

## Taurocholic acid adsorption during non-starch polysaccharide fermentation: an *in vitro* study

BY INGRID C. GELISSEN\* AND MARTIN A. EASTWOOD

*Gastro-Intestinal Laboratory, Western General Hospital, Edinburgh EH4 2XU*

(Received 18 April 1994 – Revised 3 November 1994 – Accepted 15 December 1994)

The association of radiolabelled taurocholic acid with the solid fraction of a faecal fermentation mixture was measured. A human faecal inoculum was incubated with [24-<sup>14</sup>C]taurocholic acid and several non-starch polysaccharide sources (pectin, wheat bran, ispaghula (*Plantago ovata*) husk and seed), glucose or a substrate-free control. Portions of fermentation mixture were taken at 0, 3, 6, 21 and 24 h and centrifuged to acquire a supernatant fraction and a pellet containing the fermentation residue. <sup>14</sup>C was measured in supernatant fractions and pellets at all time points. Volatile fatty acids (VFA) were measured at 0 and 24 h to confirm bacterial growth. Radioactivity in the pellet increased over time for all substrates. Glucose resulted in the greatest incorporation of taurocholic acid into the pellet, followed by pectin. At 24 h the proportion of the total radioactivity found in the pellet was 92% for glucose, 79% for pectin, 60% for wheat bran, 59% for ispaghula seed, 53% for ispaghula husk and 26% for the control (mean of duplicates). Glucose and pectin produced the greatest quantity of VFA at 24 h. VFA production was highly correlated with radioactivity in the pellet ( $r=0.976$ ,  $P < 0.005$ ). These results suggest that the bile acid binding capacity of a faecal culture mixture may be strongly influenced by the fermentability of the available substrate and hence related to bacterial metabolic activity.

**Non-starch polysaccharides: Bile acids: Faecal bacteria: Fermentation**

The relationship between dietary non-starch polysaccharides (NSP) and plasma lipids has received much interest over the last few decades. Three major hypotheses regarding the cholesterol-lowering effects of some NSP are currently under investigation: interference with fat absorption, production of volatile fatty acids (VFA), and increased bile acid and neutral sterol excretion (Topping, 1991). The third hypothesis is based on the observation that some dietary NSP bind bile acids *in vitro* and may thus prevent reabsorption *in vivo*. Bile acids are secreted into the lumen as taurine and glycine conjugates. Approximately 75% of these bile acids are reabsorbed unchanged from the terminal ileum via an active reabsorption mechanism and returned to the liver. Some 25% escape this process and are deconjugated by gut bacteria. The majority of these bile acids are reabsorbed and returned to the liver for reconjugation (Sanford, 1982). There is potential for bile acids to be bound during this process, preventing them from being reabsorbed and hence possibly reducing cholesterol levels.

Bile acids have been shown to bind to several NSP sources *in vitro*. Eastwood & Hamilton (1968) found strong adsorption of bile acids by lignin while Story & Kritchevsky (1976) demonstrated binding to bran, lucerne (*Medicago sativa*), cellulose and wood lignin. Bile acids have also been shown to bind to faecal bacteria (Midtvedt & Norman, 1972). Bacteria in the gut chemically modify bile acids in various ways. The two most important

\* Present address: Cell Biology Group, Heart Research Institute, 145–147 Missenden Road, Camperdown NSW 2050, Australia.

transformations are removal of the glycine and taurine groups, and 7- $\alpha$  dehydroxylation to convert primary to secondary bile acids. Many other minor chemical transformations have been identified (Midtvedt, 1974; Hirano *et al.* 1981).

*In vitro* experiments studying bile acid binding capacity of dietary NSP and gut bacteria have so far only looked at the two in isolation. However, *in vivo* these two components are present together in the one system. The study presented here reports on an *in vitro* fermentation method, developed to study whether  $^{14}\text{C}$ -labelled taurocholic acid associates with a human faecal inoculum when incubated with NSP sources as substrates. Volatile fatty acids (VFA), which are end-products of bacterial fermentation in the gut (Cummings, 1981), were measured to confirm bacterial growth.

## MATERIALS AND METHODS

### *Fermentation*

The NSP test substances used in the main experiment were pectin (MW 0.25–0.5  $\times 10^6$ ; HP Bulmer Ltd, Hereford, Hereford & Worcester), coarse wheat bran (Chancelot Mills Ltd, Edinburgh) and ispaghula (*Plantago ovata*) husk and seed (Madaus AG, Cologne, Germany). Glucose as substrate (BDH Merck Ltd, Poole, Dorset) as well as a substrate-free control were also tested. In the main experiment, all test substances and controls were analysed in duplicate.

One healthy female subject provided all faecal samples for the experiments. Faeces were collected in plastic sling bags (Trans Atlantic Plastics, Surrey) and a 120 g/l faecal inoculum was prepared in medium (see below) within 30 min of defecation. The inoculum was then stirred for 1 h at 37° to break up the faecal matrix, and filtered through a 104  $\mu\text{m}$  filter (Spectra/Mesh, Pierce & Warriner, Chester, Ches.) to remove any coarse material.

The medium used throughout the study consisted of tryptone (pancreatic digest of casein; Unipath Ltd, Basingstoke, Hants; 2.5 g/l) and minerals (anhydrous  $\text{Na}_2\text{HPO}_4$  1.43 g/l, anhydrous  $\text{KH}_2\text{PO}_4$  1.55 g/l,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.15 g/l,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  1.65 mg/l,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  1.25 mg/l,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.125 mg/l,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  1.0 mg/l) in a buffered solution ( $\text{NH}_4\text{HCO}_3$  1 g/l,  $\text{NaHCO}_3$  8.75 g/l; Goering & Van Soest, 1979). To each litre of medium, 25 ml reducing solution was added (L-cysteine hydrochloride 6.25 g/l,  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  6.25 g/l,  $\text{NaOH}$  1.6 g/l). The medium was autoclaved (121°, 30 lb/in<sup>2</sup>, 10 min) and anaerobic conditions were created by gassing with  $\text{CO}_2$ . Resazurin (Sigma Chemical Co., Poole, Dorset) was used as a colour indicator, changing the medium from pink to clear when anaerobic conditions were attained.

Medium (40 ml) was dispensed into 150 ml medical flat bottles (BDH Merck, Lutterworth, Leics) which were then autoclaved and gassed with  $\text{CO}_2$ . To each bottle, 0.25 g of the test fibre was added. Inoculum (10 ml) was introduced to each bottle, followed by 80  $\mu\text{l}$  of  $^{14}\text{C}$ -labelled taurocholic acid (7.3 kBq) in ethanol-methanol (1:3) (Du Pont (UK) Ltd, Stevenage, Herts). The bottles were sealed and laid flat in an incubator (37°). At times 0, 3, 6, 21 and 24 h, 5 ml portions of culture fluid were transferred to pre-cooled 10 ml centrifuge tubes. The bottles were swirled beforehand to ensure a good mix. The samples were centrifuged immediately (2000 g, 0°, 30 min) (Mistral 3000I; MSE Ltd, Loughborough, Leics) to precipitate the bacteria and insoluble NSP residues. During method development, the absence of bacteria in the supernatant fraction after spinning was confirmed using a Gram stain. After centrifugation, the sample had separated into a clear brown-coloured supernatant fraction and a firm brown pellet. A 4 ml portion of the supernatant fraction was removed and the pellet was washed twice with 4 ml pre-cooled medium to remove any loosely bound bile acids. The three supernatant fractions were combined and 1 ml was added to 10 ml scintillation fluid (Pico-aqua; Canberra Packard,

Pangbourne, Berks) and counted on a liquid scintillation counter (TriCarb® model 4430; Packard Instrument Company Inc., Downers Grove, USA).

In a separate experiment the three supernatant fractions were counted individually to confirm that two washes were sufficient to remove the majority of loosely bound bile acids from the pellet (results not shown). After washing, the pellet was resuspended and transferred into a scintillation vial using 1 ml water. Tissue solubilizer (2 ml; Optisolve; Pharmacia Biotech Ltd, Milton Keynes, Bucks) was added and the vials kept at 50° overnight. The mixture was diluted with scintillation fluid (Hionic Fluor; Canberra Packard) and counted. A standard of 80 µl radiolabelled taurocholic acid was counted separately in scintillation fluid. Both 21 h samples with glucose as substrate were lost during centrifugation.

A pilot trial was carried out to assess the coefficient of variation (CV) between bottles of radioactivity in the supernatant fraction and pellet. Six control bottles and four bottles with pectin as NSP source were carried through the experiment as described above and samples were taken at 2 and 24 h. CV values are presented in Table 1. The pH of the fermentation mixture in control and pectin samples was also measured as bile acid solubility is known to be pH-dependent (Hofmann & Mysels, 1992). A portable stick pH meter (Picollo 2; BDH Merck Ltd, Poole, Dorset) was used. At 0, 2 and 24 h, the pH values were 7.0, 7.0 and 6.9 respectively for the control and 7.0, 6.9 and 6.7 respectively for pectin. In further pilot experiments, <sup>14</sup>C incorporation into the pellet (and disappearance from the supernatant fraction) was measured at 48 h, using control and pectin samples, to assess if later time points were necessary. No change in radioactivity was found in either pellet or supernatant fraction between 24 and 48 h.

#### *Volatile fatty acid analysis*

VFA were measured at times 0 and 24 h to establish bacterial metabolic activity. Samples of 2 ml culture fluid were added to 0.5 ml 1 M-NaOH and stored at -20° until further analysis. VFA were prepared according to the method of Spiller *et al.* (1980). Samples of 800 µl were extracted three times with diethyl ether (Rathburn Chemicals Ltd, Walkerburn, Borders) and analysed by GC using a packed column (Chromosorb W-AW 80-100 mesh; Phase Separations Ltd, Deeside). 4-Methyl *n*-valeric acid (Sigma Chemical Co.) was used as an internal standard.

#### *Statistics*

The recovery of radioactivity in the samples was calculated from the sum of the radioactivity in supernatant fraction and pellet, relative to the standard. Differences in radioactivity between fibres were assessed by one-way ANOVA followed by the Newman-Keuls procedure (Snedecor & Cochran, 1980). Pearson's correlation coefficients between variables were calculated by linear regression analysis. A *P* value of < 0.05 was considered significant. A statistical computer software package was used to perform comparisons (Minitab, release 7.1, State College, PA, Pennsylvania State University, 1989).

## RESULTS

The recovery of radioactivity in the samples was on average 100.2 (SD 4.3) %. In Fig. 1 the radioactivity in the pellet over time is represented for the control, glucose and four NSP types as substrates. The radioactivity increased in all pellets over time; the smallest increase was seen for the control and the largest was seen for glucose. This trend became apparent after 6 h fermentation. At this time point the control and glucose were significantly different from all four NSP. Between 21 and 24 h the radioactivity in the pellet did not increase any

Table 1. Coefficients of variation (CV) between replicates for radioactivity measured in the supernatant fraction and pellet of faeces – substrate mixtures incubated for 2 h and 24 h\*  
(Values are for control (no substrate) and pectin incubations)

		CV (%) for radioactivity in supernatant fraction	CV (%) for radioactivity in pellet
Control (n 6)	2 h	7.5	12.2
	24 h	4.6	9.1
Pectin (n 4)	2 h	4.1	11.5
	24 h	7.7	5.1

\* For details of procedures, see pp. 222–223.

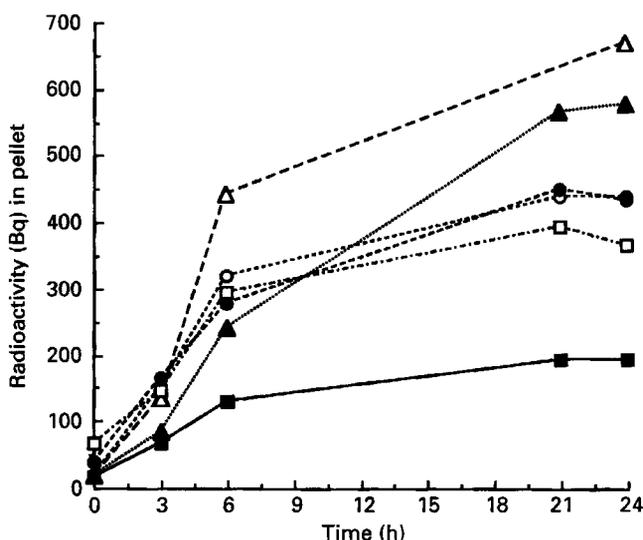


Fig. 1. Increase over time in the radioactivity (from  $[24\text{-}^{14}\text{C}]$ taurocholic acid) of the solid fraction after incubation of human faecal bacteria with glucose (—Δ—), pectin (···▲···), wheat bran (—○—), ispaghula (*Plantago ovata*) seed (—●—), ispaghula husk (—□—), or no substrate (control; —■—). Values are means of duplicate determinations. At 24 h, all six treatments were significantly different from each other ( $P < 0.05$ ) except wheat bran and ispaghula seed. For details of procedures, see pp. 222–223.

further. At 24 h all six treatments were significantly different from each other, except wheat bran and ispaghula seed. Glucose as substrate resulted in the highest radioactivity in the pellet followed by pectin, wheat bran and ispaghula seed, ispaghula husk and the NSP-free control. At 24 h the proportion of the total radioactivity found in the pellet was 92% for glucose, 79% for pectin, 60% for wheat bran, 59% for ispaghula seed, 53% for ispaghula husk and 26% for the NSP-free control. The variation between the duplicate values at this time point was on average 3.3 (SD 1.4)%.

At 0 h, no VFA were detected in any of the samples while at 24 h substantial amounts were found (Table 2). The major fatty acid produced was acetate, followed by propionate and butyrate. Isobutyrate, valerate and isovalerate were detected in smaller quantities.

Table 2. Production of volatile fatty acids (VFA; mmol/l) by human faecal bacteria incubated for 24 h with no substrate (control), glucose, and four non-starch polysaccharide sources\*

(Values are means of duplicate determinations)

	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total VFA
Control	18.8	3.4	1.9	4.3	3.0	2.6	34.0
Glucose	57.9	10.0	1.7	10.4	2.1	3.1	85.2
Pectin	65.1	6.7	1.9	6.8	2.3	2.8	85.6
Wheat bran	36.5	6.1	2.3	8.3	3.3	3.1	59.7
Ispaghula ( <i>Plantago ovata</i> ) husk	33.3	9.1	2.0	4.9	2.6	3.0	54.8
Ispaghula seed	35.3	7.4	2.3	6.8	3.0	3.1	57.9

\* For details of procedures, see pp. 222–223.

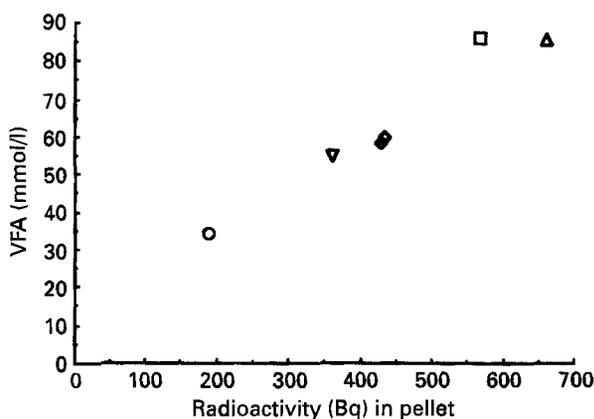


Fig. 2. Relation between radioactivity (from  $[24-^{14}\text{C}]$ taurocholic acid) in the solid fraction, and total volatile fatty acid (VFA) production by human faecal bacteria incubated for 24 h with glucose ( $\Delta$ ), pectin ( $\square$ ), wheat bran ( $\diamond$ ), ispaghula (*Plantago ovata*) seed ( $\blacklozenge$ ), ispaghula husk ( $\nabla$ ) or no substrate (control;  $\circ$ ). Values are means of duplicate determinations.  $r=0.976$ ,  $P < 0.005$ . For details of procedures, see pp. 222–223.

Total VFA production was greatest for glucose and pectin, which also showed the highest incorporation of radioactivity into the pellet at 24 h. Wheat bran and the two ispaghula fibres produced similar amounts of VFA whilst the smallest quantity was detected in the control. The variation between duplicate VFA values was on average 4.5 (SD 3.1) %.

In Fig. 2 the relation between the radioactivity in the pellet and total VFA concentration at 24 h is represented. The two variables were highly correlated with each other ( $r=0.976$ ,  $P < 0.005$ ).

#### DISCUSSION

There is evidence that supplementing the diet with certain NSP results in a decrease in serum cholesterol. However, not all NSP act in this way. For example, wheat bran has very little effect on serum cholesterol levels, whereas pectin is associated with a 5–10% decrease in serum cholesterol (reviewed by Truswell & Beynen, 1992).

One of the proposed mechanisms of action is an increased faecal bile acid excretion by

binding of bile acids to the NSP in the gut. In the present study the association of radiolabelled taurocholic acid with the solid fraction of a faecal fermentation mixture was measured using different fermentation substrates. It was shown that, of the NSP sources tested, pectin resulted in the greatest percentage of  $^{14}\text{C}$  in the pellet. Pectin, apart from lowering serum cholesterol levels, also raises faecal bile acid excretion in human subjects (Truswell & Beynen, 1992). Our *in vitro* results support the idea that pectin has the potential to interfere with bile acid reabsorption.

Compared with pectin, wheat bran and the two ispaghula fibres showed a lower association of  $^{14}\text{C}$  with the pellet fraction. Wheat bran, which has no effect on cholesterol levels, has been found to bind taurocholic acid to a lesser extent than lignin and cholestyramine (Story & Kritchevsky, 1976). Ispaghula husk, however, has been found to lower cholesterol levels in hypercholesterolaemic patients (Burton & Manninen, 1982; Anderson *et al.* 1988; Miettinen & Tarpila, 1989), although in subjects with normal starting cholesterol levels the results are not clear cut; we recently showed no change in cholesterol level or faecal bile acid excretion in a small group of healthy young men after supplementation with 10 g husk/d for 3 weeks (Gelissen *et al.* 1994). We suggested that the dose of ispaghula husk and starting cholesterol levels were important in determining the effectiveness of the treatment. In the same experiment an increase in ileostomy bile acid output was measured in a parallel group of ileostomy patients. Ispaghula husk may prevent reabsorption of bile acids in the ileum which are subsequently reabsorbed in the colon. Ispaghula husk forms a gel in water, trapping a significant amount of liquid and possibly aqueous components such as bile acids. In the present study the gel-like nature of ispaghula husk was also noted, resulting in a bigger sized pellet compared with the other substrates.

Ispaghula seed, a fibre supplement which is less well known than the husk, has been shown to lower cholesterol without increasing faecal bile acid excretion (Gelissen *et al.* 1994). The seed is therefore not likely to act by interference with bile acid metabolism. The results of the present study support this finding as the amount of radioactivity in the pellet was similar for wheat bran and ispaghula seed.

Glucose as substrate resulted in the largest percentage of  $^{14}\text{C}$  recovered in the pellet. Glucose is unlikely to bind bile acids by itself or create a matrix effect for trapping bile acids. However, glucose is an excellent source of energy for bacteria and may subsequently lead to bacterial growth. As bacteria themselves have the potential to bind bile acids (Midtvedt & Norman, 1972), it is possible that substrate fermentability, indicated by VFA production, and subsequent bacterial growth may be important factors in determining whether a NSP source can lead to trapping of bile acids. The high correlation found between VFA production and radioactivity recovered in the pellet support this idea. The bacterial fraction of faeces can be as high as 50% (Stephen & Cummings, 1980) and is therefore a quantitatively important component. This has so far received little attention with regard to cholesterol and bile acid metabolism. A report by Eastwood *et al.* (1986) provides further support for the above theory. These workers concluded that NSP which have a cholesterol-lowering effect also increase the amount of  $\text{H}_2$  excreted in the breath.  $\text{H}_2$  is also an important end-product of fermentation in the gut and breath  $\text{H}_2$  levels have been shown to reflect  $\text{H}_2$  production in the colon (Levitt, 1969).

VFA themselves have also been suggested to affect serum cholesterol levels. Chen *et al.* (1984) concluded from a study in rats that propionate may mediate some of the cholesterol-lowering effects of NSP, through inhibition of cholesterol synthesis in the liver (Wright *et al.* 1990). However, Illman *et al.* (1993) suggested this to be an unlikely mechanism as the *in vivo* concentrations required for these effects are much higher than physiological levels. In their study they showed that propionate concentrations in rat caecal contents were negatively related to cholesterol levels in the rat but also positively related to coprostanol

and secondary bile acid concentrations. In agreement with our present report, they suggested from their results that propionate and butyrate are indicators rather than regulators of sterol metabolism in the caecum.

This is the first report to link bile acid binding capacity of a faecal culture mixture with the fermentability of the available substrate. However, these results need further exploration. First, the faeces of only one subject were used throughout all experiments to minimize donor variation. Further work is needed to repeat the experiments with faeces from a range of donors. In addition, determining whether the bile acid is bound to the cell wall or internalized will help to elucidate the role of bacteria–bile acid interactions in sterol metabolism.

We would like to thank Callum Buchanan for helpful discussion during the design of the project.

#### REFERENCES

- Anderson, J. W., Zettwoch, N., Feldman, T., Tietzen-Clark, J., Oeltgen, P. & Bishop, C. W. (1988). Cholesterol-lowering effects of psyllium hydrophilic mucilloid for hypercholesterolemic men. *Archives of Internal Medicine* **148**, 292–296.
- Burton, R. & Manninen, V. (1982). Influence of a psyllium-based fibre preparation on faecal and serum parameters. *Acta Medica Scandinavica* **668**, Suppl., 91–94.
- Chen, W.-J. L., Anderson, J. W. & Jennings, D. (1984). Propionate may mediate the hypocholesterolemic effects of certain soluble plant fibers in cholesterol-fed rats. *Proceedings of the Society for Experimental Biology and Medicine* **175**, 215–218.
- Cummings, J. H. (1981). Short chain fatty acids in the human colon. *Gut* **22**, 763–779.
- Eastwood, M. A., Brydon, W. G. & Anderson, D. M. W. (1986). The effect of the polysaccharide composition and structure of dietary fibers on cecal fermentation and fecal excretion. *American Journal of Clinical Nutrition* **44**, 51–55.
- Eastwood, M. A. & Hamilton, D. (1968). Studies on the adsorption of bile salts to non-absorbed components of diet. *Biochimica et Biophysica Acta* **152**, 165–173.
- Gelissen, I. C., Brodie, B. & Eastwood, M. A. (1994). Effect of *Plantago ovata* (psyllium) husk and seed on sterol metabolism: studies in normal and ileostomy subjects. *American Journal of Clinical Nutrition* **59**, 395–400.
- Goering, H. K. & Van Soest, P. J. (1979). *In vitro* rumen digestibility determination. In *Agricultural Handbook* no. 379, pp. 12–15. Washington DC: Government Printing Office.
- Hirano, S., Masuda, N., Oda, H. & Imamura, T. (1981). Transformation of bile acids by mixed microbial cultures from human feces and bile acid transforming activities of isolated bacterial strains. *Microbiology and Immunology* **25**, 271–282.
- Hofmann, A. F. & Mysels, K. J. (1992). Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca<sup>2+</sup> ions. *Journal of Lipid Research* **33**, 617–626.
- Illman, R. J., Storer, G. B. & Topping, D. L. (1993). White wheat flour lowers plasma cholesterol and increases cecal steroids relative to whole wheat flour, wheat bran and wheat pollard in rats. *Journal of Nutrition* **123**, 1094–1100.
- Levitt, M. D. (1969). Production and excretion of hydrogen gas in man. *New England Journal of Medicine* **281**, 122–127.
- Midtvedt, T. (1974). Microbial bile acid transformation. *American Journal of Clinical Nutrition* **27**, 1341–1347.
- Midtvedt, T. & Norman, A. (1972). Adsorption of bile acids to intestinal microorganisms. *Acta Pathologica et Microbiologica Scandinavica B* **80**, 202–210.
- Miettinen, T. A. & Tarpila, S. (1989). Serum lipids and cholesterol metabolism during guar gum, plantago ovata and high fibre treatments. *Clinica Chimica Acta* **183**, 253–262.
- Sanford, P. A. (1982). Liver and secretions of pancreas and duodenum. In *Digestive System Physiology*, pp. 65–68. London: Edward Arnold Ltd.
- Snedecor, G. W. & Cochran, W. G. (1980). *Statistical Methods*, 7th ed., p. 235. Iowa: The Iowa State University Press.
- Spiller, G. A., Chernoff, M. C., Hill, R. A., Gates, J. E., Nassar, J. J. & Shipley, E. A. (1980). Effect of purified cellulose, pectin, and a low-residue diet on fecal volatile fatty acids, transit time and fecal weight in humans. *American Journal of Clinical Nutrition* **33**, 754–759.
- Stephen, A. M. & Cummings, J. H. (1980). The microbial contribution to human faecal mass. *Journal of Medical Microbiology* **13**, 45–56.
- Story, J. A. & Kritchevsky, D. (1976). Comparison of the binding of various bile acids and bile salts *in vitro* by several types of dietary fiber. *Journal of Nutrition* **106**, 1292–1294.

- Topping, D. L. (1991). Soluble fiber polysaccharides: effects on plasma cholesterol and colonic fermentation. *Nutrition Reviews* **49**, 195–203.
- Truswell, A. S. & Beynen, A. C. (1992). Dietary fibre and plasma lipids: potential for prevention and treatment of hyperlipidaemias. In *Dietary Fibre – A Component of Food*, pp. 295–332 [T. F. Schweizer & C. A. Edwards, editors]. London: Springer-Verlag.
- Wright, R. S., Anderson, J. W. & Bridges, S. R. (1990). Propionate inhibits hepatocyte lipid synthesis. *Proceedings of the Society for Experimental Biology and Medicine* **195**, 26–29.