther (1971), who found that viable counts of the dominant anaerobes in undiluted faeces were reduced significantly after 48 h at 37°C. It is not clear why organisms should survive better in a 25% suspension than in a 33% suspension, but dilution of bactericidal factors such as organic acids is one possible explanation.

Unlike the faecal studies, incubation of ileostomy effluent resulted in the multiplication of organisms, showing that the system is capable of supporting bacterial growth. This increase in bacterial numbers obviously occurs in vivo, but to a much greater extent since the counts of anaerobic organisms in the colon or faeces are considerably higher than those of either the intact terminal ileum or ileostomy effluent.

Our incubation system differs from the situation in the living colon in that metabolites accumulate instead of being absorbed or excreted. An ideal incubation system would have the modifications to remove the metabolites which normally pass through the colonic mucosa and to add nutrients which normally reach the lumen by secretion or passage through the ileo-caecal valve. The progressive fall in pH of our incubated samples is not a feature of colonic contents in situ (Meldrum, Watson, Riddle, Bown & Sladen, 1972) and can be attributed to (1) accumulation of organic acids, which in the colon are absorbed to some degree through the mucosa (Dawson, Holdsworth & Webb, 1964), and (2) absence of the bicarbonate secretion, which is a normal characteristic of the colonic mucosa (D'Agostino, Leadbetter & Schwartz, 1953). The rise in Eh was to be expected as it is known to increase as pH falls, by 58 mV for a reduction of 1 pH unit (Hentges & Maier, 1972).

Yet despite these differences from the situation in vivo, our incubation system has several advantages. It is inexpensive and simple to set up and use, and it contains all the substances normally found in faeces that might influence bacterial metabolism, which it would be impractical or even impossible to incorporate into a synthetic medium. The incubation procedure can be used to study the behaviour of colonic contents from different groups of subjects, healthy or otherwise, eating normal or specially formulated diets. Substances that might be unpleasant or too toxic to take orally may be added to the system, and various manipulations are possible (e.g. alterations of pH), which will allow a study of the factors that might influence production of metabolites by colonic bacteria.

Sources of intestinal ammonia

These incubations have confirmed and extended the observations of Gamble (1915) that faecal material obtained from various levels in the intestine is capable of generating large amounts of ammonia. In addition we have observed simultaneous generation of large amounts of organic acid. Of the two systems studied in depth, the 25% and 33% faecal incubations, the less viable 33% suspension produced significantly more ammonia. This relationship held whether ammonia production was expressed in absolute terms or as a percentage of the total faecal nitrogen converted into ammonia, and is probably due to the greater availability of enzymes released from lysed cells. The studies with faeces sterilized by gamma-irradiation show clearly that dead bacteria can produce ammonia as rapidly and in as large amounts as are produced by living stool suspensions. That this ammonia production is dependent on the preservation of bacterial enzymes was shown by the experiment with autoclaved stool in which no ammonia was generated, presumably because bacterial enzymes had been denatured by heat.

Urea was not detected in any sample of faecal material except ileostomy effluent. In the presence of ammonia the Conway technique used here is not reliable for measuring urea concentrations below 0·5 mmol/l, but Owens & Padovan (1975), using column chromatography, have shown that the normal concentration of urea in faecal diffusate is of the order of 0·1 mmol/l. We can therefore exclude urea as the source of the large amounts of ammonia generated in these incubations. The probable source is the amino nitrogen of faecal proteins, polypeptides and amino acids, for it is unlikely that other forms of faecal nitrogen are present in sufficient amounts. Intestinal bacteria can deaminate amino acids (Sabbaj, Sutter & Finegold, 1970) and have been shown to produce ammonia from urea-free substrates in vitro (Vince, Dawson, Park & O'Grady, 1973). Indeed, a far greater proportion of colonic organisms are able to deaminate peptides and amino acids than can hydrolyse urea. E. coli, the dominant aerobic organism in the colon, readily deaminates peptides and amino acids, but does not normally hydrolyse urea; similarly the majority of Gram-negative anaerobic intestinal organisms tested in this laboratory form ammonia from non-urea substrates, whereas less than 10% hydrolyse urea.
Faecal generation of ammonia

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The source of faecal ammonia has usually been assumed to be urea, because over 100 mmol of urea is broken down in the body daily (Walser & Bodenlos, 1959; Jones, Smallwood, Craigie & Rosener, 1969; Gibson, Park, Sladen & Dawson, 1976) and there is considerable evidence that this destruction is the result of bacterial hydrolysis in the intestine. Wolpert, Phillips & Summerskill (1971) and Bown, Gibson, Fenton, Snedden, Clark & Sladen (1975) have shown that the colonic mucosa is relatively impermeable to urea, but calculations from even the lower figures for permeability found by Bown et al. show that the amounts of urea diffusing through the mucosa would be sufficient to account for the small concentrations of ammonia in faecal diffusate. Support for urea as the origin of faecal ammonia has come from the observations that urea is normally absent from faeces, that it appears in concentrations close to plasma levels during administration of intestinal antibiotics (Wilson, Ing, Metcalfe-Gibson & Wrong, 1968a) and that the ammonia concentration of caecal contents and faeces is increased in the presence of renal failure (Bourke et al., 1966; Wilson, Ing, Metcalfe-Gibson & Wrong, 1968b).

Our present findings cast doubt on urea as the immediate source of faecal ammonia. Regardless of the incubation system used, ammonia generation from non-urea sources was quite sufficient to account for the small amounts of ammonia present in faecal diffusate or centrifugate. Indeed if we are right in assuming that our faecal incubation system is a reflection of normal events in the colon in situ, then the amount of ammonia in freshly passed faeces is so small in relation to the amount which would be generated by faeces in the colon that we can conclude that substantial amounts of ammonia of non-urea origin are normally absorbed through the colonic mucosa.

Our conclusion that faecal ammonia obtains an important contribution—perhaps the major contribution—from non-urea sources does not necessarily imply that urea might not be the original source of faecal ammonia. If ammonia from hydrolysed urea is available for bacterial metabolism (and this depends on the precise site of urea destruction) it would provide an excellent nitrogen source in a form which can be utilized by intestinal bacteria for their own protoplasmic needs. We have already obtained evidence (unpublished work), from the incubation system described here, that intestinal bacteria actively utilize ammonia if provided with sufficient energy.

We envisage that in the normal colonic lumen ammonia is constantly being generated from urea and non-urea sources (the latter largely bacterial protoplasm) and is simultaneously being removed by incorporation into bacterial protoplasm and by mucosal absorption.

Faecal diffusate in vivo, or faecal centrifugate, as samples of faecal fluid?

In the present work we showed that high-speed centrifugation of faecal samples and dialysis in vitro gave almost identical concentrations of ammonia when applied to diluted faecal samples, if allowance was made for the different water content of the two fluids. Yet Tarlow & Thom (1974) have shown, and we have confirmed, that high-speed centrifugation of freshly passed whole stool gives significantly higher values (mean 33 mmol/l, compared with 17 mmol/l) of ammonia and various organic solutes than diffusate in vivo of the same sample of stool. Tarlow & Thom attributed this difference to the failure of dialysis capsules to reach equilibrium with stool solute, and regarded high-speed centrifugation as yielding more valid data for the composition of faecal water. The results of the present study show, however, that the observed differences are largely the result of changes in faecal composition after faeces have left the body.

Fresh stool is a bacterial system containing from $10^{10}$ to $10^{14}$ living organisms/g, which together with large numbers of dead bacteria make up more than 30% of its total wet volume. Any analytical procedure requiring manipulation of faeces outside the body encounters the problem of bacterial multiplication, metabolism and death, as these processes must inevitably alter the chemical composition of the fluid phase of faeces. Tarlow & Thom recognized this problem, and sought to get around it by cooling faeces rapidly. In some studies they initially froze faeces, a procedure which we do not regard as acceptable as the formation of ice crystals is known to disrupt bacterial cells. In other studies fresh stool was homogenized and then spun at 40,000 g in a centrifuge set at 0°C for 30 min without preliminary freezing. In our hands this procedure takes over an hour to complete, at the end of which time the specimen temperature is 7°C, which is high enough to allow bacterial metabolism to continue. Inspection of Fig. 4 shows that only 1 h of continued faecal metabolism would be sufficient to account for the ammonia difference between faecal diffusate and centrifugate.
Over 90% of the living bacteria in faeces are obligatory anaerobes, which are killed by exposure to air, so the aerobic preparations used by Tarlow & Thom can be further criticized as likely to cause bacterial death, which must also have influenced their results. In the present study we found that although stool always generated ammonia on incubation, faster generation took place with faster rates of bacterial death. The effect of an aerobic environment on the composition of faecal fluid has been particularly thoroughly studied by Owens & Padovan (1975, 1976) in relation to amino acids. They have established the normal amino acid profile of faecal diffusate in vivo and have shown that faeces incubated aerobically liberate large amounts of the same amino acids which are liberated when faecal bacteria are disrupted by ultrasonication. Bacteriological studies would be necessary to determine the extent of bacterial death during aerobic centrifugation, but until contrary evidence is produced we are satisfied that dialysis is the better method for obtaining samples of extracellular stool water for determination of ammonia and small organic solutes such as urea and amino acids. Either centrifugation or dialysis in vivo can be used for non-volatile inorganic solutes, as they give very similar results. Bicarbonate concentrations in centrifugate are only 25% of those in diffusate (Tarlow & Thom, 1974), a difference explicable as the result of loss of volatile carbon dioxide during prolonged manipulation of faeces.

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