

Site, Rate and Extent of Starch Digestion in Weaning Infants

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“Growing bodies have the most innate heat; therefore they require the most food, for otherwise, their bodies are wasted.”

Aphorisms, Hippocrates

Declaration

I declare that I have composed this thesis. The original research upon which the thesis is based is my own. Where I have received help and support, I have indicated it in the acknowledgements section. The work has not been submitted for any other thesis.

Martin Tremayne Christian

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Summary

Background

The colon is believed to salvage energy from unabsorbed starch especially when the capacity of the small intestine to digest it is limited. The extent to which this occurs is not known.

Aims

The aim of this thesis was to determine site and relative extent of starch digestion and fermentation in young children using the individual and combined approaches of stable isotope breath tests and *in vitro* stool fermentation models.

Stable Isotope Breath Test Methods

Thirteen children (10m, 3f), median (range) age 11.8 mo (7.6-22.7 mo), took a starchy breakfast containing ^{13}C labelled wheat flour following an overnight fast. Duplicate breath samples were obtained before breakfast and every 30 min for 12 h. Breath $^{13}\text{CO}_2$ enrichment was measured by isotope ratio mass spectrometry and results were expressed as percentage dose recovered (PDR) for each 30 min. PDR data were analysed and mathematically curve fitted either assuming a constant estimate of CO_2 production rate or adjusted for physical activity.

Stable Isotope Breath Test Results

Mean \pm SD cumulative ^{13}C PDR (cPDR) at 12 h was 21.3% \pm 8.4% for unadjusted data and 26.5% \pm 11.6% for adjusted data. A composite fit of two curves fitted significantly better than a single curve. Curve fitting allowed estimation of cPDRs of

small intestine ($17.5\% \pm 6.5\%$ and $22.7\% \pm 9.3\%$ for unadjusted and adjusted data respectively) and colon ($4.6\% \pm 2.9\%$ and $6.3\% \pm 5.4\%$). From these results it is speculated that the colon may account for up to 20% of starch digestion in young children.

In Vitro Colonic Fermentation Methods

A simulated colonic environment was used to account for the fate of raw and cooked starch that was fermented in the colon of young children. A slurry was prepared from faecal samples of 6 infants (7 - 10 mo), 6 toddlers (16 - 21 mo) and 7 adults (24 - 56 years). Each slurry was anaerobically incubated with raw or cooked maize starch in MacCartney bottles in a shaking water bath. Parallel incubations were stopped at 4 and 24 h. The headspace gas volume was analysed for CO₂ and methane. The culture supernatant was analyzed for the volatile short chain fatty acids acetate, propionate and butyrate (SCFA), lactate and residual starch.

In Vitro Colonic Fermentation Results

There was a decreasing trend of SCFA production with age at 4 h which was not evident at 24 h. At 4 h, toddler stools produced the most CO₂ followed by infants and then adults, but this trend was not seen at 24 h. Methane was detected in 3 adults only. Lactate was detected mainly at 4 h in children only. The production of SCFA at 4 h generally declined with age but the differences at 24 h were less marked, suggesting fermentation is a more rapid process in young children than in adults. A highly efficient energy salvage process may take place in the colon of young children.

Calculations Using Both Data Sets and Conclusions

Using data from studies described in both parts of the dissertation, it has been possible to derive stoichiometric equations for the whole gut digestion of starch, and thereby calculate its potential energy. There are a number of limitations to the methodology and from assumptions that have been made, but this provides an attractive means to calculate relative roles of small intestine and colon to starch digestion in young children which in turn may form the scientific basis for nutritional advice given to mothers.

Acknowledgements

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I designed the study and collected all of the breath samples for the preliminary and definitive ^{13}C breath test studies apart from three definitive experiments where the data were collected by Ms Cristina Bettati. I am grateful to Dr Sergio Amarri of the University of Modena for his help with subject recruitment and for his advice and support with study design and data interpretation. The breath samples were analysed at the Scottish Universities' Environmental Research Centre and Clinica Pediatrica at the University of Modena. I am grateful to Dr Tom Preston and Ms Christine Slater from the former laboratory and Mr Fabio Franchini from the latter for their help. I carried out the curve fitting with supervision and advice from the supervisors and Dr Douglas Morrison and Dr Brian Dodson of Bell College, Hamilton.

I designed the fermentation studies based on previous experiments by Dr Christine Edwards and Ms Alison Parrett at the Department of Human Nutrition, University of Glasgow. Around half of the fermentation experiments and analytical determination of products were carried out by Ms Rebecca Varley and Ms Leanne Johnston. I am

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For the work described in both parts of the dissertation, I collated the data, presented and analysed it statistically.

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CHAPTER 1: BACKGROUND

1.1 Weaning

1.1.1 Definitions

In infancy the diet undergoes a change, from a single food in which fat and lactose are the major energy sources, to an increasing variety of foods with starch as the principal source of energy. The word *wean* derives from an Anglo-Saxon word *wenian* meaning *to accustom*. It has traditionally been understood to mean the time of introduction of non-breast milk food. Today in the Western World many babies receive formula milk rather than breast milk from birth and for the purpose of this thesis it will be defined as the period from first introduction of non-milk diet to the cessation of breast (or formula) feeding. Many weaning foods are cereal-based and starch represents a novel nutrient to the digestive system of the hitherto exclusively milk-fed infant. Much of the thrust of recommendations on infant feeding has focused on the benefits of breast feeding in establishing optimal nutrition in early life (1). The importance of weaning has received less emphasis (2,3). Two broad practical questions encapsulate the health professionals interest in the introduction of starch into the young child's diet: when first to introduce starch into the diet and which starches should be introduced?

The aim of this introduction is to summarise what is known about the weaning process, the digestibility of starch, and the ontogeny of starch digestion in early life. It will also highlight the areas where a greater understanding of starch digestion in

early life will provide the foundations upon which the practical guidelines for weaning can be based.

1.1.2 Weaning in other species

Animal models have provided a useful focus for the study of intestinal ontogeny at weaning. Changes in intestinal structure that have been observed include rapid intestinal growth and enterocyte proliferation as well as villi and crypt lengthening (4). Functional changes include the appearance or increase of sucrase-isomaltase activity (5). In higher mammals, adult intestinal functions appear perinatally, whilst for altricial species, the age of weaning is delayed because adult intestinal functions are not acquired for some time (6). The changes seen at weaning do reflect the adult feeding patterns and digestive strategies of the species concerned (7). This intestinal maturation can be delayed if suckling is prolonged (4). To draw significant extrapolations for the development of intestinal function at weaning in humans requires an animal model with a similar rate of maturation; the miniature pig has been suggested as such a model (8), however such comparisons are limited by the differences in adult diets and intestinal characteristics (7).

1.1.3 Timing of weaning

Various authors have said that the exact time for weaning in humans cannot be pinpointed and the optimal age depends on social, sociological, developmental and psychological considerations (9,10). However, developmental factors would seem to suggest the existence of an optimal time period: the changing nutritional requirements matched to maturation of gastrointestinal function with room for some

functional adaptation (11). This optimum period theory finds some support in animal work showing a mass of changes in gastrointestinal structure and function around the time of cessation of suckling (12).

Current recommendations, documented in the 1982 ESPGAN guidelines on infant feeding (13) and endorsed in the 1994 COMA report (3), is that this period occurs between 4 to 6 months of age. Historically infants have at various times been weaned earlier or later than this (14). Studies and clinical observations reporting adverse effects of such practices form the basis of ESPGAN and COMA's advice, but still there remain controversies about timing.

1.1.3.1 late weaning

The limiting factor for the length of time infants can continue to receive breast or formula milk is the necessity to meet nutrient and particularly energy requirements. A conundrum previously existed in which estimated energy requirements meant that breast milk only met energy needs until 2 months of age based on an average mother's daily breast milk output of 850 ml (15). However, a meta-analysis of 16 studies of the measured energy intakes of healthy infants showed that the energy requirements were less than previously thought (16) and the use of doubly labelled water ($^2\text{H}_2^{18}\text{O}$) to measure energy requirements by CO_2 production rate demonstrated a further decrease (17). As a result of this research, the age at which it is believed that the average infant's energy requirements cease to be met by an exclusive milk diet was revised to the current recommendations of 4 to 6 months (15). These guidelines are only for the developed world; in the developing world the

energy needs of an average infant growing along the 25th centile would be met by an exclusive breast milk diet up to 6 months (18). The particular needs of the weanling in the developing world are discussed below.

The 4 to 6 month guidelines are based on the energy needs of average breast fed babies. There is controversy over the existence of differential energy requirements for breast and formula fed infants. Butte et al (19) found significantly higher energy intakes for formula fed infants at 4 months, whilst Whitehead et al (20) found similar growth patterns of breast and formula fed infants before weaning.

Formula feeds contain iron but this is less bioavailable than the iron in human milk and therefore the depletion of iron stores is an important consideration in the commencement of iron-containing solid foods for formula-fed infants (21).

Studies of successful prolonged exclusive breast-feeding (22,23) have been cited as evidence for the sufficiency of breast milk alone for longer than 6 months (24).

However, other studies suggest that prolonged breast-feeding may be associated with malnutrition (25). Studies favouring prolonged breast-feeding have been criticised for self-selection and unhelpful in the context of generating guidelines for weaning in the developing world where weight faltering around weaning is a more common phenomenon (26). Although the micro-nutritional status and growth of these children does not remain wholly clear, it is generally accepted that with a balanced food such as breast milk, if energy requirements are observed to be met then it is safe to assume that other nutrient requirements will be similarly met (27).

1.1.3.2 early weaning

Before 4 months: head control and oral neuromuscular co-ordination are generally not fully developed to enable the infant to form a bolus of solids, transfer it to the oro-pharynx and swallow. Compared with the adult, the newborn kidney has immature tubular function and a reduced glomerular filtration rate rendering it unable to cope with high solute loads particularly in times of illness (28) although this is less important with lower protein and lower salt manufactured weaning foods (29).

Reduced concentrations of pancreatic and gastrointestinal enzymes mean that some carbohydrate, fat and protein within non-milk foods may not be well digested (3).

Other problems described in relation to early weaning include obesity, increased episodes of gastroenteritis, increased episodes of respiratory symptoms and the introduction of potentially allergenic foods. However, many of these problems remain controversial (30-32).

1.2 Starches

1.2.1 Starch classification and structure

Complex carbohydrates are polymers of single sugar molecules (monosaccharides). Those with ten or less sugar residues are called oligosaccharides and those with ten or more are called polysaccharides. Polysaccharides can be very large polymers, which are natural or synthetic. Examples of natural polysaccharides include dietary fibre (non-starch polysaccharide) and starch. Synthetic glucose polymers, made by modifying natural products, can be used to increase the energy content of formula feeds.

Starches are plant storage complex carbohydrates with molecular weights often exceeding 100 kD, which comprise glucose polymers with $\alpha 1,4$ (amylose - straight chain) and $\alpha 1,6$ (amylopectin - branched chain) linkages. The proportions of amylose and amylopectin vary between different starches as does the size and shape of the storage granules. Three crystalline forms of starch, A, B and C, are recognised which differ in their digestibility (33). Type A (e.g. raw wheat or rice starch) has an easily digestible, open helical structure. The densely packed hexagonal pattern of the double helices of the type B (e.g. raw potato starch) reduces digestibility by denying access to amylases (33,34) (Figure 1a). Schematic models of starch crystals helps understand the variation in digestibility of differing starch granules (35). Type C (e.g. peas or other legumes) is considered to be a mixture of types A and B.

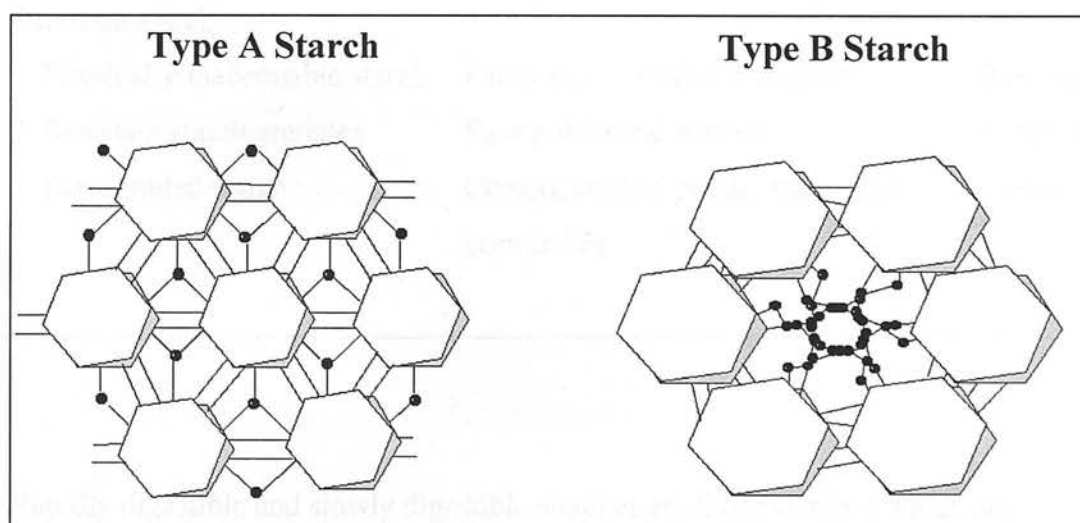


Figure 1a. Types A and B starch. Each hexagon represents a helix of amylose/amylopectin. In type A there is a lattice of starch helices with water spread throughout the molecule structure rendering it easily digestible. An example is wheat starch. In type B there is super helix with water molecules tightly bound within the centre which makes it resistant to digestion by human amylase. Examples include raw potato starch and retrograded starch.

1.2.2 Starch digestibility

Starches have also been classified empirically by their digestibility *in vitro*, into rapidly digestible, slowly digestible and resistant forms (34) (Table 1a).

Table 1a. *In vitro* classification of starch based on adult foods and digestive physiology (from Englert and Kingman 1990 (34))

Type of starch	Example of occurrence	Probable digestion in small intestine
Rapidly digestible starch	Freshly cooked starches	Rapid
Slowly digestible starch	Most raw cereals	Slow but complete
Resistant starch:		
1. Physically inaccessible starch	Partly milled grains and seeds	Resistant
2. Resistant starch granules	Raw potato and banana	Resistant
3. Retrograded starch	Cooled, cooked potato, bread and corn flakes	Resistant

Rapidly digestible and slowly digestible starches are those starches which are digested completely in the small intestine. Resistant starch is defined as the sum of starch and the products of starch degradation that are not absorbed in the small intestine of healthy adults (36). The digestibility of starch is determined by its structure, the kinetics of oligosaccharide release from the starch following hydrolysis

(37), and the degree of inhibition of α -amylase *in vitro* by other nutrients such as leguminous glycoproteins and anti-nutrients such as tannins (38). All three starch types (A, B and C) can be rapidly digestible in the cooked state. Types B and C are more likely to form retrograded starch on cooling or during processing.

Retrogradation is starch recrystallisation to an indigestible product, which may occur during cooking and cooling cycles, or under processing conditions of high moisture, pressure and temperature. The digestibility of an individual starch foodstuff can be expressed as the starch digestion rate index (SDRI) which is defined as (39):

$$SDRI = \frac{\text{rapidly digestible starch}}{\text{total starch}} \times 100$$

Since resistant starch includes starch and oligosaccharides escaping small intestinal absorption, its digestibility *in vivo* can only be estimated by experiments which mimic the intestinal conditions closely (40). Studies of the digestibility of starches *in vivo* have been largely hitherto confined to adults (41,42). In infants the lower luminal pancreatic α -amylase concentrations give rise to an increased ratio of resistant to digestible starch.

There is a wide range in digestibility of commonly used first weaning foods *in vitro*. Rice starch is rapidly digestible. Freshly cooked potato is also rapidly digestible but may become retrograded and resistant if cooled after cooking. Sterilising techniques in the canning of commercial weaning foods may considerably increase the resistant starch content of the diet of young children (43), and the consequent effects on energy absorption and growth potential are unknown (44).

1.2.3 Properties of resistant starch

Recently, some benefits of resistant starch in infant diets have been identified. In preterm infants, formulas that contain malto-dextrins are associated with increased calcium absorption (45), believed to be due to enhancement of passive absorption (46). In infant pigs this same effect, attributed to resistant starch, also leads to increased absorption of iron, and possibly zinc (47). Others have found varying results on the effect of starches on mineral absorption in human infants (48,49).

There is a concern that resistant starch, particularly the lower molecular weight portions escaping digestion in the small intestine or following hydrolysis by the colonic microflora, can generate an excessive colonic osmotic load (50), leading to diarrhoea in infancy and hence dietary manipulations to increase the resistant starch content may be harmful. However, this does not take account of the fermentative ability of the colonic flora which is discussed further below.

1.3 Starch digestion

1.3.1 Upper gut

Breakdown of starch begins in the mouth under the action of the glycoprotein enzyme α -amylase, which is secreted in saliva and human milk, and cleaves the α -1,4 linkages in the starch molecule. Alpha-amylase is inactivated by gastric acid, but digestion continues in the alkaline duodenal lumen where the salivary isozyme is reactivated and further α -amylase is secreted by the exocrine pancreas. The products of this digestive step are maltose, isomaltose, maltotriose and maltodextrins (branched chain oligosaccharides) which undergo further digestion in the brush

border of the jejunal mucosa where free glucose is liberated by the action of glucoamylase, maltase and isomaltase. Glucose is thereafter actively transported across the mucosa.

1.3.1.1 breast milk amylase

Human milk contains α -amylase, which may help breast-fed infants to digest starches in early weaning. It is structurally identical to salivary amylase, but has a broader pH optimum and retains its activity in the stomach (51). There are large inter-individual variations in breast milk concentrations but associations have been found with gestational age at birth (mothers who deliver prematurely have highest activity (52)) and parity (decreased with increasing parity (53)). Human breast milk amylase is found in its highest concentrations in colostrum (52) and decreases thereafter during the course of lactation (53). Starch supplements have been shown to be better tolerated by breast fed than formula fed infants (54).

1.3.1.2 salivary amylase

Salivary amylase is detectable from 20 weeks gestation (55). Its activity increases rapidly after birth to reach near adult levels from 6 months to 1 year (56), but there is large inter-individual variation (57). It is inactivated by low pH but remains active in the poorly acidified neonatal stomach where the pH may be >4 until 3 to 4 weeks of age (58). In the stomach it is also protected by binding to small chain glucose polymers (59). Salivary amylase may serve an important role in the young infant where there is a physiological deficiency of the pancreatic isozyme and it has been

proposed that the small amounts of amylase detected in the duodenum of young infants (60) may be of salivary origin (61).

1.3.1.3 pancreatic α -amylase

The physiology of the neonatal gastrointestinal tract is different from that of the adult (Figure 1b) with most digestive enzymes found at lower concentrations. Pancreatic α -amylase concentrations in the neonatal duodenum are much lower than in adults (60,61) (Table 1b).

Table 1b. Duodenal aspirate volume and pancreatic α -amylase activity in full term neonates who were fed a standard formula, containing 7g lactose per 100ml. Shown for comparison are values from healthy children age 9 months to 13 years (from Zoppi et al, 1972 (60)). Values expressed per kilogram body weight and represent mean \pm SD (range) of the activities during 50 min after injections of pancreozymin and secretin.

	Volume, μ l	α -Amylase, IU
Birth*	539 \pm 529 (166 - 970)	3.2 \pm 3.8 (0.1 - 9.8)
12 hours after first feeding	329 \pm 318 (96 - 800)	0.38 \pm 0.13 (0.2 - 0.5)
1 week	434 \pm 330 (96 - 1040)	1.29 \pm 1.29 (0.1 - 3.0)
Children	3900 \pm 1500 (1800 - 8100)	665 \pm 464 (160 - 2150)

* Before first feeding

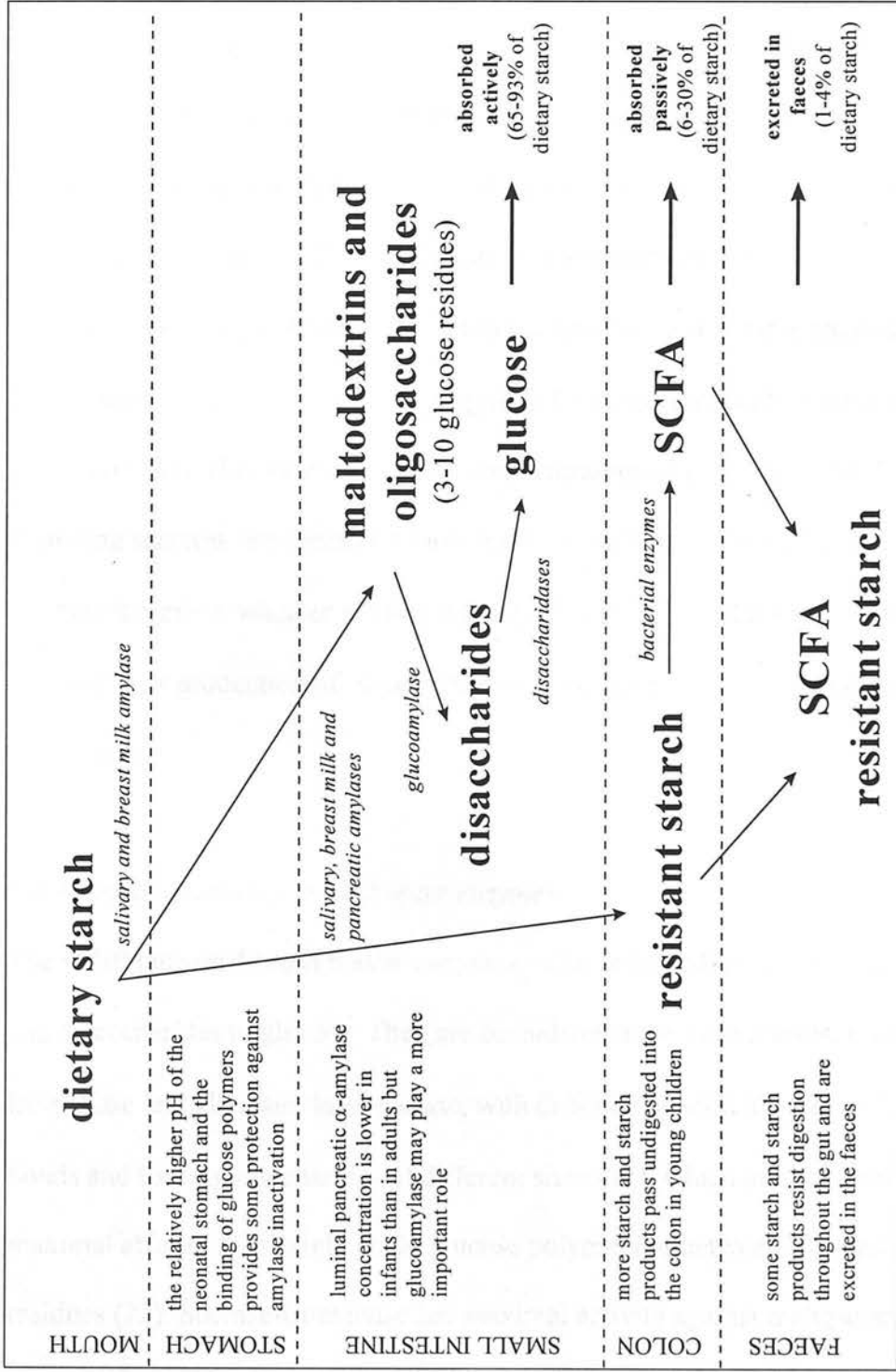


Figure 1b. The fate of starch in the infant's gut. The digestive pathways of starch throughout the whole gastrointestinal tract are shown together with the enzymes at each site. Approximate calculations for the fate of dietary starch are based on unpublished results, calculations of faecal starch in young children (62), faecal short chain fatty acids in young infants (63) and stool daily volumes for young children (64).

Serum concentrations at birth are 1.6% of adult levels and do not reach mature levels until 5 to 12 years (65). Explanation for low duodenal concentrations of pancreatic α -amylase may be low synthesis or secretion of the enzyme. Seventy years ago the existence of α -amylase in the fetal pancreas from 22 weeks gestation was reported (66) but more recently none was found in the pancreas of three week old infants (30). Pancreatic α -amylase has been detected in the amniotic fluid from 16 weeks gestation (67) although in much reduced concentrations compared with salivary amylase (68). Pancreatic fluid and electrolyte secretion increases in response to secretin and cholecystokinin (CCK) (69) but specific assays for α -amylase show that infants under one month are unresponsive to CCK and have only a minimal response to secretin (61). However, the plasma concentrations of gastrointestinal hormones, including secretin, are themselves low until the sixth day of life (70). The question remains therefore, whether in early infancy, there is low synthesis or secretion of α -amylase, low production of, or response to secretagogues, or a combination of all these factors.

1.3.1.4 small intestinal brush border enzymes

The small intestinal brush border enzymes catalyse hydrolysis of oligosaccharides and disaccharides to glucose. They are considered as two complexes, sucrase-isomaltase and glucoamylase-maltase, with differing specificities for α -1,4 or α -1,6 bonds and for oligosaccharides of different sizes (71). Glucoamylase-maltase has a maximal affinity for straight chain glucose polymers of between five and nine residues (72). Sucrase-isomaltase has maximal activity against maltose and

maltotriose, as well as α -1,6 bonds (71), making it essential for the digestion of amylopectin since amylases cannot hydrolyse these bonds (73). Enzyme activity is detectable in fetal life (74-78) with a marked third trimester increase such that at birth their activity is comparable to that of adults (79,80). In the early months of postnatal life, glucoamylase-maltase may help compensate for the physiologically lower concentrations of pancreatic α -amylase if an infant is given starch at this age (81).

1.3.1.5 regulation of starch enzymes

Lebenthal (82) described regulation of gastrointestinal ontogeny through the influence of four factors: genetic endowment; an intrinsic developmental and biological clock; endogenous regulatory mechanisms and environmental influences. The interaction between an innate biological clock and the influence of dietary factors is unclear, but most evidence suggests that the latter may modulate the 'hard-wired' genetic programme of gastrointestinal ontogeny. The biological clock is species-dependent so the inferences to be taken from animal studies do have limitations. Magee (83) draws much the same conclusions from observations that α -amylase is present in the preterm pancreas but not apparently secreted at term. He suggests either that amylase is not synthesised immediately after birth, or secretion is inhibited. He further suggests that the infant pancreas is a model for the interactions of inhibitory hormones, a biological clock, adaptation to diet and the various factors contained within human milk. This idea is supported by animal studies which have shown that various milk factors increase prior to weaning and promote the secretion of enzymes to digest an adult diet (7).

Gut hormone surges occur in infants receiving oral feeds compared to those receiving intravenous nutrition (84). In animal studies, growth hormone and thyroxine regulate small intestine ontogeny in the post-natal period (85). Much animal work has been carried out into the effects of thyroid and corticosteroid hormones on the modulation of the pre-weaned gut. *In vitro* work does not demonstrate a corticosteroid surge prior to an increase in disaccharidase enzymes (86). One possible explanation, in humans at least, is that the corticosteroid surge may be stress-induced (82).

The observation that premature infants can adapt from placental to enteral nutrition several months earlier than term infants points to external influences on gut maturation (87). Animal studies have shown that intestinal growth is affected more by nutritional changes than are enzymatic alterations (88). Studies in humans have however concentrated more on enzymatic changes. The effects on the concentrations of pancreatic α -amylase have interested researchers: Zoppi et al (60) found that starch increased the duodenal concentration of amylase in term and preterm infants although the concentrations reached were only 1.6% of the levels in older children. However, an alternative explanation for this finding is that the higher duodenal concentrations were in fact salivary amylase which was not distinguishable from pancreatic amylase in the assay and might have been protected from inactivation in the stomach by binding with starch or starch breakdown products (59).

There is indirect evidence for the influence of hormonal and/or dietary factors on studies carried out in premature infants. Assuming an unmodified biological clock, these infants would not be able to digest starches until their *corrected* age was around 4 months. In practice, premature infants are weaned, without obvious problems, when they reach the recommended *chronological* age. Functional studies show tolerance of glucose polymers in premature neonates (89). Some believe this to be attributed mainly to salivary amylase (58,90), others to the fermentative role of the colonic bacteria (91,92). In the study of Cicco et al (89) a similar glycaemic response with lactose and glucose polymers was seen but only lactose produced a significant rise in plasma insulin. The authors were unclear as to the reason for this. One possibility may be the contribution of energy derived from SCFA in the case of glucose polymers, producing increased plasma glucose through gluconeogenesis.

In premature infants the excessive fermentation of lactose has been linked to necrotising enterocolitis (93). Kien et al (92) also point out that excessive fermentation results in a greater than usual proportion of dietary fuel in the form of SCFA which may have potential effects on protein synthesis and subsequent growth. If there are short or long term adverse consequences from early weaning then the weaning age of premature infants requires further clarification; the study of starch digestion in premature infants may lead to a greater understanding of the regulation of starch digestion.

Dietary starches may increase the concentration of pancreatic α -amylase. Higher duodenal concentrations of amylase in term and preterm infants were found in response to starch (60) but the difference in concentrations before and after ingestion

were negligible in comparison with concentrations in older children. In functional studies there are discrepant results. Shulman et al (94) found greater absorption of glucose polymers with age and increased duration of feeding. Senterre (95), however, found no increased absorption of starch following prolonged administration in low birth weight infants. The two studies used similar subjects but different techniques, making direct comparisons difficult. There appear to be no published studies on salivary amylase or glucoamylase induction by dietary starch.

1.3.1.6 glucose transport

A late gestational maturation of glucose transport is suggested by a review of several glucose perfusion studies (96). Glucose transport in the newborn small intestine per unit length is a quarter of that in adults (97). This is probably the result of a relatively small mucosal surface area, less glucose transporters or limited active transport capacity. However this does not appear to be a rate-limiting process, nor is there evidence that starch or oligosaccharides can be transferred across the intestinal mucosa during the immediate neonatal period when the gut is more permeable to some molecules (98).

Colonic fermentation of starch

1.3.2.1 fermentation

Endogenous bacteria in the large intestine, principally in the proximal colon where substrate availability is highest may ferment carbohydrate which escapes digestion and absorption in the small intestine. Many nutrients reaching the colon can be

substrates for fermentation (99), but of these, starch and dietary fibre (non-starch polysaccharide) are thought to be of most biological significance.

1.3.2.2 colonic flora

The adult colonic flora is a stable population of over 400 different bacterial species in the dominant flora (100). During infancy there is a continuous evolution of the bacterial flora (101), influenced initially by the type of milk fed. A difference is first evident between the faecal bacteria of breast and formula-fed infants by 7 days with significantly greater counts of *Bacteroides fragilis* isolated from the stools of the latter (102). Newer molecular techniques using 16S rRNA and fluorescent *in situ* hybridisation (FISH) have confirmed the dominance of bacteroides species in formula-fed infants where other studies have experienced difficulty in culturing the genus (103) but have also suggested that there remain many unidentified species (104). Later the flora of breast-fed babies contains fewer facultative anaerobes and more lactobacilli and bifidobacteria (105,106) in particular the bifidobacterial species *B. longum* and *B. adolescentis* (107). Weaning introduces starch and other novel foodstuffs that are less digested in the small intestine, resulting in further changes to the colonic environment that favour the development of an adult-type flora. The microflora of breast-fed infants is more 'immature' and there is an increase in certain anaerobic species such as bacteroides following the introduction of a diet containing starch, oligosaccharides, dietary fibre and pathogens (108). This may account for the vulnerable period soon after the cessation of breast-feeding when infants are more susceptible to dehydrating diarrhoea (109), quite apart from the risk they face from contaminated complementary feed (110).

1.3.2.3 gaseous fermentation products

The products of fermentation include gases (hydrogen, carbon dioxide and methane), lactate, and short chain fatty acids (SCFA). Because the colon is the home of such a multitude of diverse and inter-dependent bacterial species, it is at present impossible to identify the contribution of each to the fermentation reactions taking place. The principal polysaccharide-degrading bacteria are bacteroides species (111) although bifidobacteria, more common in the infant colon, are also capable of degrading polysaccharides (112).

1.3.2.4 short chain fatty acids

The main SCFAs produced by colonic fermentation are acetic, propionic and butyric. Most studies have been conducted in adults where it has been calculated that they are usually generated in the respective molar ratio 60:20:18 (113). Both direct *in vivo* measurements of SCFA from dialysis bags (114), and estimates of SCFA production in the colon in combination with measurement of their faecal concentrations (115), show that they are rapidly and well absorbed in the human colon probably in their non-ionised form by concentration-dependent passive diffusion (116).

1.3.2.5 metabolism of SCFA

Butyrate has been shown to be a preferential fuel for colonic epithelial cells in adults (117,118) although much less has been detected in infant faeces (63). Significant amounts of propionate and acetate are transported to the liver, where they may be oxidised or used for gluconeogenesis (propionate) or the synthesis of long-chain fatty

acids (acetate). Almost all propionate and butyrate reaching the liver is utilised there, but acetate can be further transported and utilised in peripheral tissues, such as muscle (119).

1.3.2.6 energy salvage from SCFA

A small but significant amount of energy is gained by the colonic absorption of SCFA. In animal studies the partial utilisation of SCFAs has been shown to be 82% from intra-caecal infusions and the majority of this is retained as fat (120). In adult humans the energy is estimated to be around 2 kcal/g of non-absorbable carbohydrate (121) or a contribution of 5-10% of energy requirements (122,123). Kien et al (92) estimated that 24-74% of lactose may be converted into acetate in preterm infants with a potential 30% loss in ATP compared with its complete digestion in the small intestine (124). There appear to be no data on energy salvage from fermentation of starch in infancy at a time when the limited digestive capacity of the small intestine means that more starch will pass undigested into the colon.

1.3.2.7 properties of SCFA

Studies of the effects of SCFAs have been carried out almost exclusively in animals or in adult humans where some properties are related to all the SCFAs, and some attributed to specific ones (125,126). All SCFA appear to have trophic effects on the gastrointestinal mucosa, both in the upper and lower gut (117,119,127,128). Butyrate is the preferred fuel for colonocyte metabolism (117,129) and may protect against colonic cancer (130), although this may not happen at physiological concentrations(131). Propionate has been associated with cholesterol-lowering

effects (132,133). Basal levels of insulin and glucagon are suppressed by increased loads of SCFA in ruminants (134), and although their metabolism differs considerably from that of humans, similar increases in insulin sensitivity have been demonstrated in man (135). Insulin has anabolic effects in the human infant, and it is possible that by this means starch fermentation may indirectly influence growth (124). SCFA may also affect intestinal transit: in animal models, prolonged antibiotic treatment has been shown to alter colonic myoelectric activity which can be restored by infusing SCFA (136).

1.3.2.8 development of the fermentative ability of the infant colonic flora

Bacterial populations and diet influence fermentation products. Different components of the diet encourage the growth of particular bacterial species. The faecal flora of formula-fed infants produce more propionate than that of breast-fed infants (137). Fermentation reactions are directly affected by the substrate; for example butyrate is preferentially produced by the fermentation of resistant starch (138,139). The capacity of infants to ferment complex carbohydrates does not increase significantly until 7 to 9 months (140). In pre-weaning infants fermentation products are mainly acetate with small amounts of propionate and n-butyrate. By 9 months the mean faecal concentrations of acetate, propionate and n-butyrate have increased significantly but are still much less than adult values (140).

The net effect of the fermentation of polysaccharides to SCFA is an increase in the osmotic load within the colon although unfermented resistant starch itself causes an increased osmotic load. If the capacity for SCFA absorption is limited then diarrhoea

results, but in young piglets the presence of SCFA in the colon enhances water and sodium absorption (141). Absorption of SCFA from the colon of adults is rapid and more than half of ^{14}C labelled SCFA appears in the breath within 6 hours (142). Lifschitz et al (143) demonstrated that colonic acetate absorption in malnourished infants increased with age but there are few other human studies of the development of colonic absorption of SCFA.

1.4 Methods to measure the digestion and fermentation of starch in infancy

In 1970 it was proposed that the digestion of starch in young infants is incomplete (144) as a consequence of a well designed experiment which showed that starch caused rapid gastric emptying in infants up to 2 months and so did not provide the osmotic stimulus to the duodenum to slow intestinal motility. However, the same infants showed a glycaemic response to the starch meal and therefore were able to digest some of the starch. Various methods have been used since to measure the digestion and absorption of starches in early life. They have been used to measure digestion in the upper gut, fermentation in the lower gut, or digestion throughout the whole gut. Methods include measurement of blood glucose, balance studies, *in vitro* fermentation of faecal bacteria and the use of ^{13}C stable isotope breath tests. Many studies have been carried out in neonates, often born prematurely, whose formula feeds have been supplemented with glucose polymers to increase their energy content.

1.4.1 Stable isotope breath tests

1.4.1.1 historical aspects

The emergence of stable isotope methodology replacing radionuclide tests over the last 25 years has offered a means of studying the fate of nutrient digestion *in vivo* (145). Stable isotope breath tests, utilising ^{13}C , the stable isotope of carbon, are non-invasive, and offer a precise and reproducible method for the study of carbohydrate and fat digestion. The disadvantages of ^{13}C tracers include the natural background of around 1.1%, the cost of producing artificially labelled substrates and the cost of the instrumentation required for measurement (146). However, the cost of a mass spectrometer in a large paediatric centre for the stable isotope diagnosis of pancreatic insufficiency can be justified in offsetting the cost of upper gastrointestinal endoscopies as well as the additional increasing use for *Helicobacter pylori* testing (145).

1.4.1.2 principles

The appearance of $^{13}\text{CO}_2$ in the breath following the ingestion of a particular substrate labelled with ^{13}C depends upon exogenous and endogenous factors that control its digestion, absorption and metabolism. The labelled substrate is chosen with a “target bond” which can be cleaved resulting in a labelled functional group which is destined to produce labelled CO_2 as a metabolic end-product. If one of the metabolic steps is known to be rate-limiting, diagnostic interpretation is possible. For starch digestion, it has been shown that the rate-limiting step is hydrolysis (42). The ratio of $^{13}\text{CO}_2$: $^{12}\text{CO}_2$ in breath can be measured by isotope ratio mass spectrometry (IRMS) and, assuming constant or complete oxidation of the products of digestion

and constant production of CO_2 , the rate and extent of substrate digestion and absorption can be quantified.

1.4.1.3 substrates

The variation in ^{13}C enrichment within plant sugars allows for the use of naturally enriched substrates as tracers. This occurs because of the existence of two pathways for the fixation of atmospheric CO_2 . One involves ribulosebiphosphate carboxylase in which a 3-carbon compound is the initial stable product (C_3 or Calvin-Benson pathway) and the other involves phosphoenolpyruvate carboxylase in which a 4-carbon product results (C_4 or Hatch-Slack pathway). C_4 plants, which include maize and sugar cane, produce carbon which is more abundant in ^{13}C than C_3 plants such as beet and potatoes. For example, the abundance of ^{13}C in sugar cane is 1.09 atom percent (At%) which compares to 1.08 At% for sugar beet.

Naturally enriched substrates provide a cheap supply of tracers, but their relatively low enrichment over baseline means that large quantities of tracer are required. One approach to ameliorate this problem has been to give a diet strictly composed of C_3 substrate for several days, thereby depleting breath CO_2 and faecal carbon to C_3 levels before administration of a C_4 substrate (147). Artificially enriched plant carbohydrates have been produced by cultivating in a controlled environment and introducing $^{13}\text{CO}_2$ at a certain stage (148,149). These not only expand the available substrates, but also provide substrates with greater enrichment which can be given in small amounts.

1.4.1.4 analysis by mass spectrometry

In isotope ratio mass spectrometry (IRMS), isolated, cleaned and dried CO₂ is introduced into a vacuum system. It is then ionised, accelerated and deflected in an arc which is proportional to the ionic mass. The two commonest masses of CO₂ reflect the incorporation of stable isotopes, ¹²C¹⁶O₂ with a mass of 44 and ¹³C¹⁶O₂ with a mass of 45. The ions of different mass are separated and collected individually. They are converted into electrical currents and measured. The ratio of ¹³C:¹²C can then be calculated. The technique is suitable for detecting small differences in enrichment, but requires a relatively large sample (of the order of micromoles of CO₂).

Continuous-flow isotope ratio mass spectrometry (CF-IRMS) was developed around 20 years ago for ¹³CO₂ analysis in expired air. It provides rapid automatic sample gas preparation using a smaller sample size than conventional IRMS but with better precision than gas chromatography / mass spectrometry (GC/MS) and precision comparable to IRMS (150,151).

1.4.1.5 technical aspects

¹³C enrichment is commonly measured by reference to an international standard. That chosen is PeeDee Belemnite (PDB), a limestone of well-defined isotopic abundance. The relative difference is then expressed as delta per mil (‰). The ¹³C:¹²C ratio for PDB is 0.0112372. In a breath test experiment the results are expressed as enrichment or delta over baseline (DOB). The units of abundance, atom percent (At%), and enrichment, atom percent excess (ape), have been chosen for this

thesis (152). Parts per million excess (ppme = $\text{ape} \times 10\,000$) yields results of a magnitude which is easier to use. Since most natural levels of ^{13}C are less than PDB, abundance expressed in $\delta^{13}\text{C} \text{ ‰}$ is usually a negative number and more difficult to work with conceptually.

Breath samples are taken in duplicate or triplicate prior to administration of the tracer and then at regular time intervals for the duration of the test. From the enrichment of expired $^{13}\text{CO}_2$ the percentage dose recovered (PDR) of the ^{13}C within the substrate can then be determined for any time period.

$$\text{PDR (\%)} = \frac{V\text{CO}_2 \times \text{ape}(\text{breath CO}_2)}{\text{dose} \times \text{ape}(\text{substrate})} \times 100$$

where: ape is the ^{13}C enrichment in atom percent excess

$V\text{CO}_2$ is the CO_2 produced (expressed in moles) for the time period in question

dose refers to the substrate dose, again in moles

To do this it is necessary to measure, or assume a value for CO_2 production ($V\text{CO}_2$).

The individual PDRs can be summated to give a cumulative PDR (cPDR) for the duration of the experiment.

Mild exercise has been shown to increase the fluctuations in the ^{13}C abundance of expired CO_2 (153) and so it is necessary to have the subject at rest, or as near as is practically possible, for the duration of the experiment. Similarly, food has been shown to increase the amplitude of fluctuations and the study should be carried out

after an overnight fast with several baseline samples taken to increase precision (153-155). These considerations are of greater importance when the expected CO₂ enrichment is not high.

1.4.1.6 clinical applications

The uses and potential uses of ¹³CO₂ breath tests in clinical practice are many and include measurement of digestion, intestinal motility, hepatic function and detection of infection. The main areas of application are shown in table 1c. The application of stable isotopes in clinical medicine was the subject of a supplement to the journal Gut in 1998 (volume 43, supplement 3).

Table 1c. Clinical applications of ¹³C breath tests

1. Digestion and metabolism

Fat (156,157)
 Glucose (158)
 Starch (41,159)
 Protein (160)

2. Fermentation

Lactose (92,161)

3. Gastrointestinal motility

Lactose-¹³C-ureide (162)
 Gastric emptying (163,164)

4. Hepatic function

Caffeine breath test (165)

5. Detection of infection

Small bowel bacterial overgrowth (166)
Helicobacter pylori (167)

1.4.1.7 ^{13}C breath tests in paediatric practice

Stable isotopes are non-radioactive and so ^{13}C breath tests are safe, and the non-invasive nature of sampling breath rather than blood makes them particularly attractive for the study of infants and young children (168,169). Breath samples can be collected using a soft plastic face mask connected to a reservoir bag, such as that designed as an oxygen reservoir (Laerdal), by two one-way valves (Amdi Paedi Valve). Samples of expired air can then be taken from the reservoir via a sampling port and stored in evacuated tubes. (168). Samples are reliable for up to eight weeks if stored in Exetainer[®] sampling tubes (151).

1.4.1.8 ^{13}C breath tests to measure upper gut digestion

^{13}C breath tests were used in 1983 by Shulman and co-workers (147) to investigate the digestion and absorption of carbohydrate of differing complexity in young infants. They described their technique as a new direct approach in contrast to the inconsistent results that had been obtained by prior indirect studies such as the measurement of a glycaemic index (see below). Using naturally enriched corn starch, they demonstrated a clear $^{13}\text{CO}_2$ rise above baseline and concluded that that young infants were able to digest and absorb starch as a result of glucoamylase.

The scope of the ^{13}C starch breath test was defined more clearly by Hiele and co-workers (42,170). In the first of these experiments they used naturally-enriched maize starch in healthy adults and adults with chronic pancreatic disease, and expressed their results in PDR for experiments of 6 hours duration. The breath $^{13}\text{CO}_2$ recovery after ingestion of starch was less than the breath $^{13}\text{CO}_2$ recovery after

ingestion of glucose. They concluded that the rate-limiting step in starch digestion is starch hydrolysis, challenging the then existing view that it was monosaccharide absorption. Comparing healthy patients and patients with pancreatic disease, they found differences in PDR for both starch and glucose. They concluded that the ratio of $^{13}\text{CO}_2$ recovery for starch to $^{13}\text{CO}_2$ recovery for glucose represented pancreatic exocrine function as an independent factor.

The same group used starch breath tests with naturally enriched substrates to investigate differences in starches of differing structure (41). Since differences in $^{13}\text{CO}_2$ recovery had already been shown to reflect differences in hydrolysis rates, they were able to demonstrate a faster rate of digestion for amylopectin as compared to amylose.

^{13}C starch breath tests have been used to address some practical issues of starch digestion within paediatrics. Weaver and co-workers (159) used naturally enriched maize starch to study the effect of amylase rich flour on starch digestion in weaning infants in Gambia. Children with cystic fibrosis (CF) who have known pancreatic insufficiency have been subjects of starch digestion tests with ^{13}C substrates. Dewitt and co-workers (171) found that in children with CF there was no consistent improvement in starch digestion with the addition of pancreatic enzymes to the diet. Amarri and co-workers (172) by contrast, found a significant increase with the addition of pancreatic enzymes. In this paper, comment was made about the shapes of the $^{13}\text{CO}_2$ recovery curves; a second peak was observed in six children with CF,

leading to speculation that colonic fermentation of starch malabsorbed in the small intestine was responsible for this phenomenon.

1.4.2 Other methods to measure upper gut digestion

1.4.2.1 measurement of duodenal concentrations of pancreatic enzymes

Several investigators have measured the concentrations of amylases, in the duodenum (60,61,173), plasma (65,174), saliva (57,58) and breast milk (51,52). The intestinal concentration of glucoamylase has been measured from small bowel biopsy specimens (175). Hiele and co-workers' (42) finding that starch digestion reaches a maximum, even at low concentrations of amylase, limits the usefulness of enzyme concentrations.

1.4.2.2 functional studies

A glycaemic response following the ingestion of corn starch by three day old infants was found by Anderson and co-workers (176). However, it was less than that for glucose. These findings were endorsed by Lebenthal and co-workers (177) in a group of ten infants recovering from acute gastroenteritis. However, Cicco and co-workers (89) found glucose polymers consisting mainly of 8-30 glucose residues produced a similar glycaemic response to lactose in 2 to 3 week old pre-term infants. These inconsistent findings have led others to mitigate the findings of carbohydrate tolerance studies of glycaemic response (147).

The disappearance and hydrolysis of glucose polymers from the duodenum has been measured in infants by duodenal intubation and perfusion (94,177). The widespread

use of tolerance and perfusion studies, particularly for the investigation of starch digestion in healthy infants is limited by their invasive nature.

1.4.3 Fermentation in the lower gut

The approaches to the study of colonic fermentation are protean and disparate. They include the hydrogen breath test, *in vivo* intubation studies, post-mortem studies, *in vitro* modelling and further use of stable isotopes.

1.4.3.1 hydrogen breath test

Hydrogen is a bacterial fermentative product and cannot be produced or utilised by mammalian cells. It is absorbed into the bloodstream and excreted in the breath.

Administration of a non-absorbable carbohydrate produces a rise in hydrogen production after a period of time which is detectable in end tidal breath measurements. This is the basis of the hydrogen breath test and it can be used to measure both small intestinal carbohydrate malabsorption and oro-caecal transit time. It was first described for carbohydrate malabsorption 30 years ago (178) and has been used to estimate the degree of malabsorption by the amount of substrate being fermented (179). It has also been used in young infants to measure the fermentation of lactose (91).

However, the hydrogen breath test has several limitations. Hydrogen is an intermediary product with several possible disposals (180). The amount which is excreted in the breath is not a constant proportion of the amount produced (181) and may therefore be difficult to use quantitatively against a lactulose standard. Different



starches have different fermentation rates (182) and it would be difficult to estimate the overall extent of fermentation or calculate a percentage dose recovered with this method. In young infants the proportion of hydrogen excreted in the breath has been shown to be greater than in adults (183), therefore a comparison of subjects of differing ages would be problematic.

1.4.3.2 *in vivo* models

Accessibility of the proximal colon is a problem for the *in vivo* study of fermentation. The concentration of fermentation products has been measured in fresh post-mortem examinations (119) though this method is clearly not suitable for use with young children. Perfusion studies have been used to measure the disappearance of substrate from the lumen in both adults with jejunoileal resection by intestinal intubation (184) and children with malnutrition by naso-anal intubation (143). Again the invasive nature of such experiments limits their widespread use in healthy children.

1.4.3.3 *in vitro* models

Models based on stool incubation have been used previously to simulate fermentation in the colon (185). *In vitro* studies circumvent some of the problems encountered with *in vivo* studies: they are cheap, the collection of faeces is simple (if unpleasant) and retention of fermentation products within the system makes their measurement easier. However, the validity of *in vitro* models has been questioned on several counts: the degree to which faeces are representative of colonic flora has been debated (186,187); the inaccessibility of the colon makes precise mimicking of its conditions very difficult (112), but more importantly, removal of faeces from the

colon inevitably changes bacterial composition and metabolic activity. Because fermentation products are not absorbed, their accumulation in the incubation chamber can itself be bacteriostatic.

Continuous culture systems better mimic the steady state of the human colon more closely, but are expensive and labour-intensive (188). Different approaches have been made using continually replenished media (189) or media which is introduced at set intervals to mimic the entry of ileal chyme (190). Some models have a single chamber (190), others multiple chambers (189) therefore better modelling the pH gradient seen *in vivo*. Continuous models still lack the removal of SCFA, whose increasing concentration may inhibit further fermentation. A complex dynamic model for stomach and small intestine has been developed which simulates peristalsis, physiological pH curves and absorption of water and nutrients (191) and it is to be hoped that the development of a complex dynamic colon will not be far behind. There still remains, however, the need for a cheap system for the larger scale study of fermentation in different subjects and using different substrates.

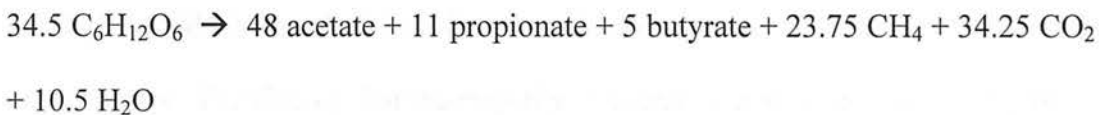
In other models the physiological conditions of the proximal colon are simulated by incubating at 37 °C, using a shaking water bath and ensuring that the simulated conditions produce good growth of anaerobic organisms (192). The difficulty in comparing results between laboratories has been circumvented to some extent by a common agreed methodology (185).

1.4.3.4 stable isotope breath tests

There are few available substrates to investigate fermentation in isolation using stable isotope breath tests. Use of the methodology has been made in an indirect way. Kien and co-workers (92) developed a model for estimating colonic acetate production in premature infants. They infused ^{13}C labelled bicarbonate and ^{13}C labelled acetate orogastrically, and used the ^{13}C enrichment of breath CO_2 to determine the presence of a plateau in enrichment at which point they assumed the concomitant presence of an enrichment plateau in plasma acetate. In an animal model, the same group of investigators (193) calculated that 28% of carbohydrate in the colon is fermented to acetate using a similar stable isotope tracer dilution method.

1.4.3.5 stoichiometry

Another way of quantifying the colonic fate of starch is by stoichiometry, that is the calculation of the numerical relationship between the molecular species entering a chemical reaction and the amount and type of molecules produced. Stoichiometric equations for carbohydrate have been produced for adult humans and ruminant animals (121,194), based on known proportions of SCFAs in faeces and known production of carbon dioxide and methane. From these, the energy derived from fermentation reactions can be calculated, such as the example for adult humans (194):



1.4.4 Whole gut digestion

1.4.4.1 balance studies

Measurable amounts of starch have been detected in the faeces of children up to 3 years old (62). However, in a functional balance study, the stools of young infants fed 2 - 3.5 g/kg per day of starch have been shown to contain very little starch (50). This gave rise to mean absorption coefficients between 99.4 - 99.8% for different starches. The authors suggested that glucoamylase accounted for this but included no discussion of colonic fermentation. Senterre (95) found a lower mean absorption coefficient of 88% when low birth weight infants up to four weeks old were fed 3.55 g/kg per day of starch. It is not clear whether these were pre-term or small for gestational age infants. Nevertheless the absorption coefficient is of the same order.

Although young infants possess the functional capacity to digest starch, this may be associated with a relatively decreased absorption of energy and nitrogen in infants of 1 to 2 months (195) but not older infants (196), highlighting the limitations of studying the digestion of a single nutrient alone. This is in keeping with others who have found that starch maldigestion in the small intestine of adults is multi-factorial (197).

Such balance studies require meticulous measurements, do not usually take into account losses from methane and CO₂ production, and total gut balance studies mean that the individual contributions of upper and lower gut to starch digestion cannot be distinguished. The finding that the majority of faecal carbohydrate energy may be in

the form of large molecular weight bacterial products (184,198) is similarly not accounted for in simple balance studies.

1.4.4.2 combination studies

Calculations of intake and stool carbohydrate output are made easier by the use of combination studies, for example balance studies combined with the hydrogen breath test, or using stable isotopes to more clearly identify the fate of the tracer.

Stable isotope breath tests, hydrogen breath tests and stool analysis have been used to show that small intestinal lactose absorption by full-term formula-fed infants is nearly complete and that colonic salvage of carbohydrate is variable (161).

Shulman and co-workers (147) used the H₂ breath test to measure oro-caecal transit and give information about the site of utilisation. They were able to conclude that all ¹³CO₂ recovered before a rise in H₂ was as a result of digestion in the small intestine. Kien and co-workers' finding of up to 90% faecal carbohydrate in the form of large molecular weight compounds was confirmed with a subsequent stable isotope method (199).

1.4.4.3 interpretation of breath test curves

The potential of stable isotope breath tests in combination with curve analysis as a tool for the study of the digestion of starch in the whole gut is under-realised.

Shulman et al (147) reported ¹³CO₂ recovery curves over time without remarking on

their shape, which showed later maximal abundance of $^{13}\text{CO}_2$ and the suggestion of a more twin-peaked curve with increasing carbohydrate complexity (Figure 1c).

Amarri et al (172) noted a similar twin peaked curve in their study of starch digestion in children with cystic fibrosis using ^{13}C -labelled wheat flour biscuits, and speculated that the second peak represented $^{13}\text{CO}_2$ derived from oxidised SCFA as a result of colonic fermentation of the starch.

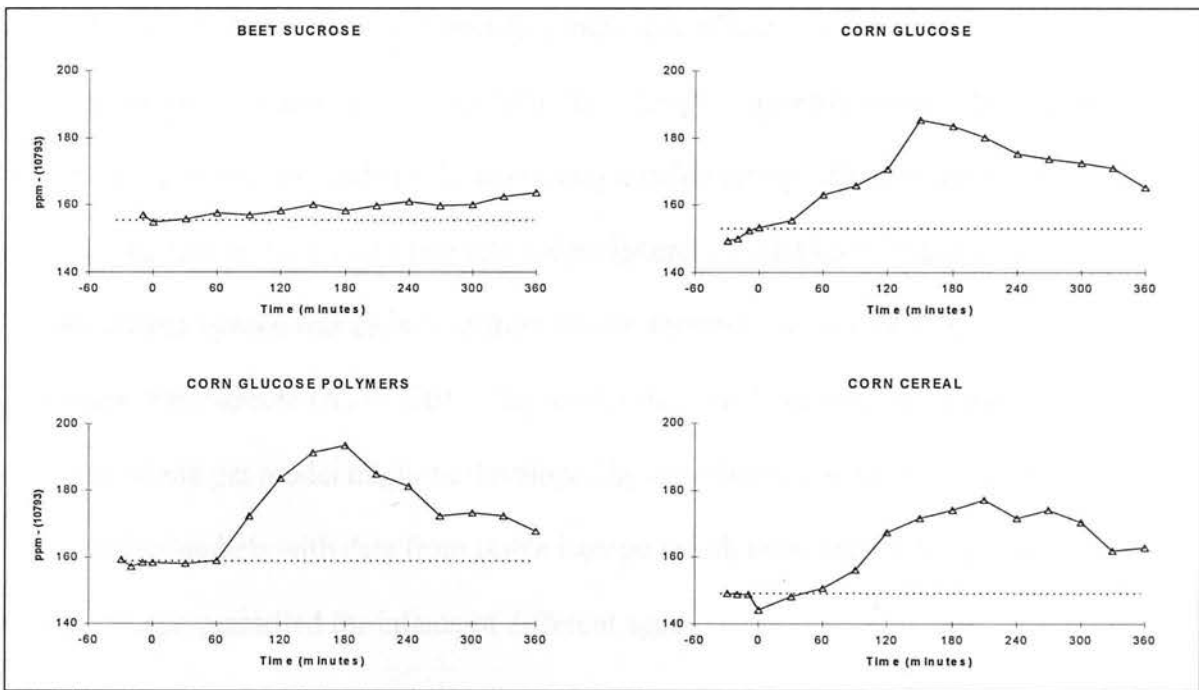


Figure 1c. The utilisation of dietary starch by young infants, from Shulman et al 1983 (147). The enrichment of breath $^{13}\text{CO}_2$ over 6 hours following the ingestion of various carbohydrate preparations by infants aged one month. Beet sugar contains little natural ^{13}C , but maize and glucose products which are derived from maize are naturally enriched.

Stable isotope PDR curves allow for the use of mathematical curve fitting to analyse whole gut starch digestion data. Curve fitting has previously been used in gastric emptying studies (164), adapting an equation which forms the basis of the χ^2

distribution. Assumptions about curve interpretation would be strengthened if a gold standard for colonic absorption could be found. There is some hope that lactose [^{13}C] ureide, a synthetic condensation product of lactose and ^{13}C labelled urea, which resists breakdown by small intestinal enzymes (162), might provide such a standard (200).

1.4.5 Energy calculations

Energy calculations have been made on the whole gut by balance studies, separating the carbohydrate from the fat and protein component of stool and then calculating carbohydrate energy absorption (195,201). An attempt to quantify energy absorption by site of digestion was made for lactose using a stable isotope dilution method to measure the rate of entry of acetate into the peripheral circulation (92) and from the results, energy uptake was estimated from known thermal losses of potential adenosine triphosphate (ATP) (201). The results show wide variability. A more complex whole gut model might be developed by combining stoichiometry from fermentation models with data from stable isotope breath tests, and carbohydrate energy source quantified for infants of different ages.

Using a stoichiometric approach, Livesey (121) has calculated dietary energy values for resistant starch in adults, what he terms unavailable carbohydrate. No such unavailable carbohydrate data exist for children.

1.5 Importance of digestion of starch in early life

There is much still to be learned about the ontogeny of the gastrointestinal tract during the weaning period and its capacity to make use of dietary starch. Reviewers have noted the importance of matching nutritional needs to the functional development of the gut (11,202). Existing work has focused mainly on the site or mechanism of starch digestion but there is a need to define the contributions of the small and large intestines, and to quantify the various fates of different starches in infants of different ages.

1.5.1 Weaning guidelines

In the most recent UK guidelines (3) it is recommended that weaning should begin between 4 and 6 months and the diet should contain adequate energy. In spite of these recommendations, guidelines are still less than ideally adhered although there have been improvements over the last 25 years (table 1d).

Table 1d. Percentage of mothers who had given their children solid food by different ages. (203,204)

	6 weeks	3 months	4 months
1975 (England and Wales)	40	85	97
1990 (Great Britain)	9	68	94

Recommended first solid foods are non-wheat cereals. Cooked starches are advised in preference to raw starches because of their greater digestibility, and rice in particular is recommended. Many mothers will use manufactured weaning foods

initially. Home prepared weaning foods, even in affluent societies may often be less than nutritionally adequate with insufficient energy, protein, fat and micronutrients, but high in non-starch polysaccharide and sodium (205). Mothers' concepts of a nutritious infant diet often place a higher ranking to the importance of a high fibre intake than to liberalising calories (206). Clearly, there is still a large need to communicate effectively current weaning guidelines.

Given that there is much variation in weaning practice, Morgan (207) has suggested a weight (6.8 to 7.0 kg) instead of age as a guideline for the introduction of complementary feeding. There is some data to suggest that the energy requirements of breast and formula fed infants differ (19) and there is a difference in weaning practice between the two groups with formula fed infants given solids foods on average 1 week before breast fed infants (208), prompting her to also suggest that consideration be given to different guidelines for breast and formula fed infants (207).

In comparison with the space given to recommendations for other nutrients, the small amount devoted to starches reflects the relative paucity of data in this field. Yet, the dramatic changes in the source of dietary energy at weaning and its potential metabolic effects suggest that this is an area that requires fuller understanding. A greater insight into the physiology of the infant gut, in particular the small intestinal digestibility of starch *in vivo*, its fermentability in the colon, the properties of SCFA, and the metabolic fate of starch, is needed to evaluate the overall nutritional value of different starches in order to define sensible and evidence-based weaning advice.

1.5.2 Weaning in the developing world

1.5.2.1 the weanling's dilemma

The unique problems associated with weaning in the developing countries deserve special mention. In contrast to the first few months of life when infants, mostly exclusively breast-fed, are relatively healthy, there is an increase in morbidity and growth faltering associated with weaning. The main problems lie in the nutritionally inadequate weaning foods used and the unhygienic ways in which they are prepared. There is in turn a deficit of knowledge about how to prepare weaning foods that are hygienic and nutritionally adequate (209) and many local traditions about weaning to overcome (210). Other problems include the higher number of small for gestational age babies born in the developing world which has an impact on depletion of nutritional stores in the fast catch up growth seen over the first few months (211). This means that infants in the developing world may be vulnerable even before the onset of weaning.

Despite a reluctance to introduce potentially nutritionally inadequate and contaminated supplementary feeds, if they were not introduced energy needs would begin to fail to be met by human milk alone. Rowland has termed the need for careful timing of the onset of weaning in this unique situation "the weanling's dilemma" (110) (Figure 1e).

He subsequently described three broad areas in which effective strategies are required (26): in improving lactational performance; avoiding unnecessarily early

weaning by defining the point at which breast milk alone becomes inadequate; and improving weaning foods nutritionally and hygienically. To this may be added the prevention and treatment of diarrhoeal disease in infancy (212).

In practice the most effective strategy has been that of developing nutritious and hygienic weaning foods. Milk output has been found to be more associated with characteristics of the mother-infant pair than with improved maternal nutrition (213). The precise point at which energy needs fail to be met by breast milk has demonstrated only marked inter-individual variation (214). Early weaning before 3 months of age has been shown to be associated with growth faltering. However, prolonged exclusive breast feeding in the developing world continues to be an area of controversy which will not be resolved until there is greater understanding of normal infant growth patterns (25).

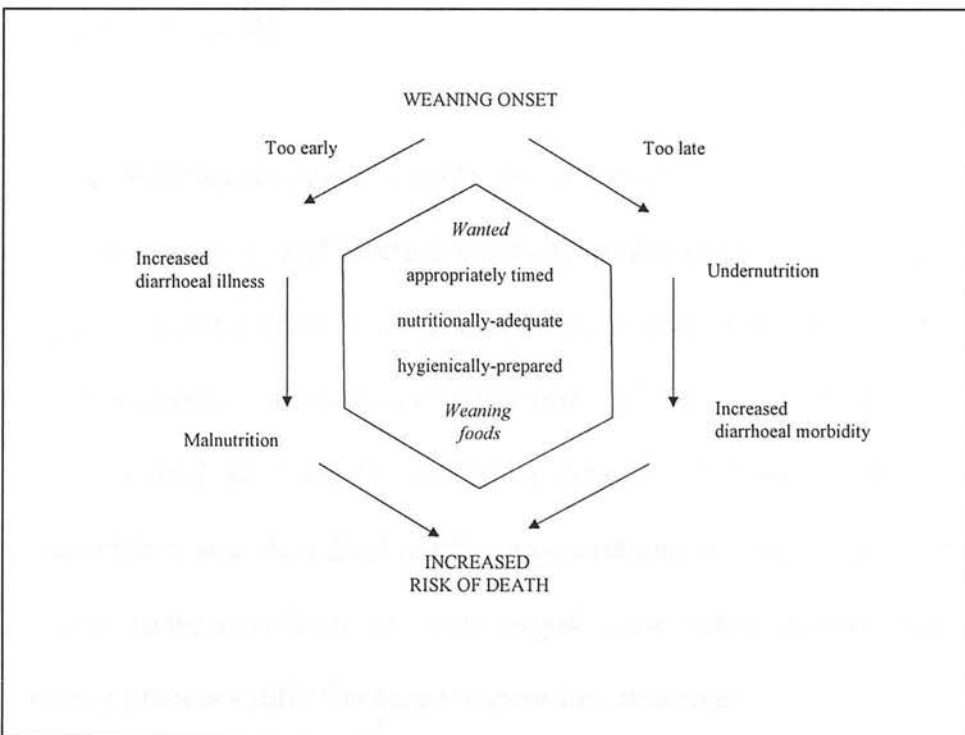


Figure 1e. The Weanling's Dilemma (26)

1.5.2.2 weaning foods for the developing world

Weaning foods in the developing world are often simple gruels. The amount of water required to be added to form a suitable consistency for a young infant results in gruels with low energy densities. Heating compounds this problem by starch gelatinisation causing increased viscosity, but the result of poorly cooked weaning foods is a failure to eradicate microbial contamination. Clearly strategies are needed which result in weaning foods of high energy and protein density, resistant to infections, prepared from locally available ingredients, and culturally acceptable (212). Traditional low-technology food preparation techniques such as roasting, germination and fermentation do counter both these problems to some extent (215-217). Adding flour prepared from germinated grains which contains amylolytic enzymes is a further cheap and locally available option that has been shown to improve starch digestibility (159) and may lead to a significant improvement in weaning foods (218).

1.5.2.3 consequences of peri-weaning malnutrition

The immediate results of severe acute malnutrition around the weaning period are kwashiorkor and marasmus; the consequences of chronic undernutrition are stunting, impaired immune and psychomotor function, and a greater mortality risk (219), problems which are evident in developing countries. Although much has been achieved in recent years there remains an enormous challenge to reduce infant mortality to the same levels as the developed world. Infant nutrition, and in particular weaning practices still offer scope to meet this challenge.

1.5.3 Importance of energy salvage

Energy from SCFA produced as a result of colonic fermentation of starches is variously considered as loss (220) or gain, as though the infant small intestine were an inefficiently functioning organ (124,201). However, the small intestinal digestion and colonic fermentation, which occur in the infant, are interrelated physiological processes that together ensure efficient overall gut function. The need to understand the individual proportions of carbohydrate energy derived from small intestine and colon is underlined by observations that SCFA may have important effects on energy balance and growth by influencing insulin concentrations (124).

1.5.4 Nutritional programming

The hypothesis that manipulations of a nutritional nature at a critical point in development can permanently program events in later life is not a new idea. For a long time it had been recognised that testosterone secretion at a certain stage of fetal development programs the development of male genitalia, and similarly that the development of a normal visual pathway requires normal binocular visual input during a critical developmental stage. Nutritional programming has been demonstrated in animals and proposed in humans.

Dating back to the sixties, McCance and Widdowson showed that rats raised in large litters were smaller at weaning, continued to eat less and remained smaller as adults (221). Work in primates has demonstrated that baboons which are overfed as infants are programmed to later obesity and there are long-term differences in cholesterol

and lipid metabolism between those who were breast fed and those who were formula fed (222).

In humans, breast feeding, long chain polyunsaturated fatty acids and iron status in infancy have been proposed to influence later childhood and adult intelligence quotient but the study sizes are small and power calculations of the study sizes needed have shown that much larger studies are required to demonstrate a convincing link (223).

Much of the evidence for nutritional programming in humans is not through experimental work but through epidemiology and is nutritional indirectly through anthropometric studies. By tracing regional infant health records from the early part of the twentieth century, Barker showed that size at birth or 1 year is associated with an increased risk in later life of ischaemic heart disease, type II diabetes, hypercholesterolaemia and hypertension (224). Follow up studies of Dutch adults born during the famine as a result of German occupation of the western Netherlands at the end of the Second World War have showed that those whose birth weight was low and those whose mothers were starved during the first half of their pregnancy were more likely to be obese (225).

Work is emerging demonstrating a link between bacterial colonisation of the colon and immunomodulation of the naïve immune system (226). It is conceivable that this association, so far studied in terms of initial colon colonisation, could be relevant

when the colonic flora changes during weaning, suggesting that weaning diet may have a role to play in subsequent resistance to infection and atopic disease (227).

Through the work of Barker and Hales, the “thrifty phenotype” concept was proposed as an explanation for the association (228); that adaptations to malnutrition in fetal or early infant life can alter metabolism in such a way that it is beneficial under continued conditions of malnutrition but deleterious under abundant nutrition. However, the mechanisms underlying the programming, which may be important in a better understanding of the pathogenesis of these adult diseases are still not clear (229). How the memory of early events is stored and expressed later also remains unclear. Lucas (230) has proposed three cellular mechanisms to explain this: that it occurs at the level of an individual gene, that there is permanent reduction in cell numbers or that specific individual cells might be deleted or clonally expanded.

Animal studies have shown weaning to be a critical period in programming and studies in rats have shown long term effects such as increased plasma cholesterol with premature weaning (231). In human studies timing of weaning is controversially associated with the development of adult obesity with some investigators proposing that early weaning is related to obesity (232) whilst others have found the reverse (233-235). The effect of complementary feeding on later health has recently been reviewed (236) with the conclusion that there is currently no evidence for an effect of weaning practice and diet on other long term health factors including the development of diabetes, cancer, hypertension, coronary vascular disease and atopy.

However the author acknowledged the need for well-designed long term studies to address whether any links may be present.

Much remains to be learned about the mechanisms controlling growth and metabolism in the infant and weaning is a time when there is a changing pattern of energy derived from the upper gut digestion of glucose and the lower gut fermentation to SCFA. The effects on metabolic control of this remain to be more clearly elucidated and in this may lie answers to programming effects of the weaning period and weaning foods. One animal study has demonstrated a relationship between weaning and weight and appetite in adulthood (237). They conceptualised weaning as reversal of the lipid to carbohydrate ratio and a switch from the predominance of glucose production to a predominant state of glucose utilisation. Rats who were “weaned” artificially and prematurely by intraperitoneal injections of glucose were lighter as adults and had decreased appetites compared with controls. The authors proposed that weaning is a critical period in development when the hypothalamic feeding centre is set.

1.6 Hypotheses and aims of the thesis

1.6.1 Hypotheses

Much of this thesis is concerned with the development of a novel methodology to study starch digestion and fermentation in young children. Several hypotheses were generated and tested using the methods described below:

- Fermentation to SCFA in the colon accounts for a measurable amount of starch digestion in young children.

- The relative role of the colon in starch digestion is numerically most important in infants soon after the weaning period.
- The absolute fermentative ability of the colonic flora is most developed in adults.
- The colonic flora of young children results in less butyrate than the colonic flora in adults.

1.6.2 Aims

The aim of this thesis is to account for the fate of dietary starch in the small intestine and colon in young children around the time of weaning by defining the rate, site and extent of starch digestion in healthy children aged up to 2 years and comparing this to the rate, site and extent of starch digestion in healthy adults.

1.6.3 Study design

1.6.3.1 stable isotope breath tests

Two approaches will be used. The first will utilise stable isotope breath tests and curve fitting techniques to attempt to define the relative contributions of small intestine and colon to starch digestion. ^{13}C labelled wheat starch breath tests will be used, both alone and in combination with labelled lactose [^{13}C] ureide breath tests to demarcate the timing of the colonic fermentation. The data will be plotted and interpreted both qualitatively and quantitatively using a mathematical curve fit to define parameters for each data set, thus making possible comparisons between different subjects and subsequently comparisons between different age groups.

1.6.3.2 in vitro stool fermentation model

A separate attempt to define the function of the colon in young children as compared to adults will utilise *in vitro* stool cultures as a model for colonic fermentation.

Fermentation products will be measured and a stoichiometric approach will be used to predict the fate of starch carbon which is fermented in the proximal colon. Further calculations will be carried out to determine potential energy salvage from this process.

1.6.3.3 combining data from both parts of the thesis

Data from each of the two approaches will be combined to derive whole gut stoichiometric equations. From these the whole gut energy value of starch will be calculated for young children.

PART I: *IN VIVO* STUDY OF STARCH DIGESTION BY CURVE FITTING OF DATA DERIVED USING STABLE ISOTOPES

CHAPTER 2. SUBJECTS, MATERIALS AND METHODS

The aim of this thesis was to account for the fate of dietary starch in the small intestine and colon in young children around the time of weaning by defining the rate, site and extent of starch digestion in healthy children aged up to 2 years and comparing this to the rate, site and extent of starch digestion in healthy adults. This chapter describes the stable isotope methodology employed to curve fit the whole gut digestion of starch.

2.1 Subjects

Fourteen children (10 male; 4 female) with an age range of 7.6 - 22.7 months (mean 13.0 months) were studied. All children lived in the Emilia Romagna region of north Italy. Twelve infants had been breast-fed exclusively for a minimum of four months. One child received mixed feeds from birth and one was exclusively formula-fed from birth. Starch was introduced into the diet of all 13 children between four and six months, and at the time of the experiments wheat starch comprised the major starch of their midday and evening meals. No child had received antibiotics in the month preceding the experiment. One child was re-studied with wheat flour at 9 and 12 months, and another child was re-studied with lactose ureide at 11 and 15 months. In both cases the second set of data has been included as a separate subject.

2.2 Substrates

The substrates used were ^{13}C enriched wheat flour and lactose [^{13}C] ureide (^{13}C -LU).

In addition, a baseline experiment (that is, breath collection without administration of tracer) was carried out in four subjects.

2.2.1 Wheat flour

The wheat had been enriched with ^{13}C by cultivating it in an environment to which $^{13}\text{CO}_2$ was added at a time to coincide with the laying down of starch within the grains. The grains were then milled and sifted to give a flour with a ^{13}C enrichment of 3.4 At%, that was composed of 54% starch, 15% protein and 9% non starch polysaccharide (NSP) (148).

2.2.2 Lactose ureide

Lactose ureide is a synthetic glycosyl ureide which is not broken down by small intestinal endogenous enzymes, but rather is dependent upon colonic bacteria for its metabolism and could therefore demarcate the time of colonic residence (162).

Lactose [^{13}C] ureide (^{13}C -LU) was produced synthetically by the condensation of lactose and ^{13}C urea (200) and had a ^{13}C enrichment of 7.7 At%. Since it was labelled exclusively on the urea moiety, the enrichment of that carbon was 99.7% ^{13}C .

2.3 Method

2.3.1 Administration of tracer

Following an overnight fast of at least 6 h, two duplicate baseline breath samples were collected. A small amount of wheat flour (100 mg in the first nine experiments and 200 mg in the remaining seven experiments) or ^{13}C -LU (100 mg) was then given mixed with milk or water in a small cup and administered with a syringe or teaspoon. The cup was rinsed out several times to ensure that the whole dose had been ingested after which the child was allowed to eat a normal breakfast, which in almost all cases included starchy food. Breath samples were collected in duplicate every 30 minutes thereafter for 12 h.

A record of food intake and periods of sleep was kept for the 12 h duration of the experiment. Children did not eat again for two hours, after which they were allowed to eat normally. For the duration of the experiment they did not consume foods that are known to be naturally enriched in ^{13}C , such as maize products and cane sugar. The major source of sugar in Italy is beet sucrose and so no restriction on sweet foods was made. For the baseline experiments, paired pre-breakfast samples were collected before a usual breakfast and for 12 h thereafter. No tracer was given with breakfast.

2.3.2 Lactose ureide induction

Previous unpublished data from our laboratory have shown that the lactose ureide curve shape changes if an induction dose of lactose ureide is given when a sharper

rise to peak results. An induction dose of unlabelled lactose ureide 100 mg was therefore given with breakfast on the morning before the ^{13}C -LU test day.

2.3.3 Breath collection

Breath was collected using two methods. Whilst the infants were awake, a “bag and mask” with a one-way valve was used to collect expired air (168). The breath collection took several seconds and caused minimal distress in most children. Whilst they were sleeping, breath was collected using a piece of naso-gastric tubing attached to a syringe which was placed close to, but not touching, the nostrils. Every time the child expired, the syringe was aspirated by around 0.5 ml until 20 ml had been aspirated.

2.3.4 Determination of body surface area

In order to determine body surface area for $V\text{CO}_2$ calculations, each child’s weight was recorded in kilograms using a balance accurate to 10g. Their height was measured standing against a wall with a tape measure. Body surface area was then calculated according to the formula of Haycock et al (238)

$$SA = W^{0.5378} \times H^{0.3964} \times 0.024265$$

where: SA = surface area (m^2)

W = weight (kg)

H = height (cm)

2.3.5 Ethical approval

Ethical approval for this study was granted by the Ethics Committee of the University of Modena. Informed, written consent was obtained from the parents prior to each test.

2.4 Analysis of samples

2.4.1 Isotope ratio mass spectrometry (IRMS)

The samples were collected in evacuated tubes and analysed using isotope ratio mass spectrometry (20-20 IRMS and Robo pep-G preparation system (Europa Scientific, Crewe, UK)) (150). The coefficient of variation of CO₂ concentration analysis was 3.04%, and the precision of ¹³CO₂ isotope abundance analysis was 0.3‰ (standard deviation of three replicate analyses of reference gas, 5% CO₂ in N₂). The ¹³C enrichment of expired CO₂ was expressed in parts per million excess (ppme), that is the difference between the ¹³CO₂ abundance of a measured breath sample and a reference ¹³CO₂ abundance, the mean baseline sample. Expression as ppme rather than atom per cent excess (ape) is conventionally chosen (ppme = 10⁴ ape) to scale the results to a convenient range.

2.4.2 Calculation of percentage dose recovered (PDR)

The PDR for each 30 min time period was calculated derived from a formula described elsewhere (239) using an assumed value of 5 ml/min/m² body surface area for basal *V*CO₂, and a height-weight estimation of body surface area (238). The results were expressed graphically over the 12 h. Cumulative PDR at the end of the experiment (cPDR) was calculated by summing the unit PDR. The time taken to

reach maximal enrichment (t_{\max}) was calculated from the first derivative of a three-point smoothed data plot as the point at which the curve first crossed the time axis:

$$\delta PDR / \delta t = \frac{PDR_{t+1} - PDR_{t-1}}{2}$$

2.5 Reproducibility studies

In two children reproducibility studies for wheat flour were carried out under as similar conditions as was practically possible. In an infant of 8 months, a second 12 h breath test was performed 5 days after the first test, with good controlling of meal timing and composition and sleep periods. In the second study, also in an infant of 8 months, the tests were carried out 11 days apart. On this occasion there was more variability in sleep periods and meal composition between the tests.

2.6 Curve fitting method

A mathematical curve fit was used to analyse the wheat flour $^{13}\text{CO}_2$ PDR plots. This enabled extrapolation of the curve to baseline and then calculation of curve parameters such as cPDR and t_{\max} . These could be calculated for the whole gut as well as individually for small intestine and colon, enabling the relative contribution to starch digestion from small intestine and colon to be estimated.

2.6.1 Equation used

The curve was based on the formula:

$$y = at^b e^{-ct}$$

where y represents PDR; t is time; and a , b and c are constants. The equation comes from the χ^2 statistical distribution and has been used previously for fitting gastric

emptying curves (164). The factor a represents a scaling factor; b and c control the shape of the curve. By increasing b , the upslope of the curve is shallower, the peak occurs later and the curve returns to baseline later. By increasing c , the upslope is steeper, the peak occurs earlier and the curve returns to baseline earlier. The factors b and c have opposing actions on the curve shape, but they change it in subtly different ways. From this equation, it is possible to describe any bell-shaped curve. The curve fitting is illustrated in figure 2a.

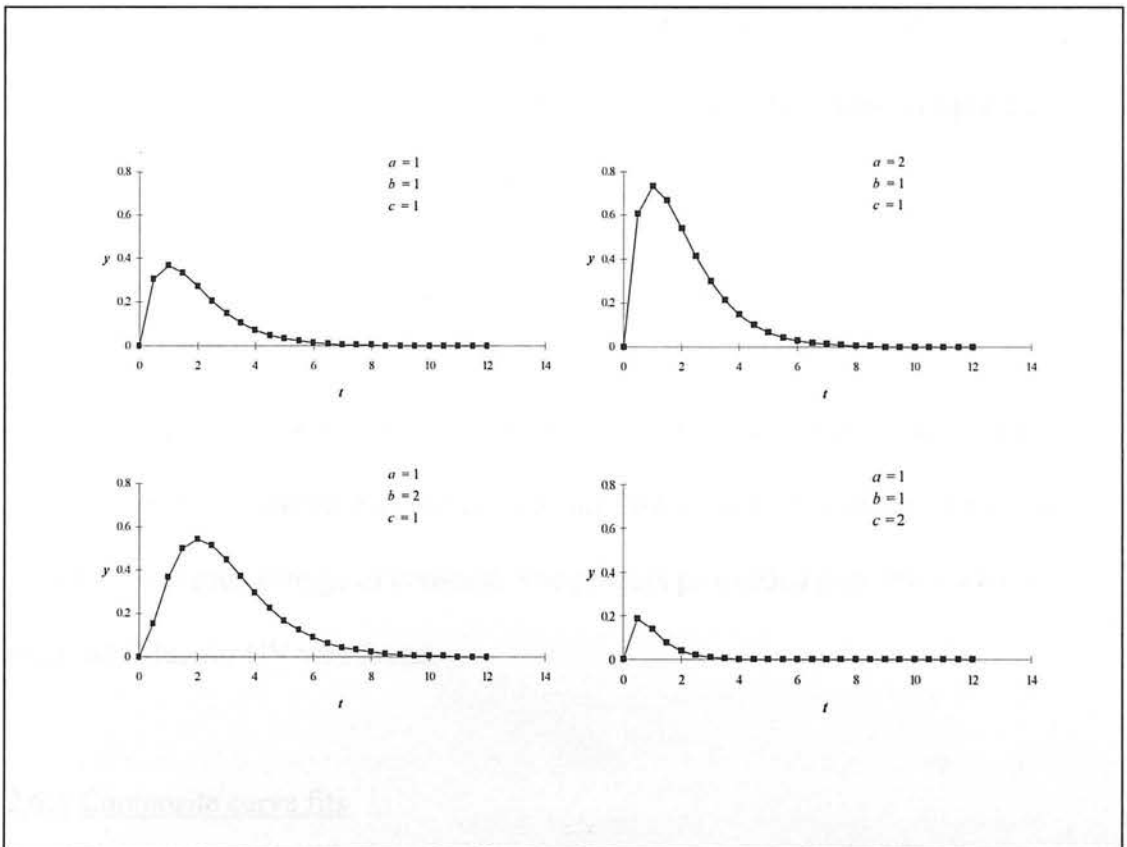


Figure 2a. Using simple values for the constants a , b and c , the effects of varying each is demonstrated.

2.6.2 Curve fitting methodology and software used

To account for intestinal passage, the curve was modified by introducing a time delay factor, d . Thus, t is replaced by $(t - d)$ and the equation becomes:

$$y = a(t - d)^b e^{-c(t-d)} \quad (200)$$

With a non-linear regression approach, using the Solver function of Microsoft Excel 95[®], values were found for the constant a , b , c and d (240). This function works by finding the minimum or maximum value of a cell that satisfies the linked constraints. In this case the value to be minimised was a coefficient of variation (CV), calculated as the square root of the sum of the squares of the difference between the y value for the raw data point (PDR) and the curve fit at each value of t (root mean square), expressed as a percentage of the mean PDR:

$$CV = \frac{\sqrt{\sum (y_{data} - y_{model})^2}}{(df - 1)} \times \frac{100}{\sum y_{data} / n}$$

where df is the number degrees of freedom, in this case four for the four variables.

The only initial constraint was that all constants were positive numbers. This was recalculated for each change of constant. The process proceeded iteratively until a minimal value for CV was found.

2.6.3 Composite curve fits

Many data plots suggested that a composite of two curves would give a better fit.

That is:

$$y = y_1 + y_2$$

where y_1 and y_2 take the same form as above, each with four constants a_1 , b_1 , c_1 and d_1 , and a_2 , b_2 , c_2 and d_2 respectively. Early curve fitting suggested that the down-

slopes of both curves were similar. Physiologically this was thought to be indicative of losses of $^{13}\text{CO}_2$ from the bicarbonate pool at a constant rate, and so the curve fit was further simplified by assuming a common value for c (200). The curve fitting method is shown schematically in figure 2b.

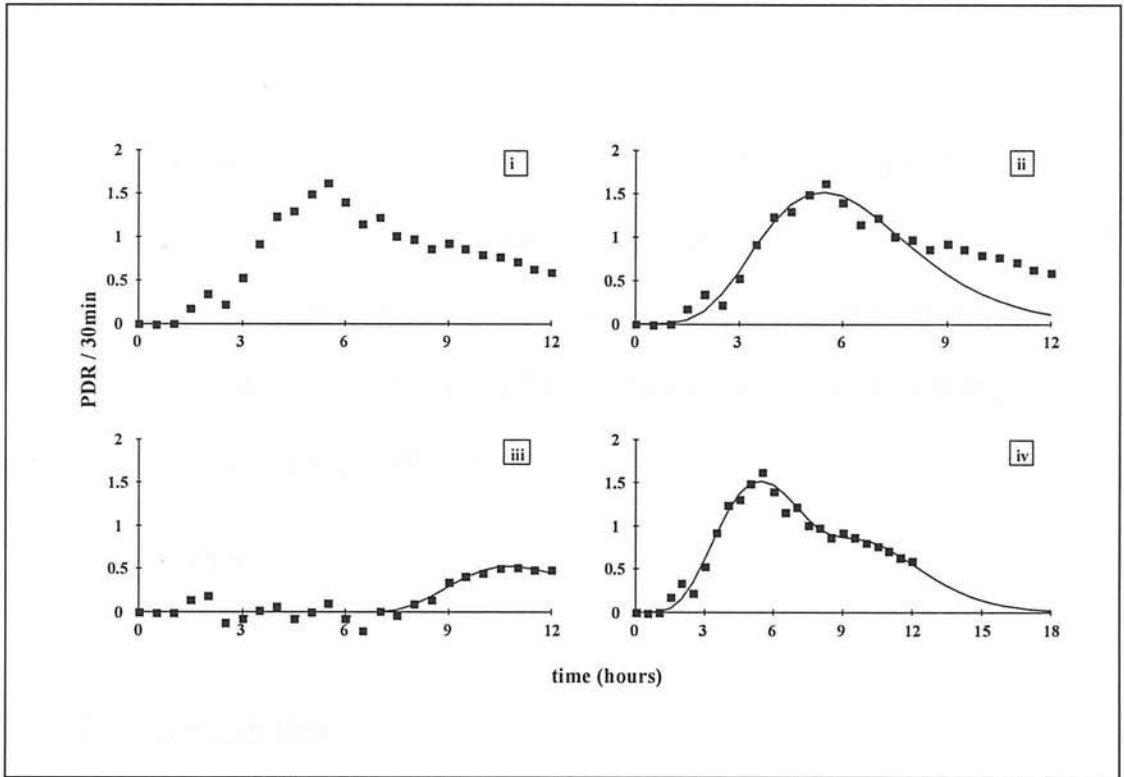


Figure 2b. A schematic illustration of the curve fitting method. Figure 2b(i) shows the PDR plot. A best-fit single curve is shown in figure 2b(ii), assumed to represent small intestinal digestion. If the single curve fit is subtracted from the original PDR plot, the residual, assumed to represent colonic fermentation, can be similarly curve fitted (figure 2b(iii)). The Solver[®] function was used to find the best fit for two curves simultaneously as is shown in figure 2b(iv) with the curve fit extrapolated to baseline at 18 h. Although this schema shows the two component curves being curve fitted in a step-wise fashion, in practice these were done simultaneously.

From the whole composite curve an overall CV with seven degrees of freedom was calculated. To compare the CV of different curves with variable magnitudes of PDR, it was necessary to scale the CV by a mean PDR value for the data set (241).

2.6.4 Analysis of curve fit data

Curve fitting in this way allowed several parameters to be calculated for each curve. The curve fit was extrapolated to baseline so that a cPDR at t_{∞} could be calculated for the whole curve, or whole gut. The first and second individual curve fits were assumed to represent small intestinal and colonic digestion respectively. For each individual curve, a cPDR was calculated by summing the individual PDRs using the trapezoidal method. Also the time taken to reach maximal enrichment (t_{\max}) was calculated from the curve parameters thus:

$$t_{\max} = d + \frac{b}{c} \quad (164)$$

2.6.5 Lactose ureide data

These were analysed both individually and in conjunction with the wheat flour results for the corresponding subject in order to define the time period of colonic fermentation.

2.7 Statistical analysis

Frequency diagrams of cPDR results approximated to a normal distribution and the single and composite approaches to curve fitting were compared using paired Student's t-tests. Individual parameters of each curve, such as cPDR and t_{\max} , could then be compared rather than amassing the individual time point data which is the

more valid statistical approach (242). Estimates of cPDR at t_{∞} , cPDRs for small intestine and colon and time to peak $^{13}\text{CO}_2$ recovery (t_{\max}) were expressed as mean \pm standard deviation (SD).

PART I: *IN VIVO* STUDY OF STARCH DIGESTION BY CURVE FITTING OF DATA DERIVED USING STABLE ISOTOPES

CHAPTER 3. PRELIMINARY EXPERIMENTS

In order to arrive at the methodology described in chapter 2 for the investigation of starch digestion, preliminary experiments were undertaken to determine the appropriate substrates, the suitable doses and administration, to validate methods of breath collection and to determine the appropriate sampling time. These preliminary experiments are described in this chapter. In addition, data sets were analysed qualitatively and early attempts to curve fit the data are described.

3.1 Study design pilot

3.1.1 Aims

The aims of this preliminary experiment were:

1. To determine the required substrate doses
2. To determine the necessary duration of sampling after substrate administration
3. To determine the optimum method for administration of the substrate
4. To describe and analyse qualitatively the shapes of the curves resulting from the data sets
5. To compare the lactose [^{13}C] ureide results with breath hydrogen data

3.1.2 Subjects, Methods and Substrates

The substrates were ^{13}C enriched wheat flour and lactose [^{13}C] ureide (^{13}C -LU). The subjects were three young children, MW (female, 10 months), CL (female, 16 months) and MC (male, 6 months). All had been breast fed for a minimum of three months and none had received antibiotics in the preceding 3 months. Ethical approval from the Yorkhill Ethical Committee was granted and informed parental consent was obtained.

Each infant was tested three times on three separate days and therefore acted as his/her own control. A baseline study was not carried out for MC. After an overnight fast a starchy breakfast was given. On the first study day, no substrate was given (baseline study). On the second study day, 100 mg ^{13}C enriched wheat flour was given with the breakfast. On the third study day, 100 mg of ^{13}C -LU was given similarly. The children were fed by spoon and after several spoonfuls of breakfast the ^{13}C -labelled flour was given on a single spoonful. The children were then allowed to eat the remainder of the rusk normally. Subsequent test meals were given after a minimum of one week to avoid contamination of $^{13}\text{CO}_2$ from previous studies. Three initial baseline breath samples spaced at 10 minute intervals were obtained prior to the test meal and thereafter every 20 minutes for 6 hours. The breath was collected using a bag and mask as described earlier. Samples were stored in evacuated tubes and analysed for $^{13}\text{CO}_2$ by CF-IRMS. Samples for H_2 analysis were stored in sealed syringes and analysed by electrochemical cell (GMI Renfrew). Both sets of results were expressed graphically with time.

3.1.3 Results

The results for each subject are shown graphically in figure 3a. The cumulative PDRs at 6 hours are shown in Table 3a.

In the two baseline studies the ^{13}C breath enrichment values varied between 0 and 17.2 ppme, and -15.0 and 7.6 ppme which was in keeping with previously published data (171,243).

Table 3a. cPDRs at 6 hours for each subject

subject	cPDR (%)	
	^{13}C wheat flour	^{13}C -LU
MW	12.3	1.4
CL	12.5	1.7
MC	11.7	0

^{13}C wheat flour produced maximal ^{13}C enrichments of 51.6, 43.9 and 53.3 ppme for, MW, CL and MC respectively. These peaks occurred between 140 and 160 minutes for MW and MC but for CL, the maximal value was the last value at 6 h, suggesting that higher results might have occurred later.

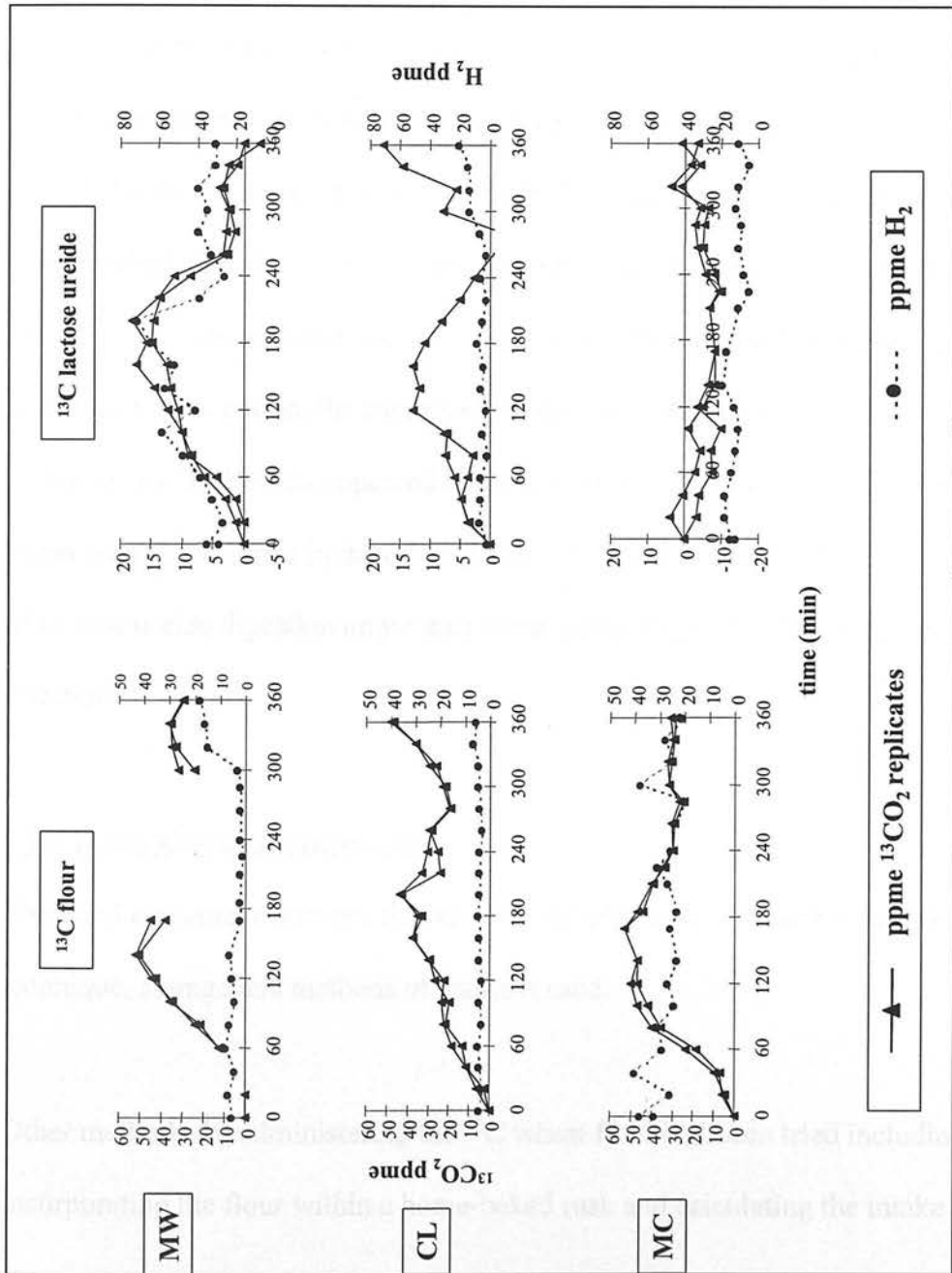


Figure 3a. For each subject, the results are shown graphically for ^{13}C flour and ^{13}C lactose ureide along with the H_2 concentration. $^{13}\text{CO}_2$ ppm is shown on the left hand axis and H_2 ppm on the right hand axis.

For MW, the suggestion of a second peak in the ^{13}C wheat flour curve was mirrored by a first increase in the breath H_2 concentration. This was not so for either MC (in whom there was a high breath H_2 concentration from the start of the study) or in CL (in whom there was no H_2 produced for the duration of the study).

For ^{13}C -LU, the maximal rises of $^{13}\text{CO}_2$ over baseline were 4.1, 12.9 and 18.1 ppme. For MW and CL these occurred at 200 and 360 minutes respectively. However, the $^{13}\text{CO}_2$ enrichment was not noticeably different from the baseline variation. For MC the ^{13}C enrichment was in fact negative for the duration of the study. For MW the maximal rise of 18.1 ppme was mirrored by increases in the H_2 concentrations but high baseline H_2 values did not make this a reliable marker of oro-caecal transit time. For CL there was an initial rise to 12.9 ppme by 160 minutes, but at the end of the 6 hour experiment period, the curve had begun to rise again and this rise was mirrored by a first rise in H_2 . This appeared to indicate bacterial metabolism in the colon rather than the first rise in the curve. However, it also suggested that a small amount of lactose ureide digestion might also occur before then, possibly in the small intestine.

3.1.4 Discussion and conclusions

The good agreement between the replicate samples endorsed the breath collection technique, storage and methods of analysis used.

Other methods for administering the ^{13}C wheat flour had been tried including incorporating the flour within a home-baked rusk and calculating the intake from the

remainder in the bowl and on the bib after breakfast. In these pilot experiments, the dose was administered by sprinkling onto an early spoon of softened rusk, or onto toast, depending upon the breakfast choice of the infant. Assumptions made about the proportion of the dose ingested were subjectively felt to be valid on observing the children eat the breakfast.

There was clear distinction between control and substrate with a dose of 100 mg wheat flour and therefore this dose of wheat flour was sufficient to produce measurable $^{13}\text{CO}_2$ breath enrichment. This clear distinction also indirectly validates the bag and mask methodology of breath collection.

The curve shapes for flour were similar. Each had an initial peak between 140 and 200 minutes. Qualitatively, each data set appeared to be more complex than a single curve. With MC and MW there was the suggestion of a second peak of lower magnitude around 300 minutes. However with MW there were data points missing due to poor CO_2 concentrations through sampling whilst sleeping. In the study with MW, the second peak co-incident with an increase in breath H_2 . These results suggested that starch digestion may not be a single process and resulted in the proposal of the hypothesis that colonic fermentation may contribute to starch digestion and may be amenable to measurement. Since the second rises in $^{13}\text{CO}_2$ occurred towards the end of the 6 h sampling period, a longer experimental period would be necessary for studying young children although the duration required was not clear. To test whether the sampling period required would be feasible in young

measurement was highlighted by this early finding. The concentration of CO_2 in

children, a prolonged pilot study was first undertaken in an adult subject (section 3.2).

There was not a clear distinction in ^{13}C breath enrichment between control and substrate for ^{13}C -LU for the duration of the studies and hence it was not possible to conclude what dose of ^{13}C -LU was required. As with the flour data, the rise in $^{13}\text{CO}_2$ enrichment seen towards the end of the 6 h period in the lactose ureide experiment with CL and mirrored by a rise in breath H_2 suggested that colonic activity was occurring outwith the timespan of the experiment and that a longer sampling period would be necessary. No digestion of the lactose ureide would have been expected in the small intestine and the significance of the observed curves in MW and CL was not certain.

The second rise that was seen at the end of the ^{13}C -LU experiments with CL was of a greater magnitude than the first peak. A small rise in breath H_2 mirrored the rise in $^{13}\text{CO}_2$ and this may have represented colonic fermentation, but a similar rise with the baseline experiment suggested the possibility of contamination by ^{13}C enriched food such as cane sugar in chocolate or sweets. In order to explain further possible contamination by ^{13}C enriched foods in this way, the need to keep a food diary during the duration of the study for further experiments was acknowledged and undertaken in all future experiments.

There was no discernible rise in H_2 in MC. Another practical difficulty in breath H_2 measurements was highlighted by this early finding. The concentration of CO_2 in

breath is known and so poor sampling of breath CO_2 , which leads to low concentrations, means the samples may be abandoned. With breath H_2 measurement, there is no normal range and a low concentration of breath H_2 may be true (little fermentation occurring) or false (poor sampling). With the definitive experiments there was no local facility to measure breath H_2 but with this consideration, the importance of simultaneous H_2 measurements was from thence devalued.

Studies of longer duration would require a technique for sampling breath whilst the children slept since poor CO_2 concentrations were obtained using bag and mask during sleep. A technique of sampling expired air whilst sleeping was evaluated in further pilot studies.

3.2 Prolonged ^{13}C starch breath test in an adult with ^{13}C -LU and baseline controls

3.2.1 Aims

This was a stable isotope breath test with breath sampling that was carried out in one adult subject over a 24 h period. Its aims were:

1. Using a prolonged sampling period which would not be possible in a child, to determine the sampling time necessary, and therefore to determine the feasibility of this study in children.
2. To study the baseline ^{13}C breath enrichment curve obtained under controlled conditions.
3. To produce data with which to pilot means of curve analysis.

3.2.2 Subjects, Methods and Substrates

Three breath tests were carried out in one healthy adult male (28 years, weight 90 kg), using the same substrates over a sampling period of 24 h. The experiment design was as described above, but with substrate doses of 400 mg ^{13}C wheat flour and 100 mg ^{13}C -LU. Foods rich in ^{13}C were avoided, and mealtimes and meal composition were standardised between the three days. Physical activity during the 24 h period was restricted in order that the variation in $V\text{CO}_2$ was minimised. The substrates were ingested with a starchy breakfast following the collection of three duplicate baseline samples over one hour. Subsequently, breath samples were collected every 30 minutes for 24 h and one further sample was collected at 30 h in two experiments. Breath was collected by blowing with a straw into non-evacuated, sealed glass tubes.

The data sets were analysed using a three-point smooth, a simple method of naked eye curve analysis. The average ^{13}C enrichment of expired CO_2 for each unit time t , $t - 1$ and $t + 1$ was calculated and then plotted at time t . This is an accepted means of studying trends in curves and has the effect of drawing out curves which may not be immediately apparent from the raw data.

Since experimental conditions were well-controlled, the data were also analysed using the data from the baseline study to act as a time-corresponding baseline for the two substrates.

3.2.3 Results

The three data sets are shown together in figure 3b. The baseline curve was negative for most of the 24 hours to a maximal negative value of -16.4 ppme at 11 h. The maximal $^{13}\text{CO}_2$ enrichments for flour and lactose ureide were 25.2 and 29.6 ppme respectively.

For wheat flour, there was a peak at 3 h with a long tail which reached baseline at 16 h but continued to fall, becoming negative to a maximum negative value of -14.8 ppme at 22.5 h. The peak for lactose ureide occurred at 8 h which was in the middle of the tail of the flour curve. There was a sharp rise to the peak, and a sharper tail than for wheat flour which reached baseline at 15 h.

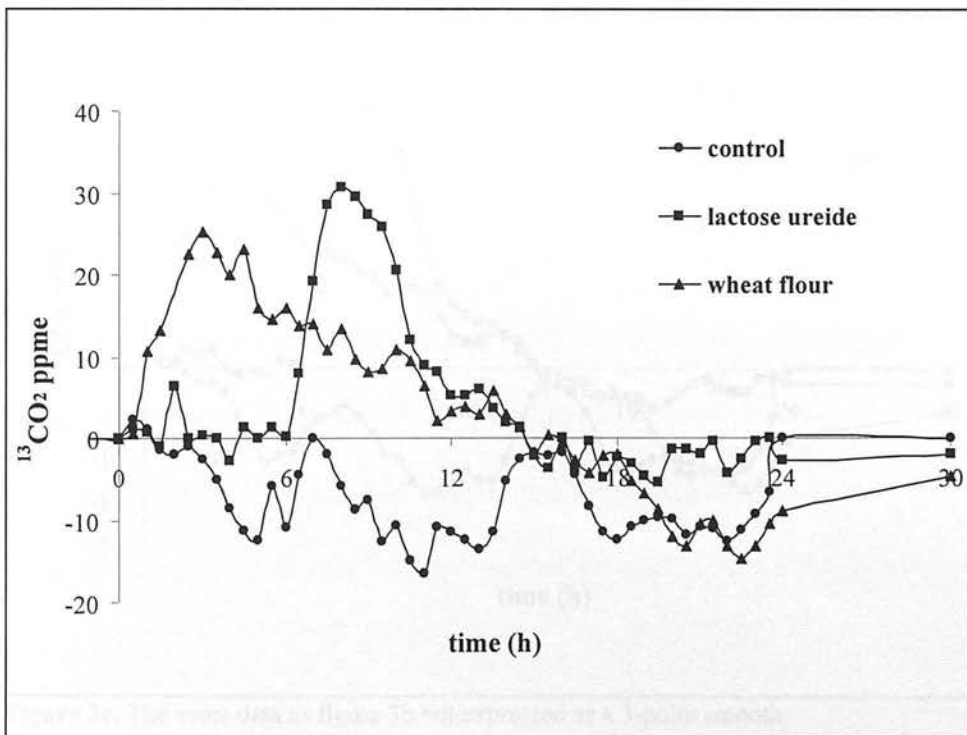


Figure 3b. $^{13}\text{CO}_2$ recovery over 30 h in a 28 year old male after ingestion of ^{13}C wheat flour, ^{13}C -LU and a control experiment

The three-point smoothed curves are shown in figure 3c. They appeared to tease out more than a single curve for the flour data. The timing of the rise to peak of the lactose ureide curve coincided with the middle of the long tail of the flour curve.

Figure 3d shows the data for lactose ureide and flour, corrected using the baseline data on a point by point basis as the substrate baseline. The three-point smoothed data was felt to be more valid for this analysis. One might argue that the ^{13}C wheat flour data set appeared to the naked eye to consist of two curves, with the second curve appearing at the same time as the lactose ureide curve (the peaks are 90 minutes apart).

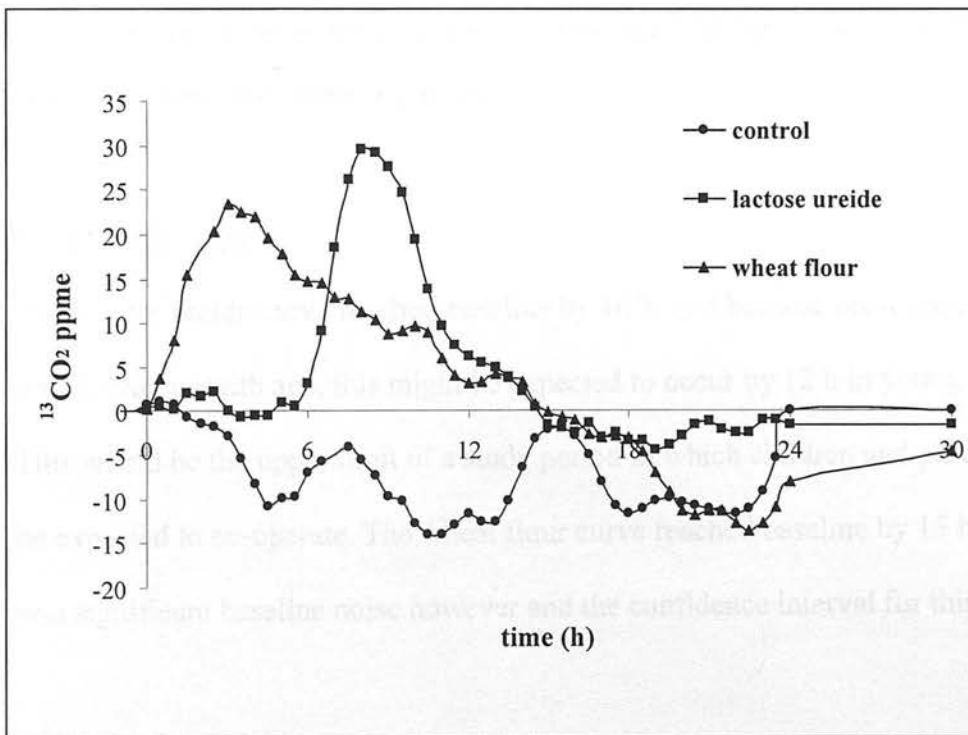


Figure 3c. The same data as figure 3b but expressed as a 3-point smooth.

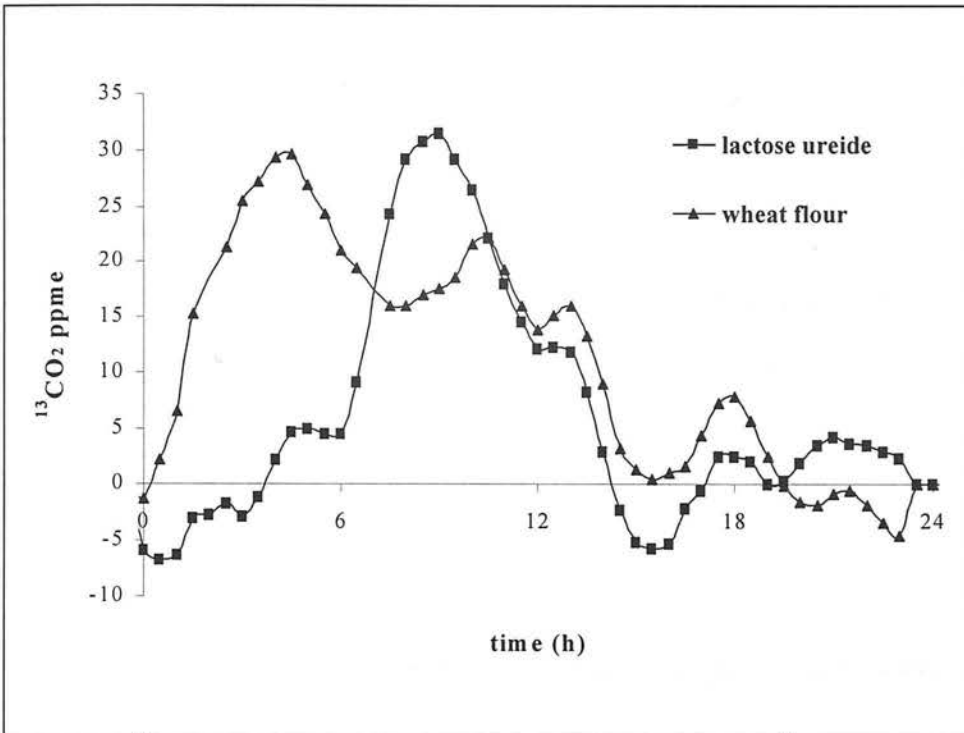


Figure 3d. $^{13}\text{CO}_2$ recovery in a 28 year old male after administration of ^{13}C wheat flour or ^{13}C -LU.

For each unit time, ppme has been calculated by subtracting $^{13}\text{CO}_2$ breath enrichment from the same time unit in a controlled baseline experiment.

3.2.4 Conclusions

The lactose ureide curve reached baseline by 16 h, and because oro-caecal transit time increases with age, this might be expected to occur by 12 h in young children. This would be the upper limit of a study period in which children and parents could be expected to co-operate. The wheat flour curve reached baseline by 15 h. There was significant baseline noise however and the confidence interval for this was wide.

The enrichment of the peaks for lactose ureide and flour were only a little over the baseline variation. The weight-adjusted doses of tracer were 4.4 mg/kg and 1.1 mg/kg for wheat flour and ^{13}C -LU respectively. Assuming a toddler weight of up to

15 kg, doses of 100 mg for both tracers would give a minimal weight-adjusted dose of 6.7 mg/kg and should result in an unambiguous rise over baseline.

The three-point smooth approach offers an attractive means of qualitatively analysing the data. It does show more clearly, for example, that the lactose ureide peak overlaps the long tail of the flour curve and suggests that this long tail may represent fermentation of the flour in the colon. However, this approach offers no numerical analysis.

The exercise in correcting substrate data on a point by point basis for the control data offers an alternative explanation of curve duration. The baseline fluctuations are difficult to explain and controlling experimental conditions would be very difficult in young children. A better approach lies in choosing sufficient substrate dose such that the baseline noise can be ignored. The baseline experiment alone is shown in figure 3e. Times of meals have been indicated. Following lunch and dinner there was an increase in the background enrichment of around 10 ppme. This may represent a change in metabolism from the utilisation of body fat stores which are known to be relatively less enriched in ^{13}C than most dietary starch. The magnitude of the change was small and within the expected baseline range, however it endorsed a need to keep dietary records during the experiments and for a tracer dose which would give $^{13}\text{CO}_2$ enrichment significantly above the baseline limits.

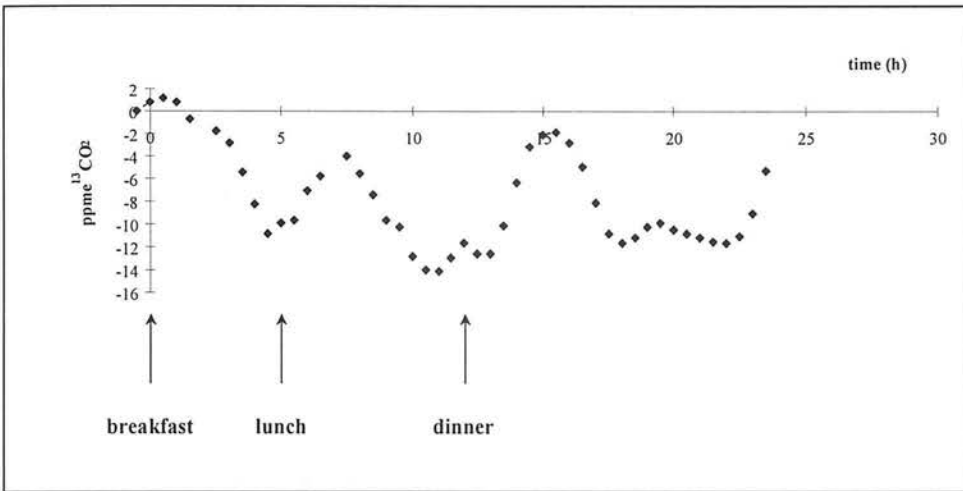


Figure 3e. Baseline data with mealtimes indicated.

3.3 Determination of length of sampling in young children and trial of breath collection by syringe aspiration

3.3.1 Aims

1. To determine if a 12 h sampling period is long enough for young children.
2. To trial an alternative method of breath collection whilst the child is sleeping

3.3.2 Subjects, Methods and Substrates

A 12 h breath test was carried out in two subjects as described above. The subjects were CL (female, 25 months) and NL (male 46 months). The substrate used was ^{13}C -LU, 100 mg (the pilot for wheat flour has been incorporated as the one of the data sets within the definitive experiments). Duplicate breath samples were taken 20 and 10 min before breakfast, and then every 30 min for 12 h. Methodology otherwise proceeded as described previously. When the children were awake these were taken as described before with a bag and mask. During periods of sleeping, breath was

collected using a piece of naso-gastric tubing cut to around 10 cm and attached to a 20 ml syringe. The tip of the naso-gastric tube was placed close to the child's nostril and the syringe was aspirated by approximately 0.5 ml with every expiratory breath. The timing of expiration was noted by chest movement and the collection of moisture at the end of the tube.

3.3.3 Results

These are shown in figure 3f.

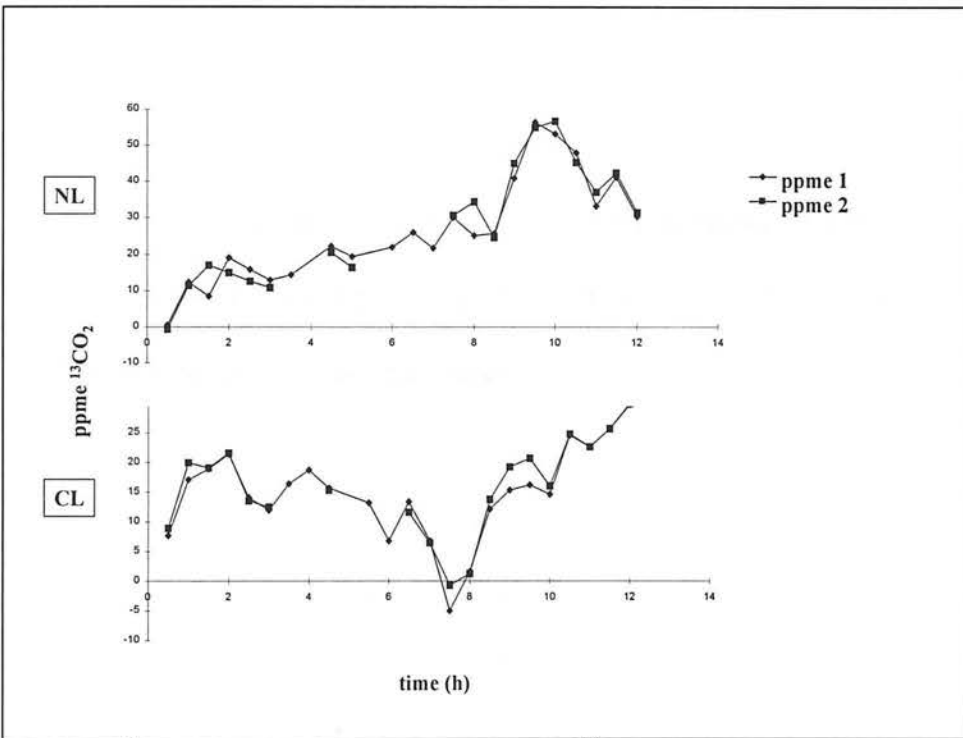


Figure 3f. ¹³CO₂ breath enrichment after ingestion of ¹³C-LU in two young children.

The CO₂ concentrations were assumed to be normally distributed. For CL the mean (standard deviation) was 2.0% (0.2%). The two naso-gastric samples had CO₂ concentrations of 2.0% and 2.8% and hence did not fall below the lower 95% confidence interval of the distribution. For NL, the mean (standard deviation) CO₂

concentration was 1.8% (0.3%) and the three naso-gastric samples were 2.6%, 2.0% and 3.4%. Moreover for both experiments, naso-gastrically collected breath samples seemed to produce better CO₂ concentration than bag and mask.

For NL there was a sustained rise in ¹³CO₂ from 3 h to a maximal ¹³C breath enrichment at around 10 h. For CL there appeared to be a small rise in ¹³C breath enrichment in the first 6 h, followed by a more sustained and greater magnitude of rise from 8 h which had not reached a maximal enrichment by the end of the 12 h study period

3.3.4 Conclusions

The naso-gastric tube method is a suitable alternative method for collecting expired CO₂ whilst a subject is sleeping. A period of 12 h may be sufficient to study lactose ¹³C ureide breath test in young children.

PART I: *IN VIVO* STUDY OF STARCH DIGESTION BY CURVE FITTING OF DATA DERIVED USING STABLE ISOTOPES

CHAPTER 4. RESULTS

This chapter documents the results to the definitive experiments whose methodology is described in chapter 2.

4.1 Subjects

Details of the children studied are shown in Table 4a.

4.2 Wheat Flour Experiments

4.2.1 Unadjusted raw data

Seventeen experiments were carried out in 13 subjects. In two subjects validation experiments were carried out (see 4.2.4). For the group analysis, only the first experiment with each child was used. In one subject, the test was carried out at 9 and 12 months. Both data sets have been included in the analysis. In one subject poor $^{13}\text{CO}_2$ enrichment over baseline was obtained using a substrate dose of 100 mg, so the test was repeated with with 200 mg wheat flour. Only the latter results have been included.

Table 4a. Subjects and experiments. The experiments were numbered by date so that the consecutive letters A – L indicated months January to December.

Subject	Sex	Age at first test (months)	Initial feeding of starch (months)	Introduction of starch (months)	wheat flour	lactose ureide	baseline
MT	M	21	Breast	6	97I01	97I02	
FR	M	11	Breast	6	97I03	97J01/98B01*	
GL	M	9	Breast	5	97J02	97K01	
MN	M	8	Breast	5	97K02	97K04	97L02
MR	M	7	Breast	5	97K03	97K05	
RU	M	16	Breast	5	97K06		
AU	M	9	Breast	6	97L01/98C01§	97L03	97L04
GR	M	15	Breast	6	98A01	98A02	
GP	F	15	Formula	6	98A03	98A04	98A06
AL	M	22	Mixed	6	98A05/98C02†	98B02	
AN	F	15	Breast	6	98C03		98C08
RI	M	7	Breast	5	98C04/98C07‡		
EL	F	9	Breast	4		98C05	
CH	F	7	Breast	6	98E01/98E02‡		

* Lactose ureide test repeated at 15 months

§ Wheat flour test repeated at 12 months

† Poor enrichment data from 100mg wheat flour in 98A05, therefore repeated with 200 mg in 98C02

‡ Validation studies

The 14 experiments included for the ^{13}C wheat flour analysis are shown in table 4b. Following ingestion of a dose of 100 mg ^{13}C -enriched wheat flour, the mean \pm SD maximal rise of $^{13}\text{CO}_2$ enrichment over baseline was 43.4 ± 19.0 ppme, and following a dose of 200 mg it was 80.9 ± 24.5 ppme. Using a constant value of basal $\dot{V}\text{CO}_2$ predicted from body surface area (239) throughout the test, the mean \pm SD cPDR over 12 h from the calculated data was $21.3 \pm 8.4\%$. Mean \pm SD t_{max} was 3.9 ± 0.9 h.

Table 4b. Subjects for the wheat flour experiments

subject	experiment	substrate dose (mg)	maximum ppme
MT	97I01	100	71.7
FR	97I03	100	41.8
GL	97J02	100	54.0
MN	97K02	100	36.5
MR	97K03	100	23.4
RU	97K06	100	28.5
AU	97L01	100	73.4
GR	98A01	100	24.1
GP	98A03	100	37.3
AU	98C01	200	67.0
AL	98C02	200	45.9
AN	98C03	200	97.6
RI	98C04	200	108.3
CH	98E01	200	63.2

4.2.2 Adjusted raw data

Children were not at rest throughout the 12 h duration of the test and an attempt to correct for physical activity level (PAL) was made. For each 30 min period whilst the

child was awake, the PDR was increased by a factor of 1.4 (244). The mean \pm SD cPDR over 12 h of the adjusted data was $26.5 \pm 11.6\%$.

4.2.3 Curve fitting

4.2.3.1 single versus composite curve fit

Precision of curve fitting was quantified by the co-efficient of variation (CV) as described in section 2.6.3. The CV for each fit, for each subject is shown in table 4c. The CV for the composite two-curve fit, was significantly lower than the CV for a single curve when compared using paired Student's t-tests ($p = 0.002$ and 0.001 for unadjusted and adjusted data respectively) and so the composite fit was adopted for further analyses.

4.2.3.2 cPDR at 12 h

The cPDR at 12 h calculated from the raw data compared favourably with the cPDR at 12 h estimated from the curve fit (figure 4a). For unadjusted data the mean \pm SD cPDR for raw data was $21.3 \pm 8.4\%$ as compared to $21.2 \pm 8.1\%$ for curve fitted data; and for adjusted data the figures were $26.5 \pm 11.6\%$ and $28.5 \pm 10.8\%$ respectively.

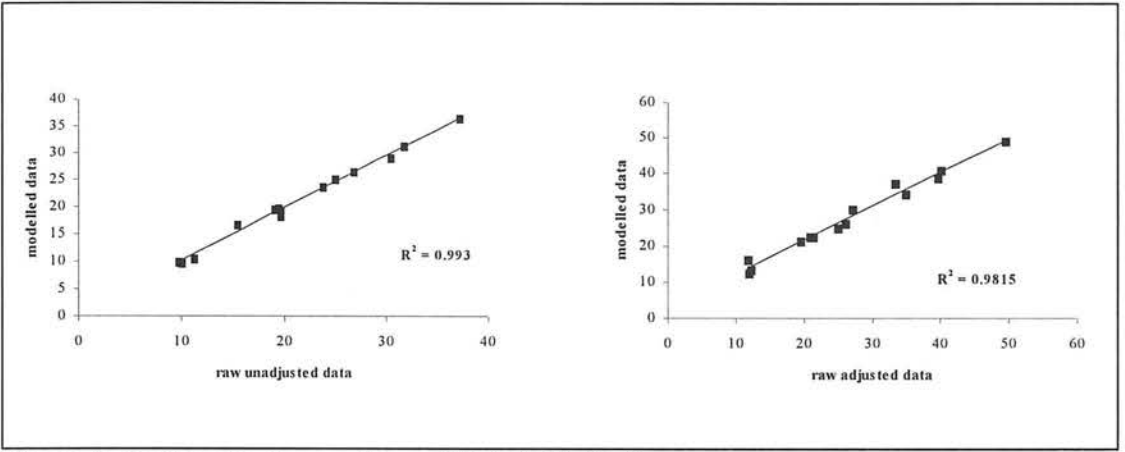


Figure 4a. Scatter plot of raw and curve fitted cPDR at 12 h.

4.2.3.3 cPDR extrapolated to baseline

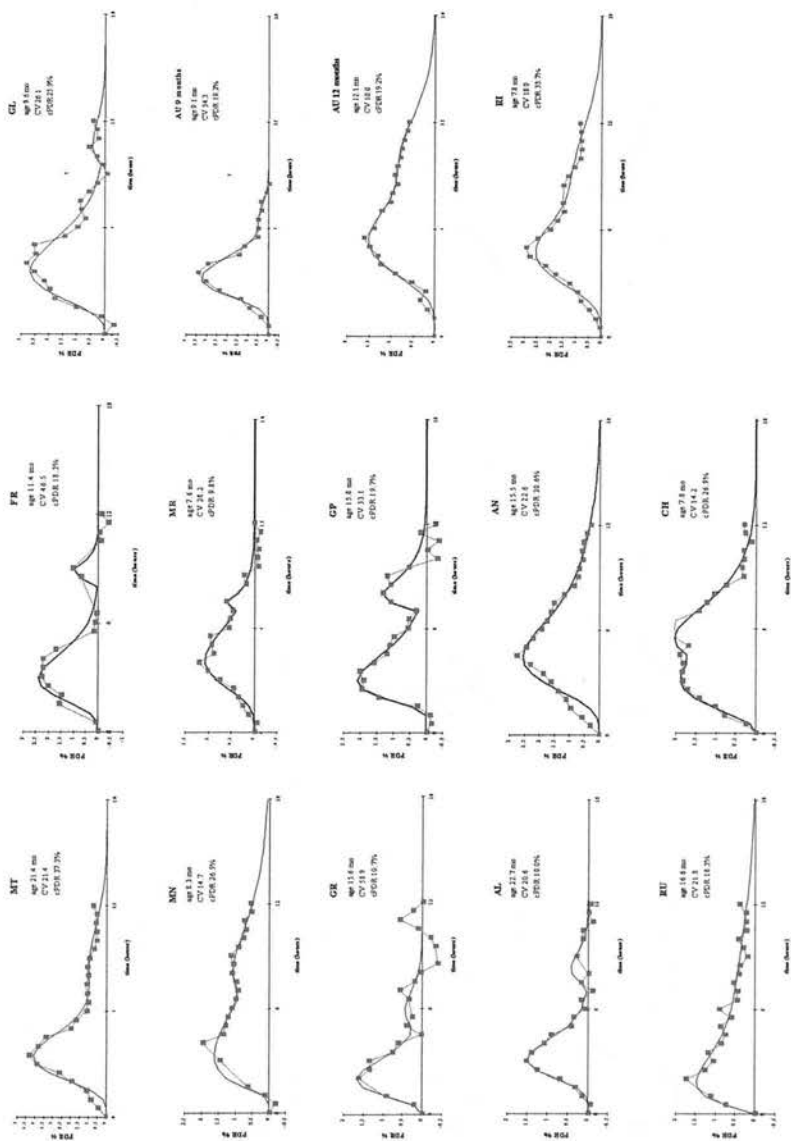
The curves were extrapolated to baseline, which gave estimates of mean \pm SD cPDR at t_{∞} of $22.1 \pm 8.6\%$ and $29.1 \pm 11.8\%$ for unadjusted and adjusted data respectively.

The individual data sets and curve fits are shown in figure 4b for unadjusted data and figure 4c for adjusted data. To compare curve shapes, the curve fits for both unadjusted and adjusted data are shown together in figure 4d.

Table 4c. Coefficients of variation (CV) for precision of curve fitting a single versus a composite two-curve fit

experiment	Coefficient of variation					
	unadjusted data			adjusted data		
	single curve	composite 2-curve	difference	single curve	composite 2-curve	difference
97I01	37.2	21.4	15.9	30.1	20.6	9.5
97I03	65.5	46.5	19.1	53.2	38.1	15.1
97J02	29.6	26.1	3.5	30.7	24.0	6.7
97K02	25.6	14.7	10.8	31.7	15.1	16.6
97K03	30.7	28.2	2.5	30.1	30.7	-0.5
97L01	36.3	34.3	2.0	42.9	46.5	-3.5
98A01	70.7	58.9	11.8	65.6	56.7	8.9
98A03	62.8	33.1	29.7	59.1	39.1	19.9
98C01	16.7	10.0	6.7	17.7	14.1	3.6
98C02	33.4	20.6	12.7	27.5	21.5	6.0
98C03	15.6	22.6	-7.0	21.0	22.1	-1.0
98E01	20.7	14.2	6.5	31.2	13.0	18.1
98C04	21.6	18.0	3.6	27.3	23.5	3.7
97K06	22.8	21.8	1.1	22.3	17.9	4.4
98E02	8.3	6.9	1.4	11.5	11.8	-0.3
98C07	23.3	6.0	17.3	22.6	9.2	13.4

Figure 4b. Graphs of raw data and modelled curves for each individual subject. Here the data that has not been adjusted for physical activity is shown. With each data set is shown the age of the child and the cPDR. In addition, the CV is shown to illustrate the meaning of the curve fitting parameter.



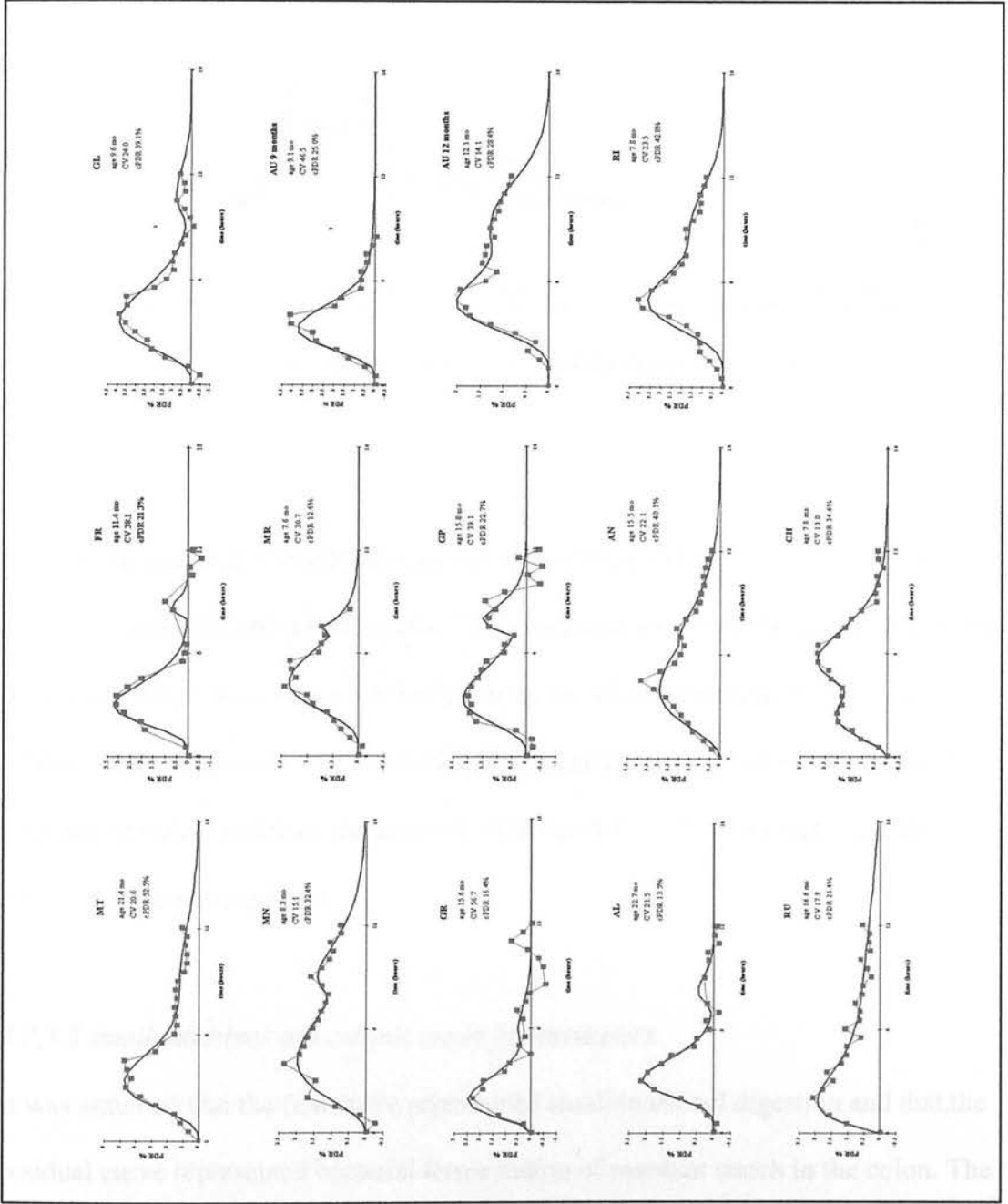


Figure 4c. Graphs of raw data and modelled curves for each individual subject. Here the data that has been adjusted for physical activity is shown. With each data set is shown the age of the child and the cPDR. In addition, the CV is shown to illustrate the meaning of the curve

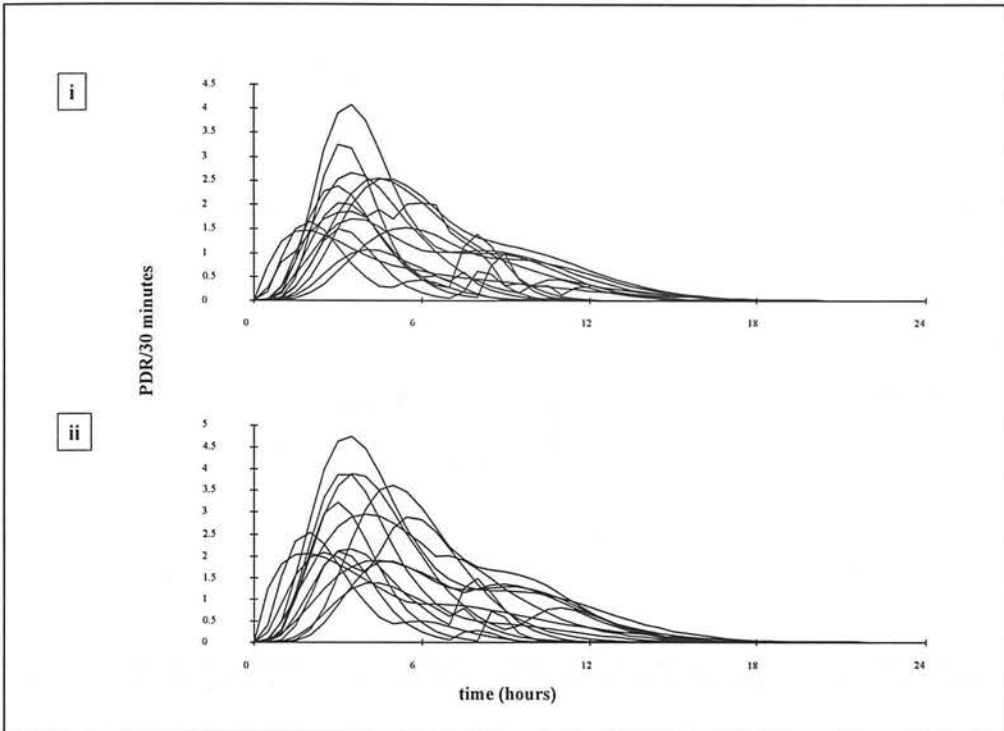


Figure 4d. The composite curve fits for all subjects are shown together. In part i, the unadjusted data is shown and in part ii, the data has been corrected for physical activity prior to curve fitting.

4.2.3.4 t_{max}

The time to maximal enrichment (t_{max}) calculated from the raw data was 3.9 ± 0.9 h for both unadjusted and adjusted data. This compared well with the t_{max} derived from the curve fitting which was 3.4 ± 1.0 h (values for adjusted data were the same). This is illustrated in figure 4e. It was not possible to calculate a t_{max} for the second peak from the raw data, but from the curve fit, this was 8.5 ± 1.6 h for unadjusted data and 8.6 ± 1.4 h for adjusted data.

4.2.3.5 *small intestinal and colonic curve fit parameters*

It was assumed that the first curve represented small intestinal digestion and that the residual curve represented bacterial fermentation of resistant starch in the colon. The

component cPDR, the percentage of the total cPDR and the t_{\max} for small intestinal and colonic curves are shown in table 4d.

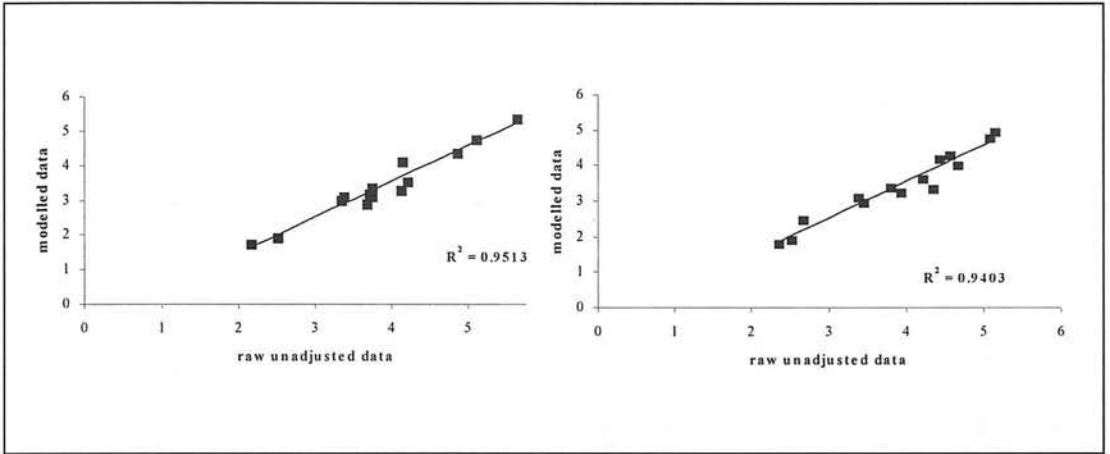


Figure 4e. Scatter plot of t_{\max} calculated from raw and curve fitted data. The data unadjusted for physical activity is shown on the left and that corrected for physical activity is shown on the right.

Table 4d. Percentage of cPDR, the percentage of the total cPDR and the t_{\max} for small intestinal and colonic curves

Site	Age (years)	CPDR (%)	CPDR (%)	t_{\max} (h)
SI	31.6	9.8	12.6	1.8
SI	31.8	26.9	26.6	1.8
SI	31.8	31.7	12.8	1.8
SI	31.3	26.5	32.6	1.8
SI	31.1	19.2	25.0	1.8
SI	31.6	23.8	39.1	2.5
SI	31.4	18.3	21.8	1.8
SI	31.1	21.9	28.4	1.8
SI	31.5	36.6	40.1	1.8
SI	31.6	16.7	16.4	1.8
SI	31.6	16.7	22.7	1.8
SI	31.6	18.3	25.4	1.8
SI	31.4	37.3	52.5	2.5
SI	31.7	10.9	11.3	1.8
SI	31.1	21.1	29.1	1.8
SI	31.6	8.6	11.4	1.8

SI: Small Intestinal; CPDR: Component of Percentage of Total cPDR; t_{\max} : Time to Maximum

Table 4d. Results of cPDR and t_{max} for the whole gut and small intestinal and colonic components

age (mo)	WHOLE GUT				SMALL INTESTINE				COLON					
	cPDR		t_{max} (h)		cPDR		t_{max} (h)		cPDR		t_{max} (h)			
	UA	A	UA	A	UA	A	UA	A	UA	A	UA	A		
MR	9.8	12.6	9.2	12.0	94	96	4.1	4.3	0.6	0.5	6	4	7.3	7.2
CH	26.9	34.6	18.7	16.6	70	48	3.1	2.5	8.2	18.0	30	52	6.1	6.0
RI	33.7	42.8	25.8	32.1	76	75	4.8	4.9	7.9	10.6	24	25	10.3	9.9
MN	26.5	32.4	19.8	25.6	75	79	3.3	4.2	6.7	6.8	25	21	9.1	9.6
AU*	19.2	25.0	18.0	24.0	94	96	3.2	3.2	1.2	1.0	6	4	6.7	8.4
GL	25.9	39.1	23.8	34.1	92	87	3.5	3.6	2.2	5.0	8	13	10.8	11.0
FR	18.3	21.3	15.9	19.6	87	92	2.9	3.0	2.5	1.6	13	8	8.6	8.5
AU*	21.9	28.4	16.3	16.9	74	59	5.4	4.8	5.6	11.6	26	41	10.8	9.8
AN	30.6	40.1	25.0	37.1	82	92	4.4	4.0	5.6	3.0	18	8	9.5	8.0
GR	10.7	16.4	8.8	14.3	83	87	1.9	1.9	1.8	2.1	17	13	6.1	6.3
GP	19.7	22.7	14.3	17.5	73	77	3.0	3.4	5.3	5.2	27	23	7.9	7.9
RU	18.3	25.4	12.6	17.8	69	70	1.8	1.8	5.6	7.6	31	30	8.3	9.6
MT	37.3	52.5	28.3	38.6	76	74	3.4	3.3	9.0	13.9	24	26	8.9	9.8
AL	10.0	13.5	8.3	11.8	84	87	3.1	3.1	1.6	1.8	16	13	8.3	8.3
mean	22.1	29.1	17.5	22.7	80	80	3.4	3.4	4.6	6.3	20	20	8.5	8.6
SD	8.6	11.8	6.5	9.3	9	14	1.0	1.0	2.9	5.4	9	14	1.6	1.4

* AU was tested twice at 9.1 and 12.1 months

Table 4e. Controlling of conditions for reproducibility studies

time (h)	RI		CH	
	98C04	98C07	98E01	98E02
	12/03/98	17/03/98	18/05/98	29/05/98
0.00	milk	milk	breakfast	breakfast
0.30				
1.00				
1.30				
2.00				
2.30	sample missed	sleeping	sleeping	
3.00		sleeping	sleeping	lunch
3.30	sleeping	sleeping	sleeping	
4.00	pureed fruit	pear	sleeping	
4.30			lunch	sleeping
5.00	lunch	lunch		sleeping
5.30				
6.00		stool		stool
6.30				
7.00				yoghurt, bread
7.30		sleeping	yoghurt, apple	
8.00	sleeping	sleeping		
8.30	yoghurt, stool		stool	
9.00		yoghurt		
9.30		stool		apple
10.00				
10.30				
11.00				
11.30		sleeping	pizza	
12.00	sleeping	sleeping		pizza
lunch	meat, pasta, cheese, olive oil	meat, pasta, cheese, olive oil	pasta, cheese, bread, apple	pasta, cheese, bread, strawberries

Table 4e. Controlling of conditions for reproducibility studies

	RI		CH	
time (h)	98C04	98C07	98E01	98E02
	12/03/98	17/03/98	18/05/98	29/05/98
0.00	milk	milk	breakfast	breakfast
0.30				
1.00				
1.30				
2.00				
2.30	sample missed	sleeping	sleeping	
3.00		sleeping	sleeping	lunch
3.30	sleeping	sleeping	sleeping	
4.00	pureed fruit	pear	sleeping	
4.30			lunch	sleeping
5.00	lunch	lunch		sleeping
5.30				
6.00		stool		stool
6.30				
7.00				yoghurt, bread
7.30		sleeping	yoghurt, apple	
8.00	sleeping	sleeping		
8.30	yoghurt, stool		stool	
9.00		yoghurt		
9.30		stool		apple
10.00				
10.30				
11.00				
11.30		sleeping	pizza	
12.00	sleeping	sleeping		pizza
lunch	meat, pasta, cheese, olive oil	meat, pasta, cheese, olive oil	pasta, cheese, bread, apple	pasta, cheese, bread, strawberries

Table 4f. Comparison of curve parameters for reproducibility studies

subject	test	unadjusted data						adjusted data					
		cPDR		% of whole gut		t _{max}		cPDR		% of whole gut		t _{max}	
		whole	gut	small intestine	colon	small intestine	colon	whole	gut	small intestine	colon	small intestine	colon
RI	98C04	33.7	76.5	23.5	4.8	10.3	42.8	75.2	24.8	4.9	9.9		
MA	98C07	37.8	70.0	30.0	4.2	10.5	52.5	68.5	31.5	4.3	11.1		
CH	98E01	26.9	69.5	30.5	3.1	6.1	34.6	47.9	52.1	2.5	6.0		
AU	98E02	23.1	44.1	55.9	1.7	3.8	31.5	90.7	9.3	2.3	3.2		

Table 4g. ¹³C-LU subjects and experiments.

subject	experiment	age	weaning age (months)	feeding	sex	min ppme	max ppme	raw data	cPDR
MT	97I02	21.6	6	BF	m	-1.3	112.1	27.4	
FR	97J01	11.8	6	BF	m	-13.0	30.8	9.7	
FR	98B01	15.6	6	BF	m	-6.4	30.9	8.4	
GL	97K01	10.0	4	BF	m	-3.4	34.1	10.2	
MA	97K04	8.6	5	BF	m	-5.3	19.1	5.3	
MR	97K05	7.9	5	BF	m	-17.3	0.6	0.0	
AU	97L03c	9.3	6	BF	m	-4.9	152.8	22.4	
GR	98A02	15.8	4	BF	m	-4.5	89.6	25.5	
GP	98A04	15.9	6	FF	f	-6.9	26.8	5.8	
AL	98B02	21.8	6.5	FF/BF	m	6.2	22.5	13.1	
EL	98C05	9.9	4	BF	f	-0.8	109.5	20.5	

BF = breast fed; FF = formula fed

4.3 Lactose ureide experiments

Eleven lactose ureide experiments were carried out in ten infants. In one infant the test was carried out at 11.8 and 15.6 months of age. The subjects and experiment numbers are shown in table 4g. All subjects received the same 100 mg dose of ^{13}C -LU. The median (range) age of the children was 11.8 (7.9 – 21.8 mo).

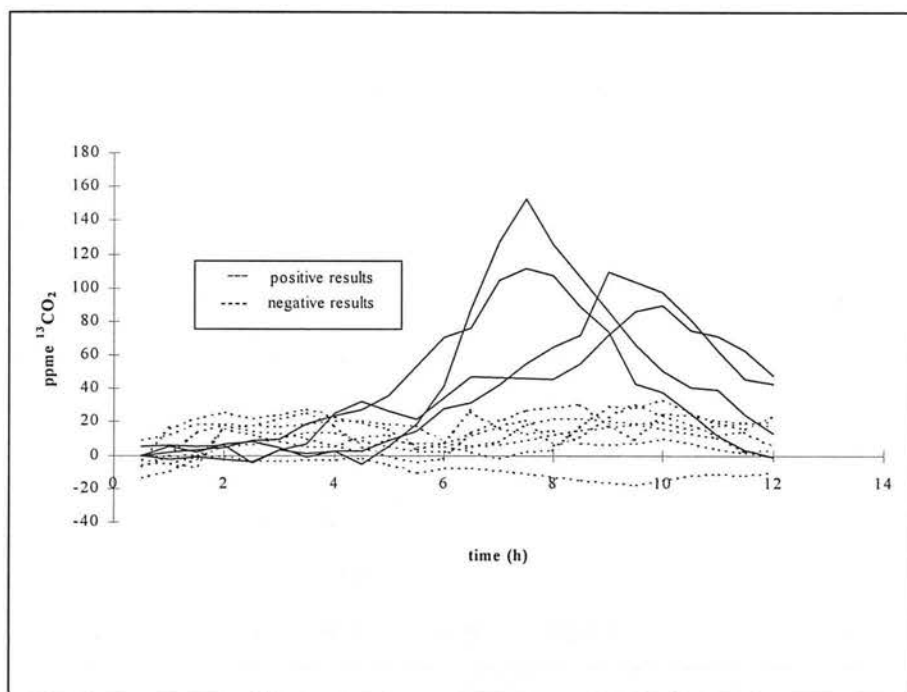


Figure 4g. Results for ^{13}C -LU showing positive and negative studies.

4.3.1 Dichotomous pattern of results

Not all children exhibited a clear $^{13}\text{CO}_2$ rise over baseline. Some children responded with a clear ppme rise over baseline (median: 111 ppme; range: 90 - 153 ppme) whilst in others, the $^{13}\text{CO}_2$ rise fell barely outside the expected baseline results (median 27 ppme; range 1 - 34 ppme) (Figure 4g). The median (range) age of the children who had a positive result was 12.8 months (9.3 -21.6) and the age of the

children who had a negative result was 11.8 (7.9 - 21.8). The sample size is too small and the range too large to make any inference from this.

4.3.2 Positive results

The median (range) cPDR of the children with the positive results was 24 (20 - 27)% for data unadjusted for physical activity (as described in section 4.2.2) and 31 (27 - 35)% for adjusted data. The median (range) first derivative-calculated t_{\max} was 8.5 (7.5 - 9.8) h for unadjusted data and 8.7 (7.7 - 9.8) h for adjusted data. The values for all children with positive results are shown in table 4h.

Table 4h. Positive ^{13}C -LU results

Subject	Sex	Age (mo)	min ppme	max ppme	unadjusted data		adjusted data	
					cPDR	t_{\max}	cPDR	t_{\max}
MT	m	21.6	-1.3	112.1	27.4	7.6	35.0	7.9
AU	m	9.3	-4.9	152.8	22.4	7.5	28.4	7.7
GR	m	15.8	-4.5	89.6	25.5	9.8	34.3	9.8
EL	f	9.9	-0.8	109.5	20.5	9.4	27.2	9.4

4.3.4 Wheat flour with positive lactose ureide

Figure 4h shows the individual results for wheat flour and lactose ureide for the three subjects who had wheat flour and lactose ureide tests and had positive results for lactose ureide.

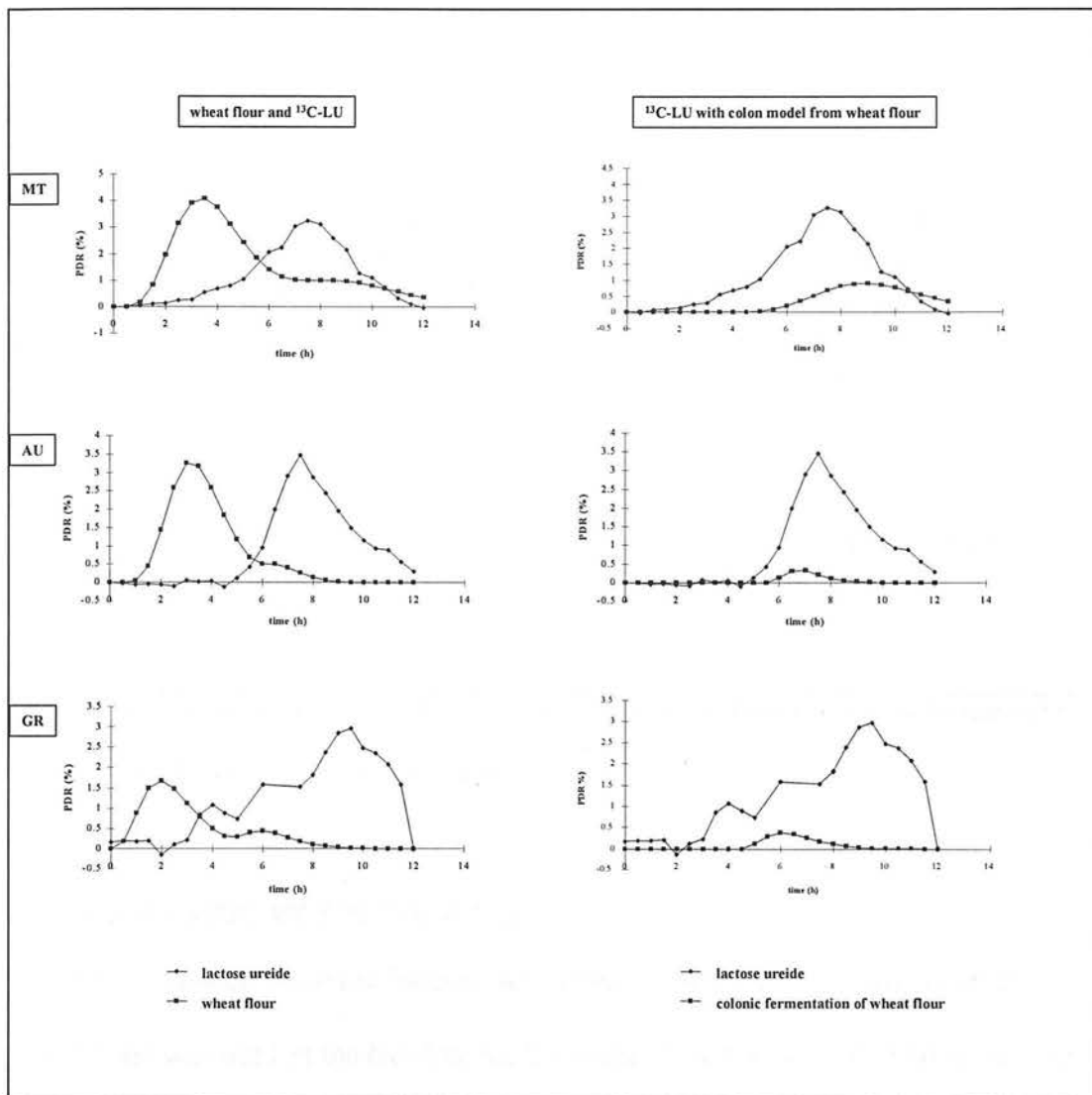


Figure 4h. The whole gut and colon component of the curve fitted wheat flour data is shown with the corresponding $^{13}\text{C-LU}$ test (shown in raw data form). Only curves for the unadjusted data is shown.

4.4 Baseline results

4.4.1 Baseline results in isolation

Baseline tests were carried out in four subjects. The ^{13}C abundance of breath CO_2 varied between 22.5 and -13.4 ppme. All four baselines are shown together in figure 4i and for each individual subject, the baseline is shown together with the wheat flour data in figure 4j.

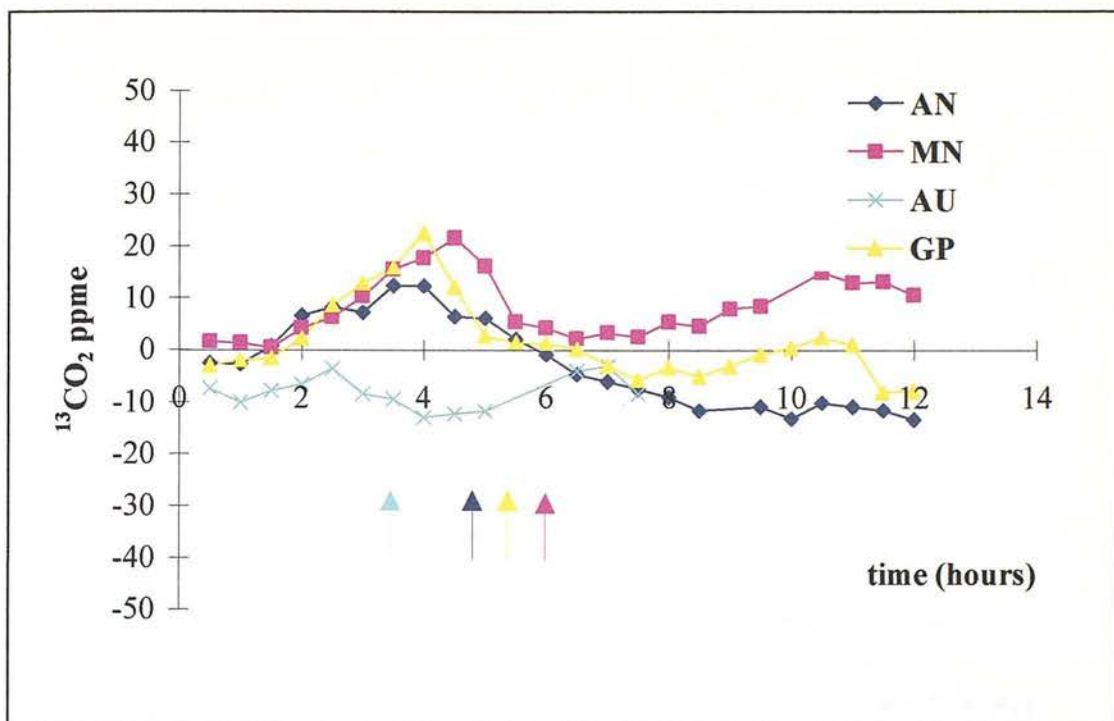


Figure 4i. Baseline studies for the four subjects in whom it was undertaken. The arrow indicates the time of the mid-day meal for the corresponding subject.

4.4.2 Baseline-corrected wheat flour results

For the four subjects in whom baseline and wheat flour tests were carried out, the baseline test was used as the baseline for the wheat flour test on a time point by time point basis, rather than using the two sets of duplicate samples that were taken before breakfast. The results are shown graphically in figure 4k which compares the two curve fits. The parameters for the raw data sets and the curve fits are shown in table 4i.

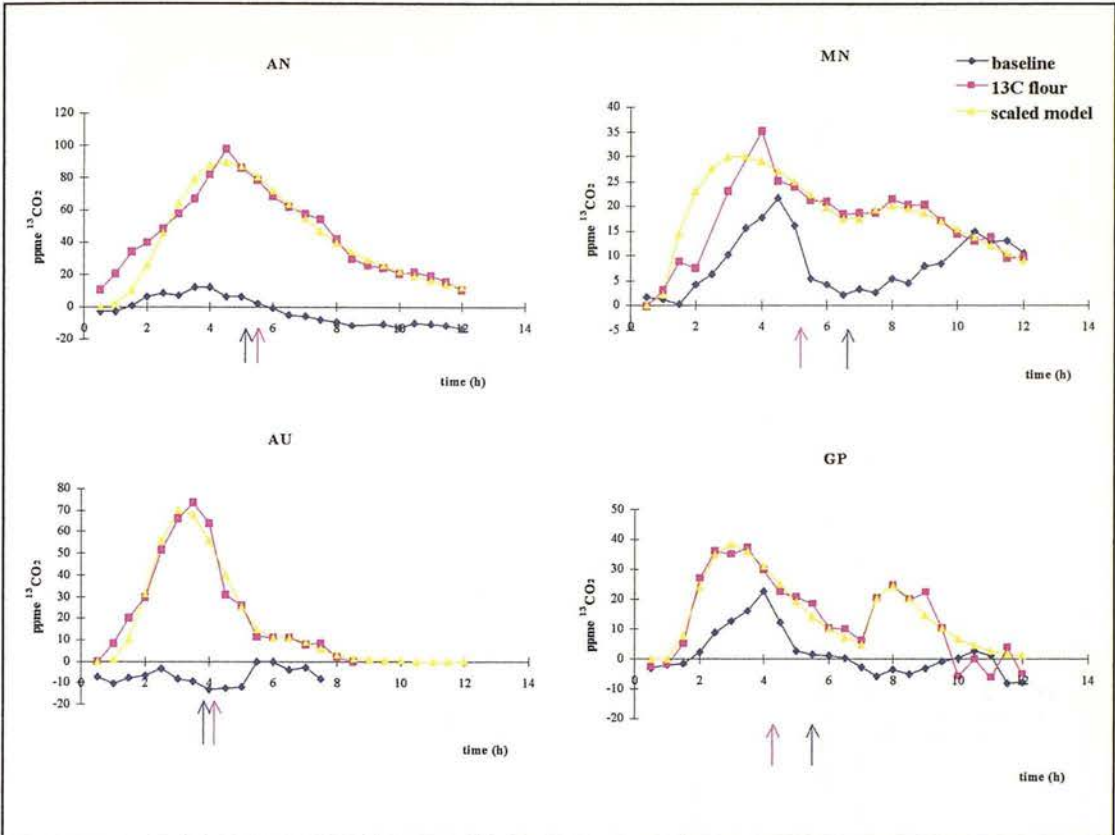


Figure 4j. Baseline studies shown with the ^{13}C wheat flour studies for the corresponding subjects. The

arrows indicate the mid-day meals for the corresponding study.

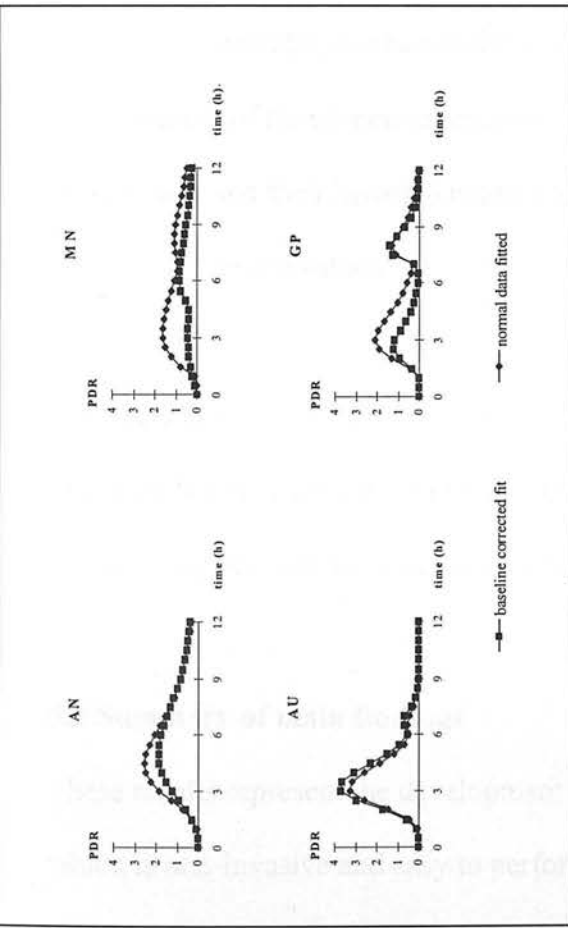


Figure 4k. ^{13}C wheat flour studies shown as point by point baseline correctd fitted data.

Table 4i. Comparison of curve parameters for normal and baseline-corrected data

	raw data			curve fitted data		
	12 h cPDR			cPDR		
	normal data	baseline corrected	normal data	baseline corrected	normal data	baseline corrected
MN	23.8	13.9	26.5	12.7	75	52
GP	19.1	12.6	19.7	11.5	73	53
AU	28.3	23.8	19.2	22.9	94	94
AN	30.4	24.3	30.6	31.4	82	79

PART I: *IN VIVO* STUDY OF STARCH DIGESTION BY CURVE FITTING OF DATA DERIVED USING STABLE ISOTOPES

CHAPTER 5. DISCUSSION

5.1 Introduction

An effective and simple means of measuring starch digestion in young children *in vivo* has long eluded researchers. Measurement of duodenal enzyme concentrations (60) gives only indirect evidence about digestion and is of limited value in the light of Hiele and co-workers (42) findings that the hydrolysis works by saturation kinetics, reaching maximal activity at low levels of enzyme.

More direct evidence can be obtained from functional studies, such as the measurement of the glycemic response to starch (89,176) but the results are inconsistent and their invasive nature has led to their abandonment for widespread use in children and infants.

Balance studies (50) are non-invasive and provide information about whole gut digestion but are time consuming and require precise calculations. As with the above studies, they also cannot separate digestion in the upper and lower gut.

5.2 Summary of main findings

These results represent the development of a means of measuring starch digestion which is non-invasive and easy to perform. Despite the long duration of the tests,

breath collection over 12 h with a bag and mask was well tolerated by young children. Wheat flour artificially enriched to 3.4 At% ^{13}C was sufficient to generate clear peaks of $^{13}\text{CO}_2$ enrichment over baseline when used in doses of 200 mg (equivalent to approximately 15-20 mg/kg body weight). All subjects were able to digest, absorb and oxidise wheat flour. The mean 12 h cPDRs of 21.3% and 26.5% for unadjusted and adjusted data respectively were comparable with those obtained from other shorter duration stable isotope studies in children and adults (42,148). The shapes of the raw data sets were variable and mathematical curve fits provided a means of quantitative analysis. A composite two-curve fit produced a significantly closer fit than a single curve. Making assumptions about the physiological basis for two curves, it was possible to deconvolve the individual curves attributable to small intestinal digestion and colonic bacterial fermentation. In some cases two curves were not immediately apparent but curve-fitting from a computer program was still often able to produce results that were physiologically plausible and consistent. The curve fits allowed for the calculation of parameters to describe the individual component curves, such as the individual cPDR and the t_{max} . The contribution of the colonic component was estimated to be up to 20% in children of this age group.

5.3 Previous studies of starch digestion using stable isotope breath tests

Stable isotope breath tests have been used before in studies of starch digestion in healthy young children (147,171) and have shown that even young infants are capable of digesting and absorbing starch.

5.3.1 Test duration

Most such studies have lasted for up to 6 h. Weaver and co-workers (159) noted the enriched CO₂ that was still present at the end of their 6 h tests, but felt it was unfair to expect mothers and children to co-operate for longer. The preliminary 6 h experiments likewise demonstrated recovery of the tracer at the end of the time limit. Since the aims of the project were to analyse curve shapes and to investigate the role of the colon, these would have been compromised by a shorter test duration. A 12 h test was approved by the University of Modena Ethics Committee and there was no difficulty in recruiting subjects in Emilia Romagna by word of mouth.

5.3.2 cPDR

The use of cPDR as a means of comparing starch digestion is widespread throughout the literature. Shulman and co-workers (147) found a mean 6 h cPDR of 34.2% in infants up to 4 weeks. However, they used a malted preparation which would be expected to be more digestible. Dewit and co-workers (171) found a 6 h cPDR between 15 and 53% which increased with age in children between 6 and 21 months. Weaver and co-workers' (159) 6 h cPDR of 13.7% was less than these results but their subjects were malnourished children (ages ranging between 7 and 16 months). In two of these papers (159,171), a constant value of 5 ml/min/m² body surface area was used to calculate $\dot{V}CO_2$ whilst Shulman and co-workers (147) measured CO₂ production in each subject. Whilst this was possible with young infants over a 6 h experiment duration, it would not have been appropriate for a free-living experiment such as mine in which the toddlers could not have been expected to remain at rest for 12 h.

5.3.3 Analysis of data form

Analysis of curve shapes may be an effective approach to estimation of the site of digestion. The approach is not new, and a mathematical curve fit for the calculation of small bowel residence has been made from gamma camera measurements of gastric emptying and colonic filling (245). In infants stable isotope breath tests have been used with curve fitting to measure gastric emptying (163) where the mathematical curve fit permitted the calculation of gastric emptying parameters.

Shulman et al (147) found no significant difference between cPDR as a marker for carbohydrate digestion for glucose, glucose polymers and corn cereal (malted starch). However the curves that they produced (figure 1c) show a qualitative difference. For corn cereal, there is a slower time to peak (greater t_{\max}) a lesser enrichment at t_{\max} , and a shallower down-slope. The corn cereal curve also hints at a second peak; none of these qualitative features were discussed by the authors.

Amarri and co-workers (172) in contrast, remarked upon the difference in curve shapes between children with cystic fibrosis (CF) and healthy controls. A dual-peaked curve was observed in five of sixteen children with CF who were not taking pancreatic enzyme supplements. The second peak coincided with a rise in breath H_2 suggesting its physiological basis as colonic bacterial fermentation. The dual-peaked curve became a single-peaked curve for four out of five of these subjects when the test was repeated whilst taking enzymes.

These authors suggested that colonic salvage by bacterial fermentation to SCFA is the explanation for the dual-peaked curve shapes, since it would be expected that a greater proportion of starch would pass undigested into the colon in children with a decreased digestive capacity of the small intestine. A physiologically diminished small intestinal digestive capacity might also be expected in young children around the time of weaning when the capacity of the small intestine to digest starch is less than in older children. The work described in this thesis has attempted to take up this argument from that point by qualitative descriptions of $^{13}\text{CO}_2$ recovery curves. Furthermore, attempts have been made to quantify relative roles of small intestine and colon in starch digestion in young children by mathematical analysis of curves from curve fitted data.

5.3.4 Investigation of the roles of small intestine and colon in starch digestion

Using stable isotope breath tests without mathematical curve fits means that it is not possible to distinguish between $^{13}\text{CO}_2$ produced by oxidation of glucose digested in the small intestinal and that produced by oxidation of SCFA derived from colonic fermentation. However, Shulman and co-workers (147) used the hydrogen breath test to define the period of small intestinal digestion. The time of the initial rise in breath hydrogen marked the arrival of undigested starch in the colon and therefore all $^{13}\text{CO}_2$ recovered up to this point was considered to have derived from small intestinal digestion. Since all subjects were shown to be H_2 producers at the outset and the limitations of the quantitative aspect of the H_2 breath test do not apply with this use, the method is a simple one to begin to define individual roles of upper and lower gut. This approach is at best only semi-quantitative since there will be an overlap period

where small intestinal digestion and colonic fermentation occur concurrently. The conclusions to be drawn are limited since the method is unable to define the full extent of digestion in the small intestine and the authors felt that separation of the contributions of upper and lower gut to starch digestion is simply not possible.

5.4 Interpretation of results

5.4.1 Interpretation of wheat flour data curve fitting

In these studies a mathematical curve fit has been used and, with assumptions made about the physiological basis for the curves, estimations of the contributions of upper and lower gut have been made. This allowed an estimation of the total starch digested by extrapolating the curve to baseline and the calculation of several parameters not amenable to direct measurement.

The equation chosen based on Ghoo's and co-workers' (164) study of gastric emptying was not based on any physiological premise, but was simply an equation chosen that can describe any bell-shaped curve with three (four in this case) parameters. A closer fit using a composite two-curve fit rather than a single curve (table 4c) implies the occurrence of more than a single digestive or metabolic process. The curve fitting approach is further endorsed by good agreement between parameters that can also be calculated from raw data such as (small intestinal) t_{\max} and cPDR at 12h (figures 4a and 4e and table 5a).

5.4.2 Alternative explanations for the second curve

From knowledge of starch digestion the most likely explanation for the second peak of a dual-peaked digestive process was through the oxidation of SCFA and bacterially produced CO_2 produced and absorbed as a result of colonic fermentation. Alternative explanations are less likely. These are discussed in more depth below.

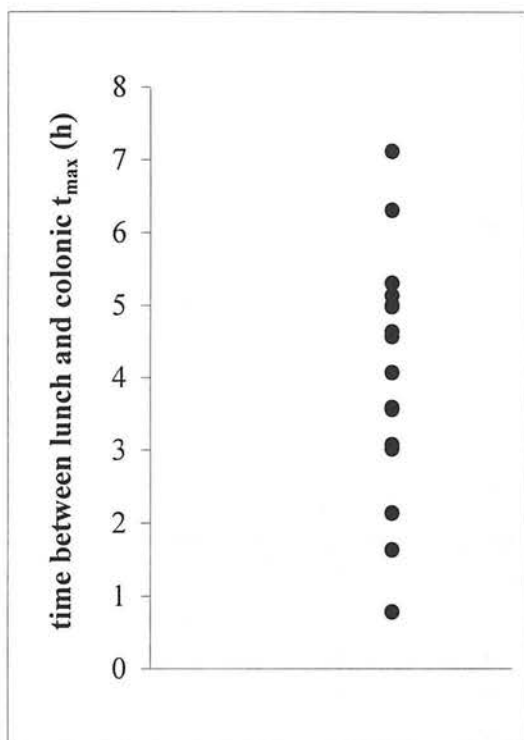


Figure 5a. Scatter plot of the time between lunch and the t_{\max} of the second modelled curve, ascribed to fermentation in the colon. The data shown is unadjusted for physical activity.

5.4.2.1 contamination from naturally enriched carbohydrate

Ingestion of naturally enriched carbohydrate was controlled. Table 5b shows the foods which were consumed by the children during the duration of the tests. The majority of food items have been analysed and their enrichment corresponds with the less enriched C3 pathway. Also, that there was no relationship between the time of the second peak and lunch-time is further evidence for no appreciable background contamination during the testing.

Table 5a. Comparison of cPDR and t_{\max} for raw and curve fitted data, using both unadjusted and adjusted data sets.**unadjusted data**

	12 h cPDR (%)			t_{\max} (h)		
	raw data	curve fit	% agreement	raw data	curve fit	% agreement
97I01	37.2	36.5	98.2	3.7	3.4	91.0
97I03	19.7	18.3	93.1	3.7	2.9	79.3
97J02	25.0	25.3	98.7	4.2	3.5	84.3
97K02	23.8	23.9	99.8	4.1	3.3	80.5
97K03	9.9	9.8	98.1	4.1	4.1	99.8
97L01	19.6	19.2	98.0	3.7	3.2	86.1
98A01	11.2	10.7	95.3	2.5	1.9	77.6
98A03	19.1	19.6	97.5	3.3	3.0	90.4
98C01	19.4	19.7	98.5	5.6	5.4	95.5
98C02	9.7	10.0	97.0	3.4	3.1	92.3
98C03	30.4	29.1	95.8	4.8	4.4	90.5
98E01	26.8	26.7	99.6	3.7	3.1	83.7
98C04	31.7	31.3	98.8	5.1	4.8	93.3
97K06	15.4	16.9	90.4	2.2	1.8	81.5
98E02	23.2	23.1	99.7	2.9	1.7	58.1
98C07	33.7	34.1	98.7	3.6	4.2	83.9

adjusted data

	12 h cPDR (%)			t_{\max} (h)		
	raw data	curve fit data	% agreement	raw data	curve fit data	% agreement
97I01	49.3	49.1	99.6	4.3	3.3	76.8
97I03	19.4	21.3	90.2	3.4	3.0	86.1
97J02	33.2	37.4	87.4	4.2	3.6	86.6
97K02	27.0	30.0	89.0	4.4	4.2	94.7
97K03	11.8	12.5	94.0	4.6	4.3	94.2
97L01	24.9	24.9	99.9	3.9	3.2	82.3
98A01	11.7	16.4	59.6	2.5	1.9	76.7
98A03	21.3	22.6	93.5	3.8	3.4	89.6
98C01	26.0	26.4	98.5	5.1	4.8	94.4
98C02	12.2	13.5	89.0	3.4	3.1	92.3
98C03	39.6	38.9	98.3	4.6	4.0	86.4
98E01	34.7	34.3	98.8	2.7	2.5	92.8
98C04	39.9	40.9	97.6	5.1	4.9	96.2
97K06	20.7	22.6	91.0	2.3	1.8	77.7
98E02	31.3	31.4	99.6	2.9	2.3	79.1
98C07	44.0	45.5	96.6	4.0	4.3	92.3

Table 5b. List of foods consumed by subjects and their natural ^{13}C enrichment where data is available (246).

	delta ^{13}C	ppm ^{13}C	Comment
Rice Krispies (Special K)	-26.99	10815.65	Special K; Kellogg's rice crispies were -26.08
Liquorice			No data
Homogenised rabbit	-27.3	10812.24	Mean, 8 meats
Vegetable soup	-27.45	10810.59	Mean, 10 vegetables (+/- 1.25)
Orange juice			Depends if it is sweetened
Ice cream			Not yet analysed; depends on sugar content
Cheese spread	-28.88	10794.87	Mean, 3 cheeses
Pasta	-28.05	10804.00	Wheat
Artichokes			No data
Milk	-29.38	10789.37	
Biscuits	-26.76	10818.18	Rich tea
Tinned tomatoes	-28.77	10796.08	
Olive oil	-29.59	10787.06	
Parmesan cheese	-28.88	10794.87	Mean, 3 cheeses
Prunes	-27.44	10810.70	Mean, 10 fruits
Yoghurt	-27.05	10814.99	
Potato	-27.94	10805.20	
Fennel			No data
Carrots	-29.5	10788.05	
Homogenised turkey	-26.41	10822.03	
Bread	-27.81	10806.63	Wholemeal, -28.05
Apple	-26.84	10817.30	
Pear	-29.81	10784.64	
Banana	-26.55	10820.49	
Courgette	-26.7	10818.84	
Pumpkin			No data; courgette -26.7
Beans (haricots)	-27.29	10812.35	
Swiss chard	-29.37	10789.48	Lettuce data given
Porcini mushrooms	-25.8	10828.74	
Camomile tea			No data
Homogenised veal	-27.3	10812.24	Mean, 8 meats; but depends on feed source
Beet sugar	-27.0	10815.54	

Figure 5b shows the raw data plots for one infant who had a rise of 38.1 ppme in both starch and baseline studies at the same time point (97L01 and 97L03 respectively). The rise was traced to a yoghurt which, unusually, contained cane sugar. A further test was carried out on this infant but omitting the yoghurt from the afternoon snack and no corresponding enrichment was observed. Its contribution was curve fitted in the same way and subtracted from the $^{13}\text{CO}_2$ recovery curve, enabling the data to be retained within the study.

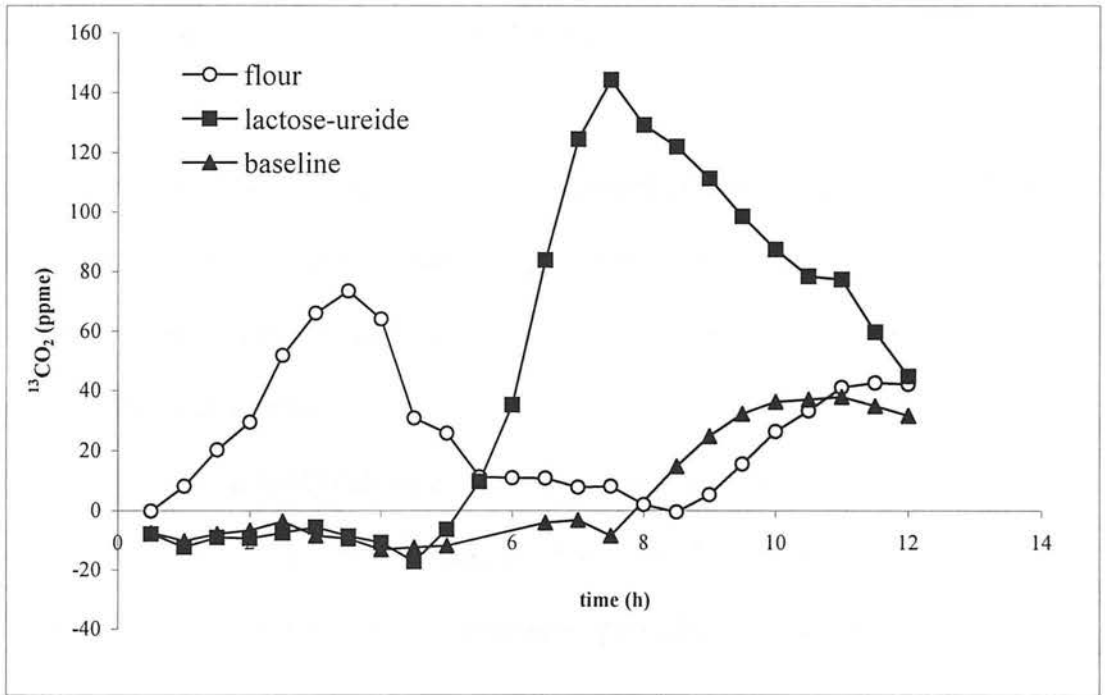


Figure 5b. Subject AU showing contamination from naturally ^{13}C -enriched cane sugar yoghurt.

5.4.2.2 subsequent breakdown from labelled glycogen stores

Subsequent breakdown of labelled glycogen from liver stores would be expected to trickle out over several hours rather than give a discrete second peak and since most

infants were sleeping or resting at the time of the second peak, breakdown of muscle glycogen stores is also unlikely.

In a recent paper (247), a Dutch group of investigators looked at liver glycogen depletion using $^{13}\text{CO}_2$ breath tests. They initially argued by means of previous experimental work that the muscle glycogenolysis in a sedentary or minimally exercising subject was minimal with respect to liver glycogenolysis. Since protein and fat stores are not enriched over period of five days feeding with ^{13}C enriched foods (248), gluconeogenesis from proteinolysis or lipolysis would be unlikely to contribute to delayed $^{13}\text{CO}_2$ breath enrichment.

They demonstrated a 'plateau' effect from oxidation of enriched glycogen reserves occurring between four to ten hours post-prandially. Such a 'plateau' effect may contribute to the second curve in this study but it is unlikely to account for all of it for the following reasons:

- i) The subjects in the Dutch study consumed a hypocaloric diet the day before the study began and then had ^{13}C enriched foods for all three meals on the first study day to achieve enrichment of a substantial part of liver glycogen stores. There was no such hypocaloric preparation in our study and only a single test meal so any 'plateau' effect may be expected to be less.
- ii) For many of the subjects described in this thesis, a clear second curve was demonstrated, that is a second rise. This would not be expected from a trickling out of liver glycogen and was not observed in Tanis's (247) work.

- iii) Given the magnitude and timing of this liver glycogen effect, the data would have been expected to be better fitted by a single curve with a long tail. The equation curve fit, without making assumptions about physiological considerations, indicates the presence of a second curve from this analysis.

It has therefore been concluded that the direct appearance of labelled CO_2 as a result of colonic fermentation of the tracer is much more likely than liver glycogen utilisation. Liver glycogen may in part account for the second peak, but cannot wholly explain it. Invasive sampling may be necessary to give a definitive answer concerning the magnitude of tracer flux from glycogenolysis, but this would have been unacceptable in the population of healthy children studied.

5.4.2.3 intestinal motility stimulus of mid-day meal

It is possible that the second peaks were related to the timing of the lunchtime meal. The time between lunch and the colonic t_{max} varied between 0.8 and 7.1 h, with a median of 4.4 h. This amount of variability suggests there was no such relationship (see also figure 5a).

5.4.3 Other approaches to the residual curve

More than a single curve may represent fermentation but wheat starch is rapidly digestible (34) and would similarly be expected to be rapidly fermentable. Curve fitting the assumed colonic fermentation might make the estimation of the colonic phase more precise, but it would be unlikely to significantly affect the estimation of contributions to starch digestion from small intestine and colon.

5.4.4 Interpretations of component approach

5.4.4.1 interpretations with regard to whole gut starch digestion

The main attraction of this curve fitting approach, however, lies in the possibility it offers to separate the contributions of small intestine and colon to the digestive process in a more comprehensive way than use of a simple time of rise in breath hydrogen concentration. The data from this thesis suggests that for children of this age, the small intestine accounts for 70 to 94% of the total starch digested using the unadjusted data, and for 48 to 96% for the adjusted data.

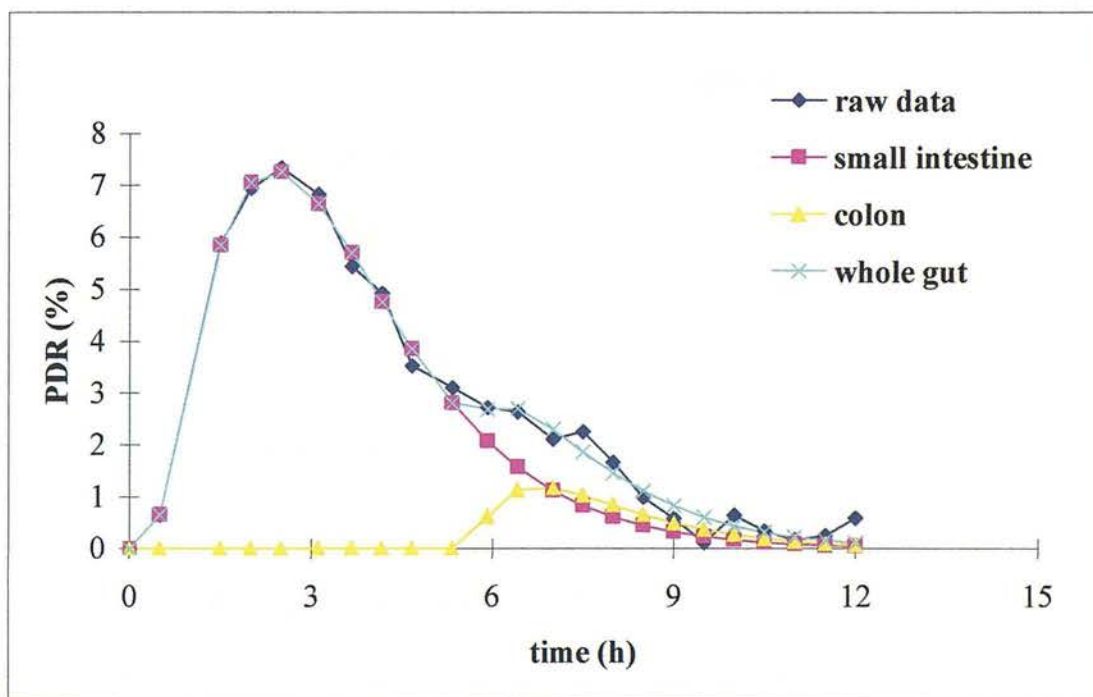


Figure 5c. Composite curve fitting applied to an adult subject with glucose as the substrate.

5.4.4.2 curve fitting of glucose digestion

The result of a glucose breath test in an adult is shown in figure 5c. The same method of curve analysis has been used to interpret the curve. The experiment was a free-

living design one, although the subject was sedentary for the duration of the test. A constant value for CO₂ production was used of 5 ml/min/m² body surface area. The curve fit suggests that only 89% of the glucose was digested and absorbed in the small intestine, with the assumption that the remainder was fermented in the colon. This is not what would be expected for a molecule that requires no hydrolysis and is well-absorbed in the small intestine. Other alternatives discussed earlier, in particular the breakdown of labelled liver glycogen need to be considered. These results suggest there is a large error margin for the estimation of the contribution of colonic fermentation to whole gut starch digestion and caution against over-interpretation of colonic contributions that are small. Extrapolating this to the data from this thesis, in three sets of unadjusted data and in four sets of adjusted data, the contribution of the colon to starch digestion may have been negligible (table 4d). ¹³C glucose and ¹³C starch tests with equivalent doses of tracer (i.e 162 g glucose for 180 g starch) would need to be carried out in the same subjects to assess this further.

5.4.5 Baselines

The 12 h baseline studies that were carried out in four subjects found the variability of breath CO₂ enrichment was between -13.4 to 22.5 ppme with respect to the first sample of the day. This is in keeping with the upper end of the reference range for background ¹³C variation quoted for adults (243). Dewit and co-workers (171) found a data spread (2 SD) of 0.8 ‰ which is equivalent to 8.8 ppme. Subjected to the same statistical analysis, this baseline data gives a 2 SD spread of 17.8 ppme. Three of the four baseline sets showed an increasing trend for the first

4 h of the study, suggesting some contamination from naturally enriched ^{13}C in the breakfast. There was no trend in background variation through the day and there did not seem to be any effect from the mid-day meal (figure 4i).

Baselines strengthen the evidence from stable isotope breath tests by demonstrating the breath enrichment following tracer cannot have been caused by background contamination and the baseline variation described here was able to do this. They may also be used as a point by point baseline for the tracer experiments. There are a number of difficulties with this approach in a free-living experiment. These are discussed below.

5.4.6 Lactose ureide data in isolation

5.4.6.1 background

Lactose [^{13}C] ureide (^{13}C -LU) offers a new attractive means of measuring oro-caecal transit time (162), for studying the metabolic activity of the colonic microflora (249) or as an aid in the interpretation of breath test curve fitting interpretation (200). The molecular bond between the [^{13}C] urea and lactose moieties cannot be metabolised by endogenous enzymes (250) but rather requires the enzymatic action of colonic bacteria. This had been proposed to be the result of the action of two enzymes, allantoate amidohydrolase (EC 3.5.3.9) and allantoate amidinohydrolase (EC 3.5.3.4) which are both present in *Pseudomonas aeruginosa* and other colonic bacterial species (251). More recently, the same group (252) conducted a careful search of more than 174 bacterial species from the colons of healthy human adults and

identified a single species, *Clostridium innocuum* as the only one capable of hydrolysing the ureide bond.

Following cleavage of the bond between carbohydrate and urea moieties, the urea is hydrolysed by urease which is also a bacterial enzyme. The resulting $^{13}\text{CO}_2$ can then be detected in breath. Adults have been found to be consistent producers of $^{13}\text{CO}_2$ following ^{13}C -LU administration in contrast to the variable production of H_2 following administration of lactulose. ^{13}C -LU needs only to be given in small quantities and does not stimulate gut motility in contrast to lactulose which acts as an osmotic laxative. It has been proposed therefore to be a superior measure of oro-caecal transit than the H_2 breath test (251). In addition, it has been validated against scintigraphic methods for use in adults (253).

Lactose ureide can be absorbed intact in the small intestine, but it undergoes no further metabolism and can be detected in the urine (162). There is greater recovery of isotope in the breath if the urea moiety rather than the lactose moiety is labelled (162). Up to half of the isotope can be recovered in the breath and the remainder is believed to be retained within the body, or excreted in the stool (254). Other investigators have found up to 13% excreted in the urine but with a maximum of 1% as urea, implying the remainder is excreted unmetabolised (255).

5.4.6.2 findings in this study

The most significant outcome of data this data was the surprise finding that only some children were able to metabolise ^{13}C -LU. The numbers are too small to draw

statistical significance, but the responders were generally older than the non-responders. However, among individual subjects there was great variation in response: one child was re-tested at 12 months, having been a non-responder at 9 months but was still a non-responder; one child of 22 months tested for the first time did not respond. Van Den Driessche and co-workers (256) have recently described the use of the ^{13}C -LU breath test to measure oro-caecal transit time (OCTT) in children. OCTT was defined as a rise in $^{13}\text{CO}_2$ of greater than 2 SD over baseline. They reported the results of breath tests in 20 children aged between 3 and 17 years showing a mean (range) OCTT of 255 (165 – 390) minutes. In addition, in 32 children aged 0 – 3 years they carried out stool incubations to look for evidence of lactose ureide hydrolysis. No $^{13}\text{CO}_2$ was produced for infants below 6 months; for children of 8 months and over, there was lactose ureide hydrolysis consistently present and for infants of 6 and 7 months, there was variable response. They concluded that bacterial enzymic activity capable of hydrolysing lactose ureide was present in the stools of children from 8 months upwards.

Van Den Driessche's results are at odds with the findings of this thesis that individual children up to 22 months had a negative response to a ^{13}C -LU breath test. However, they did not carry out breath tests in this younger group of children and there may be several explanations for a difference between these *in vivo* and their *in vitro* findings. The incubation methodology used by Van Den Driessche differed from the methodology normally used for such stool fermentation experiments. Conventionally, the stool is processed as soon as possible after passage to preserve anaerobic numbers. In Van Den Driessche's experiments, the stool was refrigerated

overnight before processing. This may have had the effect of killing strict anaerobes whilst selecting out facultative anaerobes, such as *Pseudomonas aeruginosa*, therefore giving an unphysiological representation of the colonic flora.

Another explanation for the difference in findings may be concerned with bacterial induction. This may occur both by upregulation of enzyme receptors and by selection of bacteria capable of the metabolic process concerned. Van Den Driessche et al used pre-dosing of unlabelled lactose ureide for both breath tests and stool fermentation tests. For the breath tests they used three doses of 500 mg unlabelled lactose ureide the day before. For those children of 3 years and over undergoing the stool analysis, they used a single dose of 500 mg lactose ureide one day before stool collection as an “*in vivo*” induction and compared the fermentation results to those obtained from a non-induced stool sample collected one week earlier. The induced stool sample resulted in a faster time to peak mean $^{13}\text{CO}_2$ production rate (450 minutes) compared to the non-induced stool samples (900 minutes). Unfortunately no breath tests were carried out on the same day as the non-induced stool sample was collected. Van Den Driessche also carried out an “*in vitro*” induction by incubating some of the stool slurries with unlabelled lactose ureide 1 h before the $^{13}\text{CO}_2$ collection commenced. These results were not reported however. No stool induction was carried out in the group of children aged less than 3 years.

Van Den Driessche’s use of stool fermentation and breath tests simultaneously highlights the need to consider the time for fermentation to take place when measuring OCTT. Contrary to what might have been expected, they noted that the

time for fermentation *in vitro* was in fact delayed compared to that *in vivo* and postulated that this might have been due to the refrigeration of the stool samples depressing bacterial activity though it may also have been accounted for by a lag time whilst enzymatic induction took place.

The amount of time for fermentation to take place is important and this may be altered by pre-dosing, subsequently altering OCTT. A difference in ^{13}C -LU breath test curve parameters was observed when pre-dosing with unlabelled lactose ureide was used by one group of investigators (257) but the parameters which were altered included the time from tracer appearance to peak maximum (i.e. $t_{\text{max}} - \text{OCTT}$) and the initial rate of appearance of breath $^{13}\text{CO}_2$, but not OCTT. OCTT was here defined as a rise in $^{13}\text{CO}_2$ of greater than 5 ppme. In contrast, Wutzke and co-workers (251) found an earlier rise in breath $^{13}\text{CO}_2$ following the same pre-dosing regimen as Morrison et al (257). The pre-dosing regimen used by Van Den Driessche (256) and also by me (single dose of unlabelled lactose ureide 24 hours prior to the breath test) is easier to administer to a young child. It remains to be ascertained whether an observed difference in OCTT is seen in young children and what is the minimal pre-dosing regimen required. The magnitude of incubation required to produce $^{13}\text{CO}_2$ from stool fermentations *in vitro* may not be the same as that required to produce a rise in breath $^{13}\text{CO}_2$ following *in vivo* experiments. What can be concluded however is that if it is possible to induce $^{13}\text{CO}_2$ production after administration of ^{13}C -LU to infants and young children by pre-dosing with unlabelled lactose ureide, it is likely to require more than a single 100 mg dose the day before the test such as was used in these experiments. A more intensive pre-dosing regimen may have resulted in

positive results with younger children. This is something which requires further validation both measured by both curve parameters produced and also changes in colonic flora. The bacterial species identified which can metabolise lactose ureide (252) may be pathogenic and until this can be established, there are questions to be asked about the ethics of inducing the colonic flora of young children in this way.

The median cPDR of 24% for unadjusted data (this is a better comparison since other investigators have not corrected $V\text{CO}_2$ for physical activity) is considerably lower than findings in adults, with some investigators reporting up to 80% recovery after 48 h (249). This finding in combination with the findings of Van Den Driessche et al (256) suggest a change in the composition of the colonic flora from infancy to adulthood and one that is likely to take place over the first 1 or 2 years of life.

In summary, the ^{13}C -LU breath test is an attractive means to measure OCTT that offers more potential for a result which is reproducible and valid than the currently available hydrogen breath test and more suitable for routine use in children than scintigraphic means. However it still requires further validation in young children, and in general, further work needs to be done on the role of pre-dosing and the particular regimen to use.

5.4.7 Lactose ureide in combination with wheat flour data

The use of ^{13}C -LU data to interpret starch breath tests curves is in its infancy. To date, the work has used a qualitative approach, looking for superimposition of ^{13}C -LU and deconvoluted colonic starch curves (200). Cautioning against more

quantitative analysis is the lack of precise definition for oro-caecal transit time (OCTT) from ^{13}C -LU data. Some have defined it as a rise above 2 delta over baseline (DOB) which equates to 22 ppme (251) while others have used a definition of more than 2 standard deviations over baseline range (256). Since the maximal enrichment range for those considered to be non-responders is 1-34 ppme with a median of 27 ppme, by Wutzke et al's criteria (251) five of the subjects described here would have been considered as positive, but the form of their raw data curves suggests otherwise (figure 4g). This highlights the need for simultaneous quantitative and qualitative interpretation when mathematical curve fits are used to interpret data.

In this thesis, oro-caecal transit time has been defined as the time at which 10% of the isotope had been recovered. This is in keeping with what others have used in radio-isotope studies (253). This parameter can be calculated from the curve fitted wheat flour colon curve parameters and it can also be calculated from the raw data cumulative PDR curve for ^{13}C -LU. The corresponding results for the three subjects are shown in table 5c. The curves are shown in figure 4h.

Table 5c. Comparison of oro-caecal transit time (OCTT) for colonic fitting of wheat flour data and lactose [^{13}C] ureide raw data.

subject	oro-caecal transit (h)	
	wheat flour colon	^{13}C -LU
MT	7.0	4.6
AU	6.2	6.4
GR	5.4	4.8

The agreement between colonic curve fitting of wheat flour and ^{13}C -LU for AU and GR was good, but the ^{13}C -LU suggests a faster OCTT for MT. The ^{13}C -LU graphs for both GR and MT begin to rise from the start and both would have an OCTT of less than 4 h based on a definition of a first rise over 2 DOB. It is possible that both had some metabolism of lactose ureide by small intestinal bacteria, or even *Helicobacter pylori* in the stomach.

The use of ^{13}C -LU to measure oro-caecal transit time opens up new possibilities of parameters not amenable to measurement with previously used techniques. With previous methods, a single parameter measure of OCTT has been defined as, for example rise of > 2 DOB which measures the arrival of the head of the meal in the colon. In the same way, that gastric emptying is described using three parameters – $t_{1/2}$, gastric emptying coefficient (GEC – a measure of gastric emptying rate) and a lag time (164), so might OCTT be defined in more than a single parameter. Here, it is suggested that a more comprehensive definition might define OCTT as that time at which a percentage of the total tracer dose has arrived in the caecum (say, 10%), but also include the time at which the maximal breath enrichment is seen (t_{max}) in order that the two parameters together give a measure of the rate of tracer arrival in the caecum. Further work investigating the effect of known intestinal pro- and anti-motility agents on these parameters would be helpful in elucidating their physiological relevance.

5.5 Limitations of methodology

5.5.1 Breath collection

The duration of most breath tests has been up to six hours, but pilot studies demonstrated the need for a longer time course in order to include the period of colonic fermentation. To avoid disturbing children during sleep, two methods were used to collect breath: a bag and mask, previously validated (145), was used while the children were awake, and a nasal tube while they were asleep. This latter method was new but was validated in terms of pilot studies which showed CO₂ concentrations that fell within the normal “bag and mask” range. However in the definitive experiments, while breath samples with good CO₂ concentrations were obtained in the main, proportionally more samples with CO₂ concentrations less than 0.5% were obtained using this technique than with bag and mask. These were considered unacceptable for accurate analysis (258) and this loss of data points was reflected by greater difficulty in curve fitting some data sets with a loss of precision in calculating cPDR.

Ideally, there ought to have been further validation of this technique of breath collection. To assert that the two techniques collect breath in the same way would require a direct comparison of breath collected first by the nasogastric tube technique and then immediately afterwards by the bag and mask technique. This would enable CO₂ concentrations and also ¹³C/¹²C comparisons to be made. Nasogastric tubes of different internal diameter, with and without water to minimise deadspace could then be compared for their propensity to contamination by air. A one-way valve between

syringe and nasogastric tube might also allow less CO₂ to diffuse with atmospheric air in between aspirations.

5.5.2 Tracer dose

In early experiments the dose of wheat flour used was 100 mg. A reappraisal of the technique after nine experiments suggested that in some cases the curves were close to baseline ¹³CO₂ variability and so the higher dose of 200 mg was used from then on. There was a significant increase in peak enrichment with the increased dose but no significant difference in cPDR.

The wheat flour contained 9.4% NSP and it is possible this contributed to the colonic curve. Bran comprises most of the NSP and its enrichment, measured on the first of the batches of ¹³C wheat was 2.9 At% as compared to 2.6 At% for the flour overall (M. Harding, personal communication). However, this is unlikely to have contributed greatly to the CO₂ enrichment since *in vitro* experiments with faecal cultures in our laboratory have suggested that wheat bran may not be readily fermentable *in vivo*.

Small doses of tracer were used because of a scarcity of the ¹³C-labelled wheat flour. The raw data set ideally ought to have data points for the colonic part of the curve that were clearly above the baseline variability to allow the investigator to confidently exclude natural ¹³C contamination as a cause of the apparent second peak. Using a wheat flour tracer dose of 200 mg, the mean maximum ¹³CO₂ enrichment of the data proposed to represent colonic fermentation (that is on which the second curve was fitted) was 41.1 ppme. This compared to a baseline variability

of up to 22.5 ppme. The effects of a further dose increase are shown in table 5d. This makes the assumption that there would be a direct relationship between dose and $^{13}\text{CO}_2$ enrichment. This dose, which is still a small amount of wheat flour and likely to be well tolerated, ought to have the effect of producing data points that are clearly above baseline variability

Table 5d. Experiments carried out with a tracer dose of 200 mg showing the estimated effect on $^{13}\text{CO}_2$ enrichment of the colonic part of the data of increasing the tracer dose to 500 mg. The data points are the raw uncorrected data points which overlap the peak of the second curve from the curve-fit.

Experiment	maximum ppme for second curve	Estimated second curve maximum ppme from dose increase to 500 mg
98C01	38.2	95.5
98C02	18.8	47.0
98C03	21.8	54.6
98C04	55.7	139.2
98C07	44.8	112.0
98E01	63.2	158.1
98E02	45.2	112.9

5.5.3 Reproducibility studies

Of the two reproducibility studies, that which was more closely matched for periods of sleep and mealtimes had good agreement for the small intestinal phase of digestion (tables 4d and 4e and figure 4f). The lack of agreement even with the small intestinal phase in the reproducibility study with poorer controlling of sleep periods and mealtimes demonstrates the influence of background factors on the result. With neither study was the agreement improved by correcting for physical activity,

suggesting that the variability comes from more than simple variation in $V\text{CO}_2$. Before this approach can be used for inter-individual comparison, consideration needs to be given to strict controlling of mealtimes, food consumed and sleep periods. However in free-living experiments of this duration in young children, it is difficult to control all these factors and still expect co-operation from the subject and his or her parents. Some factors, such as bowel action which may have an effect on the colonic residence time, are completely outwith experimental control.

Until better reproducibility can be achieved, the use of these experiments is appropriate for population measures but not for diagnostic purposes. This finding is in agreement with others who have attempted to use starch breath tests diagnostically (42).

There is an argument to be made here for better standardisation of mealtimes and meal content when these experiments are repeated. For example there was great variety of breakfasts consumed when a standard breakfast would have been preferable. Toddlers are not able to easily adapt to changes in routine, but a test standard breakfast (minus tracer) might have been administered in the week preceding the study to test the subjects likely responsiveness to cope with a standardised test. Any additional constraints which limited subject numbers would not have been welcome in my project when subjects were not easy to recruit. A repeat study with less difficulty in recruiting for 12 h experiments might try to introduce more stringent reproducible measures.

5.5.4 Estimation of energy expenditure

Stable isotope breath tests are usually carried out in controlled conditions, with regulation of diet and activity in order that a basal estimate of $\dot{V}\text{CO}_2$ can be considered valid. This was not possible with young children over 12 h and so the 30 minute unit PDR was adjusted for physical activity, by multiplying the $\dot{V}\text{CO}_2$ by a factor of 1.4 (244) as a physical activity level (PAL) during wake periods. This was done as follows: the sleep periods were identified retrospectively as those periods in which samples were collected by naso-gastric tube (NGT). The $\dot{V}\text{CO}_2$ for the first such 30 minute sleep period sample was corrected by a factor of 1.2, for subsequent NGT samples, the $\dot{V}\text{CO}_2$ was corrected by a factor of 1.0 and for the first subsequent bag and mask sample, $\dot{V}\text{CO}_2$ was again corrected by 1.2. All other bag and mask samples were corrected by a factor of 1.4. In many cases smooth data set curves were transformed to 'look' less physiological, and for this reason both the adjusted and unadjusted data have been presented although it is felt that use of the adjusted data offered a closer reflection of the free-living situation.

Ideally $\dot{V}\text{CO}_2$ ought to have been measured for each 30 min period but with existing techniques of directly measuring $\dot{V}\text{CO}_2$ taking 30 min to perform, this was clearly not practical. Amarri and co-workers (259) concluded that estimates of basal $\dot{V}\text{CO}_2$ were close to measured values in healthy children, and that their cPDRs were not significantly affected. The situation is different with data for curve fitting however, since hour by hour variations in $\dot{V}\text{CO}_2$ due to different levels of exercise and to diet-induced thermogenesis, the increase in energy expenditure following a meal (260), will affect the shape of the curve and the subsequent conclusions drawn. A potential

solution to this problem lies in the future prospects of energy expenditure measured by continuous heart rate monitoring (261,262).

5.5.5 Natural ^{13}C background contamination

Another limitation to the free-living study design is the variability of baseline CO_2 measurements. For the small intestinal component the CO_2 enrichment was clearly above this baseline variability. The colonic peaks often fell within this baseline variability however. The general homogeneity of the curves (figure 4d) and the absence of a corresponding peak in the baseline studies where these were carried out suggest that they are still more likely to represent a physiological phenomenon rather than artefact.

Table 5b shows that the foods consumed by the children, where data is available, do not include naturally enriched foods. However the variability of baseline to the upper limit of predicted, the relatively poor enrichment of the colonic curves and the variation in curve parameters when the baseline study is used as the wheat flour baseline (figure 4k) point to the need for a greater ^{13}C tracer dose to confirm the hypothesis of colonic fermentation as the explanation for the second curve.

For a repeat of these experiments as an additional means of standardising the tests, a range of acceptable foodstuffs might be offered where the ^{13}C abundance is known for the product and brand.

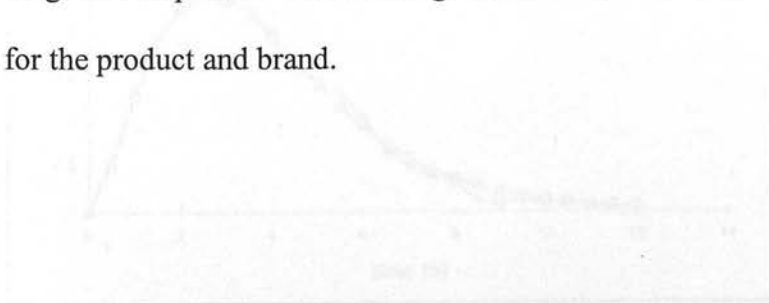


Figure 4d. The relationship of the modelling approach to the subject's ^{13}C data set.

5.5.6 Limitations of curve fitting method

Good curve fitting relies on a combination of computing and human judgement. Often the Solver[®] programme arrived at an improbable solution with nonsensical curve parameters. In these situations, a change of starting parameters almost inevitably resulted in a better fit. Figure 5d shows two interpretations of EL's wheat flour data. The composite curve fit, with a small intestinal contribution of 44% has a CV of 6.9, whilst the single curve interpretation (implying negligible digestion in the colon) had a CV of 8.3. In such a situation, a ¹³C-LU test would have been a useful interpreting tool on the most appropriate solution. Where the raw data was noisy because of background contamination and missed samples, curve fitting was often more troublesome. Solver[®] curve fits ought to be used with caution when the raw data is not smooth.

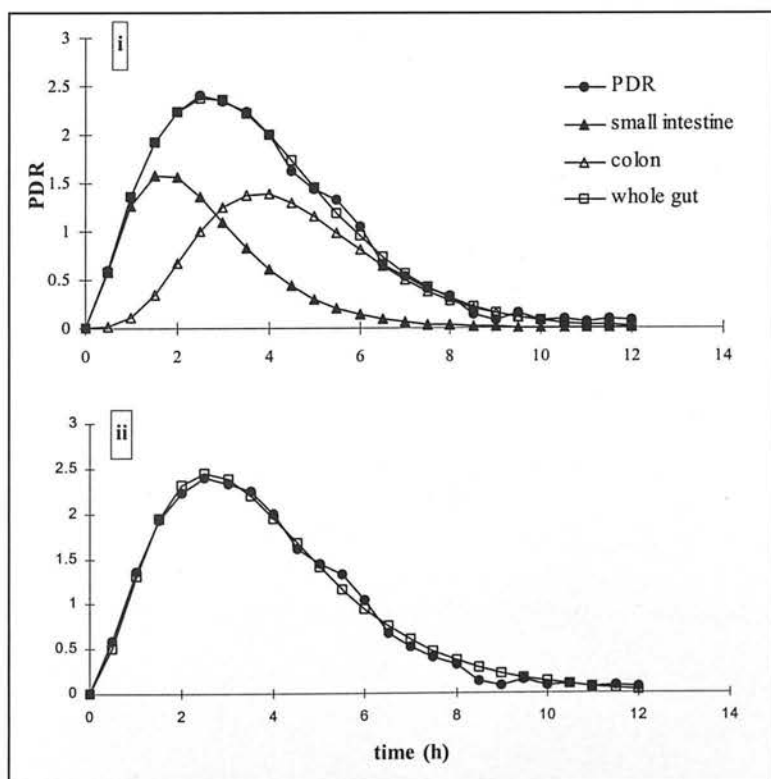


Figure 5d. Two interpretations of the modelling approach to one subject's (EL) data set.

5.6 Summary

In summary, it has been demonstrated how $^{13}\text{CO}_2$ recovery data from stable isotope breath test curves can yield more information by mathematical curve fitting of the data. These mathematical curve fits can then be used to deconvolute the raw data sets to give estimates of the contribution to digestion from small intestine and colon. There is scope for the further information still by the use of baseline studies and ^{13}C -LU breath test curves. There are a number of limitations to the methodology, some of which will be resolved with further studies controlling for activity levels and mealtimes and the use of greater tracer doses. This approach offers an attractive means for the study of starch digestion in young children. Further development of this methodology may give information about the digestibility of various starches at different stages of weaning and the capacity of the colon to utilise undigested starch in the production of SCFA with their potential benefits of providing 'unavailable' energy and in promoting colonic health.

PART II: *IN VITRO* MODELLING OF COLONIC STARCH

FERMENTATION

CHAPTER 6. SUBJECTS, MATERIALS AND METHODS

The aim of this thesis is to account for the fate of dietary starch in the small intestine and colon in young children around the time of weaning by defining the rate, site and extent of starch digestion in healthy children aged up to 2 years and comparing this to the rate, site and extent of starch digestion in healthy adults. This chapter describes the *in vitro* methodology used to model the fermentative function of the colon.

6.1 Subjects

Stool samples were collected from nineteen healthy subjects: six infants (two male) median age 9 months (range 7 – 10 months), six toddlers (three male), median age 19 months (range, 16 – 21 months) and seven adults (three male), median age 29 years (range 24-56 years). Adults were recruited from staff of the Yorkhill hospitals. The children and infants were recruited from friends and relatives of staff of Yorkhill Hospitals. Ethical approval was granted by the Yorkhill Ethics Committee. An information sheet was given to all parents and prior written consent was obtained. All infants and children had been exclusively breast fed for at least three months. No subject had taken antibiotics for at least two months prior to the study. Stool samples were collected and processed within two hours of passage.

6.2 *In vitro* model

A 5% faecal slurry was prepared by homogenising a weighed faecal sample with a 0.1 M phosphate buffer at pH 7 and filtering through a nylon mesh. A 5 ml volume of this slurry was pipetted in parallel into 30 ml McCartney bottles which contained:

- a) 25 mg raw maize starch or,
- b) 25 mg cooked maize starch or,
- c) no carbohydrate (blank)

The blank was to allow for the measurement of fermentation of endogenous carbohydrate. The incubation method was based on previous validated methods (185). The phosphate buffer was prepared as follows: 130 ml solution A ($1/15$ molar $\text{KH}_2\text{PO}_4 = 9.078 \text{ g/l}$) + 200 ml solution B ($1/15$ molar $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} = 11.876 \text{ g/l}$) + 16.5 ml reducing solution (cysteine hydrochloride 0.3 mg/ml, sodium sulphide 0.3 mg/ml, sodium hydroxide 1.9 $\mu\text{mol/ml}$) + 413 μl rezazurin. The solution was mixed and brought to the boil. It was cooled with oxygen free nitrogen and adjusted to pH 7.0 with HCl.

6.3 Substrates

The raw maize starch was a standard laboratory substrate (Corn starch – Sigma, Poole, UK). The cooked starch had been pre-prepared by boiling a 10% solution of raw starch for ten minutes and then freeze-drying to constant weight. The substrates were weighed on a calibrated balance accurate to 0.001 mg. These particular substrates were chosen to model a rapidly digestible and a resistant starch according to the classification of Englyst and Kingman (34).

6.4 Incubation

Before incubation, the tops were sealed and each McCartney bottle was flushed with oxygen free nitrogen to initiate anaerobic conditions. These were maintained with the incorporation of the cysteine hydrochloride-sodium sulphide reducing solution in the slurry. The samples were incubated in a shaking water bath at 37 °C and 60 strokes/minute, laid horizontally (263), aiming to simulate the conditions in the colonic lumen. At 4 h and 24 h, parallel incubations were stopped and the contents were analysed.

6.5 Measurement of fermentation products

6.5.1 Gaseous products

The volume of the headspace gas was measured using a syringe as a manometer (figure 6a). Samples of the headspace gas were diluted with oxygen free nitrogen (OFN) to give sufficient gas volumes for measurement. A 1 ml sample was diluted 5-fold and then analysed for carbon dioxide concentration (Servomex, Crowborough, UK); a further 1 ml sample was diluted 50-fold with OFN and analysed for H₂ by electrochemical cell (GMI, Renfrew, UK); and finally 5 ml was stored in an evacuated tube and later analysed for methane by continuous flow isotope ratio mass spectrometry. At the same time, the pH of the fermentation solution was measured by electrochemical cell.

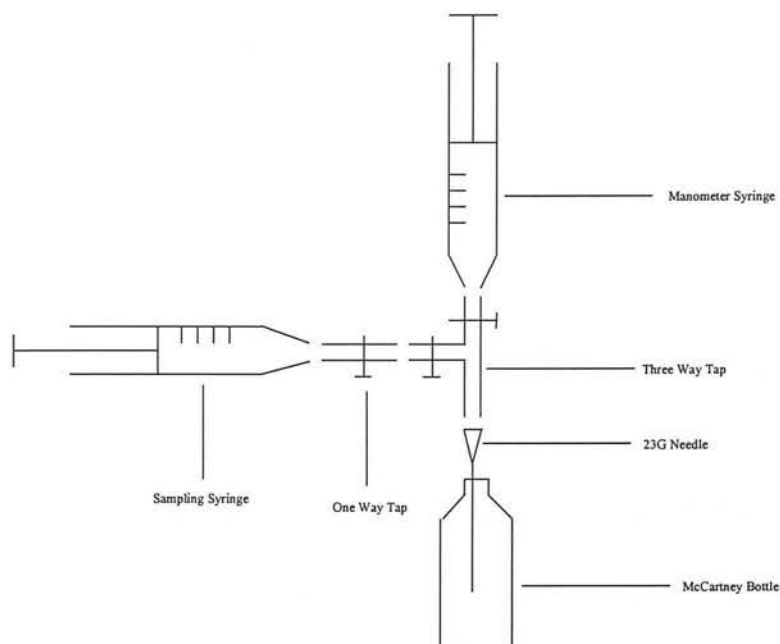


Figure 6a. Gas sampling procedure

6.5.2 SCFA and lactate

The slurry was immediately frozen to prevent further fermentation and at a later date thawed and sampled to measure SCFA and lactate content by gas liquid chromatography (GLC) of acidified ether extracts (264). A 400 μl sample of the slurry was mixed with 50 μl of a β methyl valerate internal standard, and 50 μl orthophosphoric acid. The SCFA were then extracted by vortex mixing three times with 1.5 ml diethyl ether and pipetting the supernatant between mixings. A 2 μl sample of extracted slurry was injected onto a gas liquid chromatograph (Phase Separations, Deeside. Glass column 4' $\frac{1}{4}$ " outside diameter; 4 mm internal diameter; 10% SP1000 and 1% H_3PO_4 on Chromosorb WAW 80 – 100 mesh) with the following settings which had been found to produce the clearest separation of peaks with prior experiments:

column temperature	125 °C
initial time	2 minutes
rate	10 °C/minute
upper time	1 minute
upper temperature	155 °C

The internal standard was referred back to an external standard which was analysed in concentrations of 20, 50, 200, 400 and 600 $\mu\text{l/ml}$. A standard curve was constructed and the best fit line found using Microsoft Excel[®]. The computer-calculated gradient of the curve was then used to calculate the concentrations of the samples after manual measurement of the GLC peaks.

Lactate analysis was carried out using a similar method. A 500 μl sample + 100 μl succinate internal standard + 200 μl 50% H_2SO_4 + 1 ml methanol was incubated in a water bath at 55 °C for 30 minutes to produce methylated derivatives. 100 μl orthophosphoric acid was then added and the mixture vortex mixed for 1 minute. The same three-times extraction with 1.5 ml diethyl ether was then carried out and a 2 μl sample of extracted slurry injected onto the GLC (same column as described above) at the following settings:

column temperature	117 °C
initial time	1 minute
rate	5 °C/minute
upper time	0 minutes
upper temperature	147 °C

The internal standard was referred back to an external standard which was analysed in concentrations of (25, 50, 100 μ l) with a standard curve and best fit line constructed as above.

6.5.3 Residual starch

The remainder of the slurry was then used to measure residual starch content using a method based on that first described by Englyst et al (185,265). This is illustrated in figure 6b.

6.5.3 Combustion analysis of biomass

The carbon content of solids remaining after dispersing starch in alkali was taken as bacterial biomass carbon. Samples were first neutralised with concentrated sulphuric acid, then pipetted in 100 μ l aliquots into tin boats for combustion analysis. After drying the samples were combusted and their carbon content was determined by continuous flow isotope ratio mass spectrometry (CF-IRMS) (150).

6.6 Expression of results and statistical methods

The amount of each fermentation product was corrected for fermentation of endogenous substrate. The results were then expressed both as absolute concentrations and as percentage dose recovered (PDR). This is the percentage of moles of carbon within the original starch substrate resulting in moles of carbon within the fermentation product in question. Results are expressed as median and range. Statistical significance was determined using the Wilcoxon rank sum test.

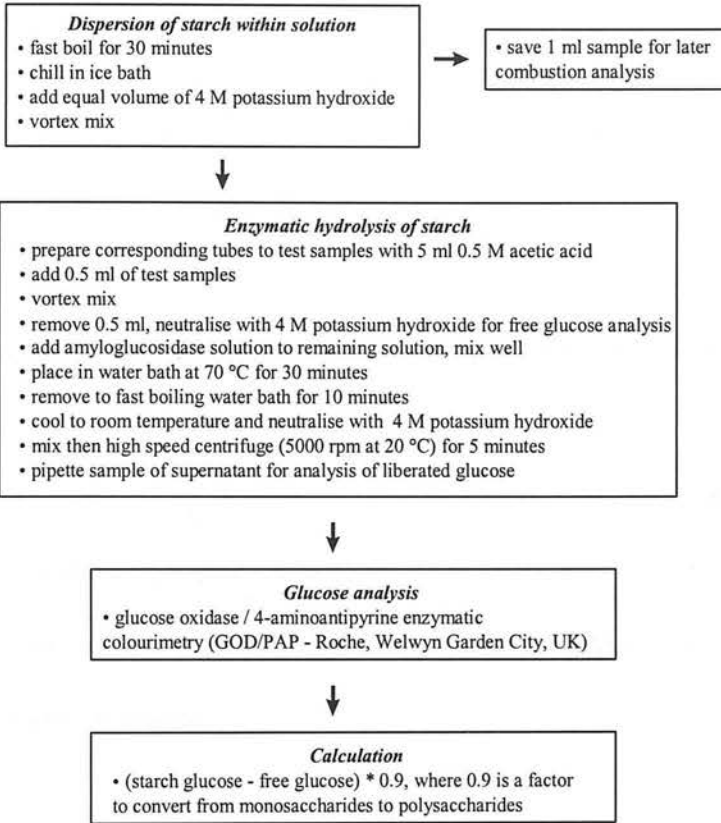


Figure 6b. Residual starch assay

PART II: *IN VITRO* MODELLING OF COLONIC STARCH

FERMENTATION

CHAPTER 7. PRELIMINARY EXPERIMENTS

This chapter describes the initial experiments that were carried out to arrive at the stool fermentation methodology of the preceding chapter.

7.1 Determination of optimum dose of substrate and faecal slurry

7.1.1 Aims

1. To determine if varying the ratio of substrate to faecal bacteria had any effect on the proportion of fermentation product.
2. To determine the optimum substrate dose for a 30 ml McCartney bottle based on viable anaerobic counts.

7.1.2 Subjects and Methods

Stool samples from two healthy young children were processed within one hour of defaecation. A 32% faecal slurry was prepared by the addition of 50 ml of a phosphate buffer to 16 g faeces. 1 ml of this faecal slurry was mixed with 9 ml of a mineral-peptide-reducing solution (192) and the substrate in a 30 ml McCartney bottle. The substrates used were glucose (Sigma Co, Poole, Dorset) and potato starch (Roquette Freres Lestrem, France) in quantities of 10, 25, 50, 100 and 200 mg (up to 100 mg only for subject 2). A rubber seal cap was fitted and anaerobic conditions initiated by flushing with oxygen free nitrogen (OFN). Colonic conditions

were simulated by incubating in a shaking water bath at a temperature of 37 °C and at 60 strokes per minute. For each experiment a faecal slurry without substrate (blank fermentation) was prepared. Parallel incubations were stopped at 2, 4, 8 and 24 h. At each time point, gas volume, H₂ concentration and SCFA concentration were measured as previously described.

After 24 h incubation, samples from each McCartney bottle were diluted 10⁻⁵, 10⁻⁶ and 10⁻⁷ and then plated onto nutrient agar and Wilkens-Chargen media which were incubated for 48 hours under aerobic and anaerobic conditions respectively. Colonies were then counted and the ratio of anaerobic to aerobic species was calculated.

The results are expressed graphically as millimoles of H₂ and SCFA produced per gramme of substrate. The proportions of individual SCFA were calculated and are expressed as histograms for sampling at 4 and 24 h.

7.1.3 Results

The results for H₂ expressed as mmol/g are shown in figure 7a. Sampling problems unfortunately meant that there were missed samples for some time points. From the available data it can still be seen that proportionally different amounts of hydrogen were produced when the substrate dose was varied though the results are not consistent. For subject 1, the most hydrogen was produced with 50 mg of both potato starch and glucose. For subject 2, H₂ production from the blank fermentation exceeded substrate fermentations for most incubations at 8 and 24 h and hence these are recorded as zero production on the graphs. For subject 2 at 4 h, 10 mg of potato starch and 25 mg of glucose produced most H₂.

The results of total SCFA expressed as mmol/mg are shown in figure 7b. For both subjects, smaller quantities of substrate produced proportionally more total SCFA.

The proportions of individual SCFA are shown in figure 7c with branched and straight chain SCFA grouped together. Different proportions of individual SCFA are seen by varying the substrate dose. In general more acetate and less butyrate is produced with smaller substrate quantities.

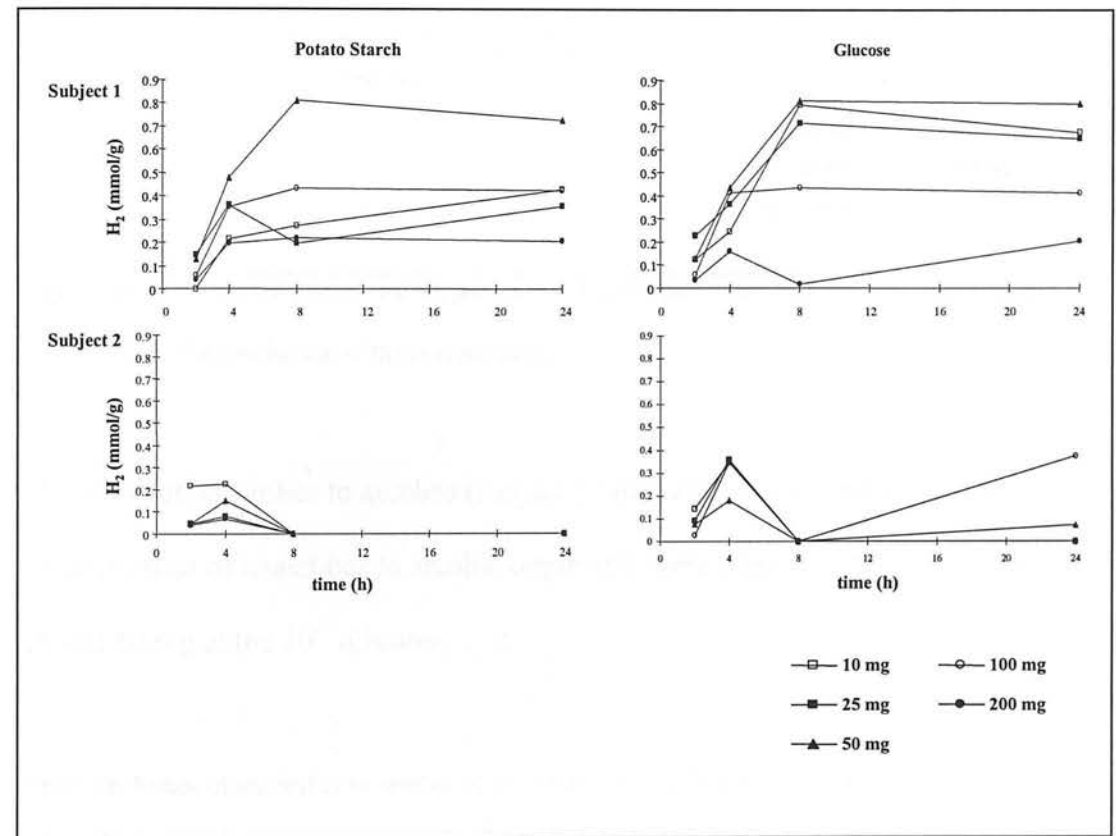


Figure 7a. H₂ results for the two subjects and two substrates expressed as mmol/g to show variation in H₂ production with substrate dose.

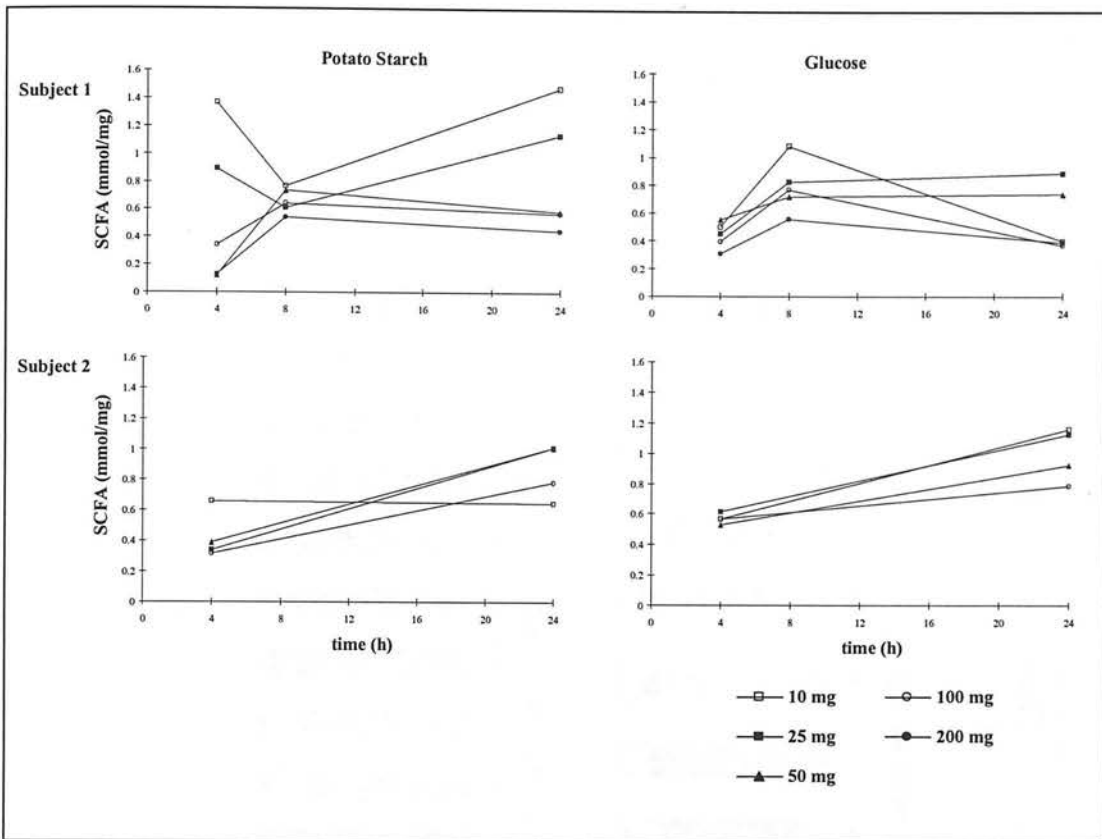


Figure 7b. SCFA results for the two subjects and two substrates expressed as mmol/mg to show variation in SCFA production with substrate dose.

The ratios of anaerobes to aerobes (subject 1 only) are shown in table 7a. The greatest ratios of anaerobes to aerobic organisms were seen with substrate doses of 25 and 50 mg at the 10^{-7} dilution.

Table 7a. Ratios of anaerobes to aerobes of at dilutions of, 10^{-6} and 10^{-7} for subject 1.

dilution	blank	glucose				potato starch			
		10 mg	25 mg	50 mg	100 mg	10 mg	25 mg	50 mg	100 mg
10^{-6}	7	3	3	2	9	17	2	3	25
10^{-7}	N/A	3	14	2	10	0	28	38	7

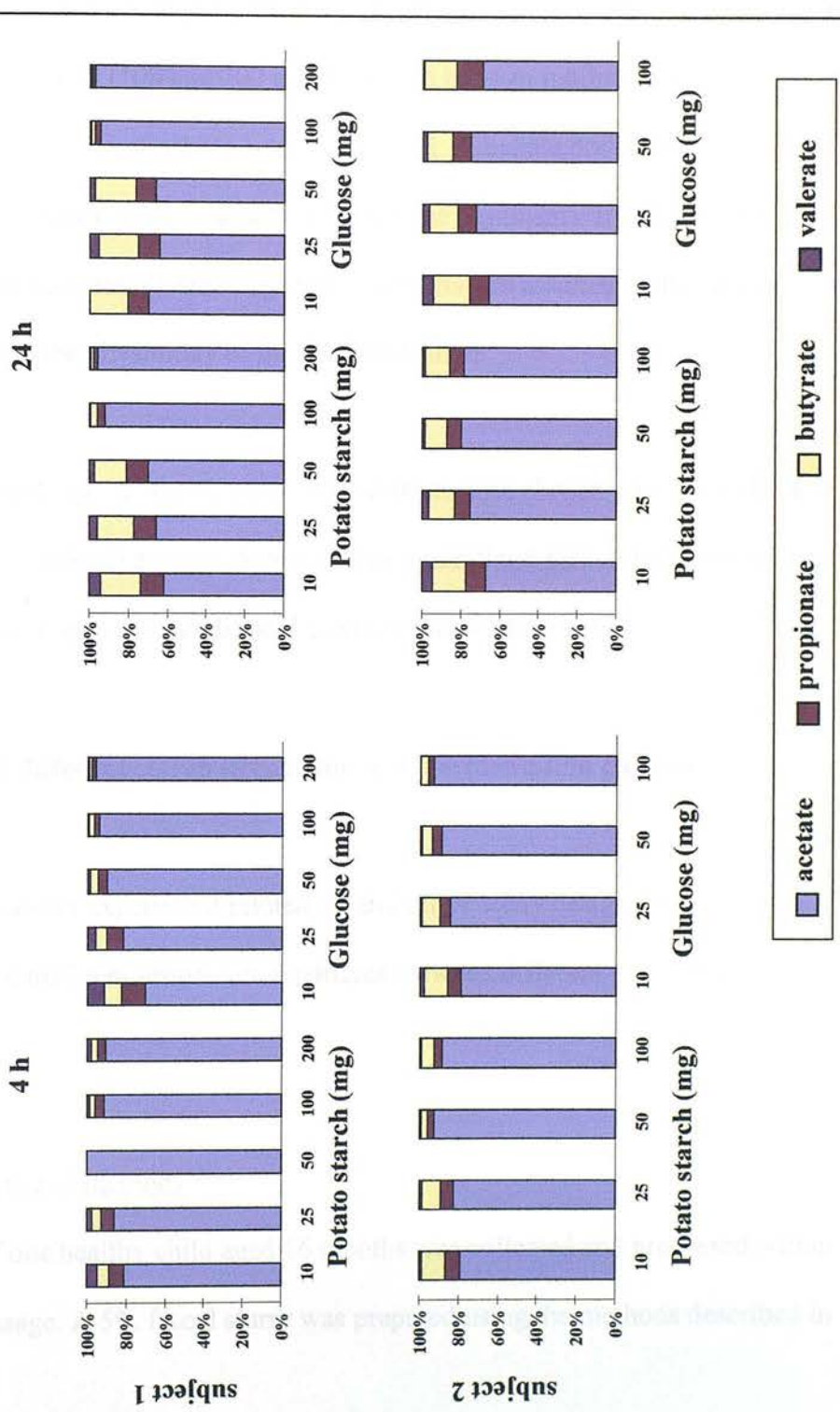


Figure 7c. Individual SCFA expressed as a percentage of total SCFA at 4 and 24 h

7.1.4 Conclusions

Varying the substrate dose does change the pattern of fermentation and therefore the substrate dose chosen is important. Considering fermentation patterns alone does not show which substrate dose produces the most physiological fermentation pattern, but large substrate doses (100 and 200 mg) appear to have an inhibitory effect on fermentation and are unlikely to be physiological. Substrate doses of 25 and 50 mg produced the greatest ratio of anaerobic to aerobic organisms. It follows that the ratio of substrate to bacteria for these substrate doses may be assumed to be the most consistent with the physiology of the proximal colon.

From these findings and conclusions, the substrate dose chosen was 50 mg for a 10 ml slurry. The definitive experimental design was refined further to 25 mg substrate with 5 ml slurry, giving an additional 5 ml volume for gas production.

7.2 Effect of different starch preparations on fermentation pattern

7.2.1 Aims

This single subject experiment piloted the definitive study design. Its aim was to determine the different fermentation patterns between different preparations of starch.

7.2.2 Subjects and methods

The stool of one healthy child aged 16 months was collected and processed within 2 hours of passage. A 5% faecal slurry was prepared using the methods described in

the previous chapter. Since the aim of this experiment was to account for the fate of the substrate, the slurry consisted of a phosphate buffer and reducing solution alone.

The substrate used was maize starch (Sigma) in various prepared states:

1. Raw (R).
2. Cooked (C). A 10% solution was boiled for 10 minutes and allowed to cool. By weighing the solution before and after, the water loss from evaporation was calculated and the weight of cooked solution required to give 25 mg of starch was determined.
3. Cooked and freeze-dried (CF). The cooked solution was freeze-dried to constant weight.
4. Autoclaved (A). After boiling for 10 minutes, the starch solution was placed through a high pressure, high temperature laboratory autoclave (121 °C and 14 psi for 15 minutes).
5. Autoclaved and freeze dried (AF). Freeze-dried to constant weight after autoclaving.

The incubations, sampling and assays were all carried out as described in the previous chapter. At the time of this experiment the measurement of methane and biomass was unavailable, but all other fermentation products were measured. After 24 h of incubation, the slurry was diluted and plated onto nutrient agar and Wilkens Chargen media as described earlier in this chapter.

The results were expressed as percentage dose recovered. Firstly the gaseous and SCFA results were corrected for that produced from the endogenous content of the stool using the corresponding blank incubation. The percentage of the original substrate recovered in the fermentation product (PDR) was calculated thus:

$$\text{PDR}_{[\text{fermentation product}]} = \frac{\text{molecular carbon content}_{[\text{fermentation product}]} \times 100}{\text{molecular carbon content}_{[\text{original substrate}]}}$$

7.2.3 Results

H₂ and CO₂ results, corrected for fermentation of nutrient within the stool, and expressed in mmol are shown in figure 7d. The PDR results for CO₂, SCFA, lactate and the residual starch are shown in figure 7e. Lactate was only seen in small quantities on the 2 h sample; the PDR for CO₂ did not exceed 1% and is not visible on the graph. The bacteriology results are shown in table 7b.

Table 7b. Anaerobe to aerobe ratios for the substrates raw starch (R), cooked starch (C), autoclaved starch (A) and autoclaved and freeze-dried starch (AF). The ratios for the blank (B) incubation are also shown.

dilution	B	R	C	A	CF	AF
10 ⁻⁵	565	175	146	293	523	184
10 ⁻⁶	N/A	N/A	73	N/A	157	166
10 ⁻⁷	N/A	N/A	N/A	N/A	N/A	17

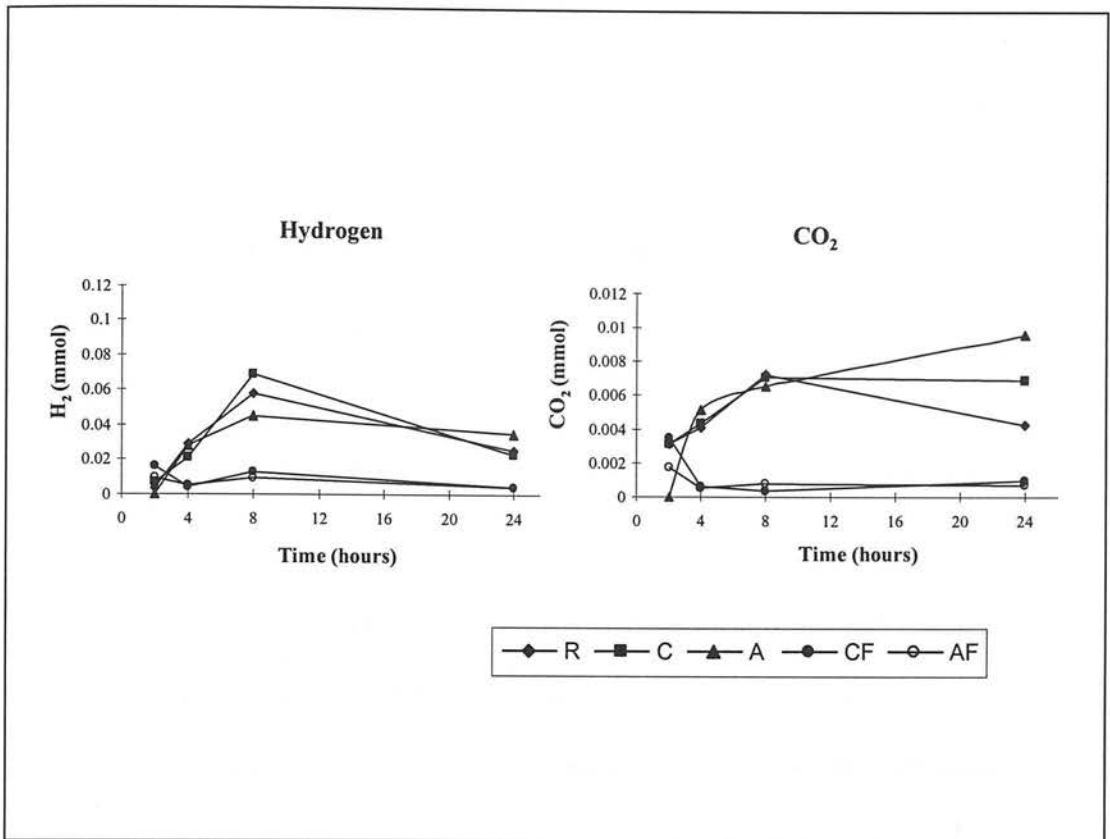


Figure 7d. H₂ and CO₂ production over time. Results are expressed in mmol.

7.2.4 Conclusions

All substrate preparations show viable anaerobic counts. There is increasing SCFA production over time. A decrease in H₂ and CO₂ production between 8 and 24 h suggests that the gases are being used for acetogenesis or possibly methanogenesis. Lactate was measurable at 2 h, but undetectable at 24 h as would be expected since it is an intermediary metabolic product. Residual starch fell over time, most rapidly between 2 and 8 h, when most SCFA and CO₂ was produced. This demonstrates internal consistency in spite of some laboratory errors such as the 4 h sampling point. There seemed to be little difference between C and A. However, the freeze-drying process appeared to result in less fermentation. No scientific explanation could be

offered for this. The low values of total PDR suggested that a measurement error had occurred. Inadequate freeze drying might have produced such results but this was not confirmed in a further pilot study (section 7.3) when similar quantities of fermentation product were produced for raw, cooked and freeze dried, and autoclaved and freeze dried substrates.

7.3 Study design pilot and determination of appropriate sampling times

7.3.1 Aims

1. To study the fermentation patterns of raw, cooked, autoclaved and freeze-dried preparations of maize starch by the stool bacteria of young children
2. To compare these patterns to that seen from the fermentation of adult stool.
3. To assess intra-substrate variability
4. To determine whether there was any difference in fermentation products between the substrates cooked and autoclaved starch
5. To determine the most informative time points for sampling

7.3.2 Subjects and methods

The subjects comprised two healthy children (both aged 16 months, one male and one female) and one healthy male adult (age 29). Both children had been breast fed initially. Stool samples were collected, incubations, sampling and assays were all carried out as described in chapter 6. In this pilot the substrate used was maize starch in various prepared states: raw (R), cooked (C) and autoclaved (A). The freeze-dried preparations of the substrate only were used. Each individual incubation was carried out in triplicate.

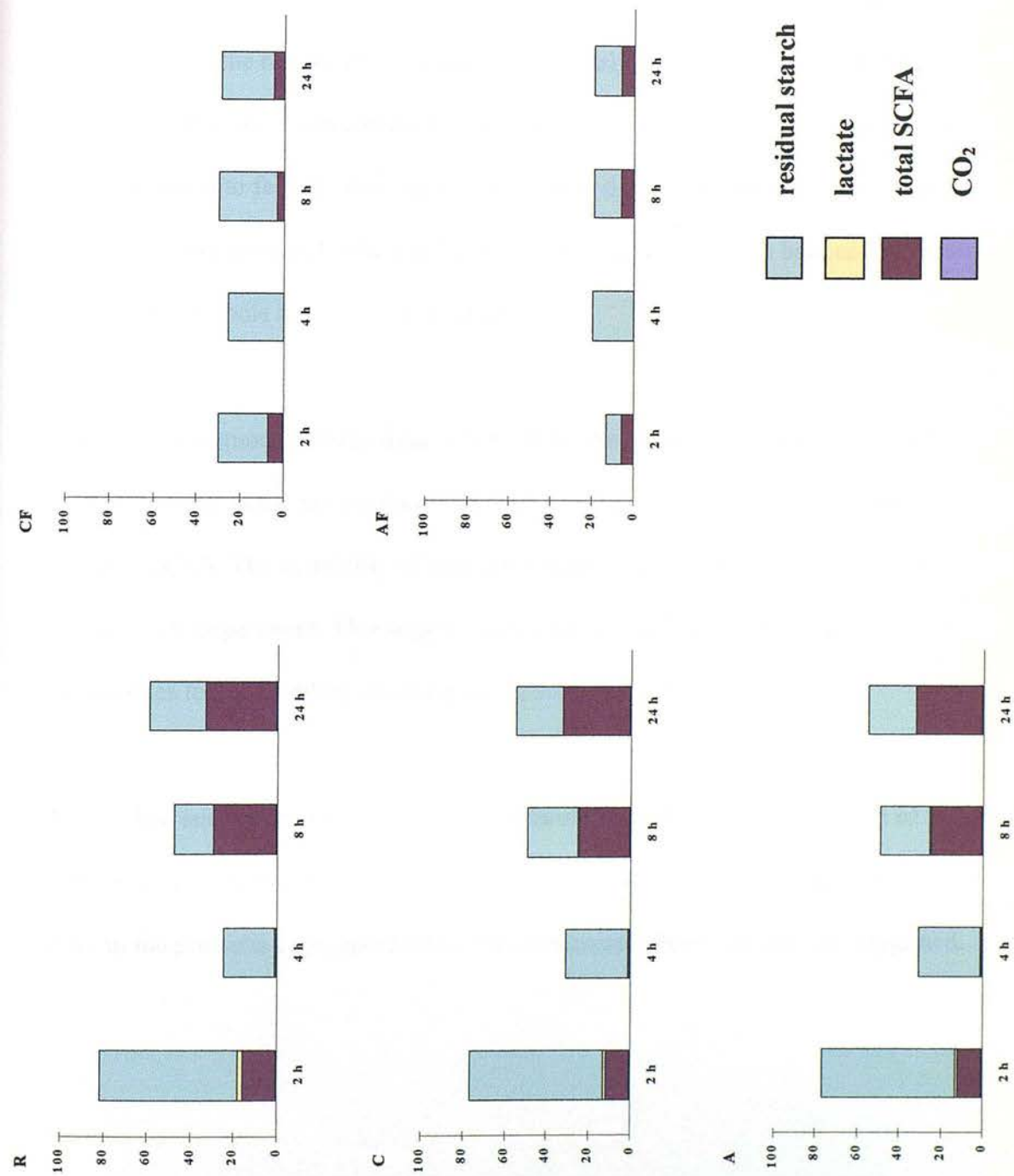


Figure 7e. PDR at 2, 4, 8 and 24 h for residual starch, lactate, total SCFA and CO₂

7.3.3 Results

The results for each incubation are shown in figures 7f, 7g and 7h. There were no H₂ results for subject 1 (figure 7f).

The children's stool fermentation showed little difference between the three substrate preparations. However for the stool of the adult, the raw starch was less well fermented than the raw starch in young children early in the experiment, but by 24 h equivalent amounts of fermentation product had been produced. In other words, adult stools appeared to ferment raw starch more slowly than the stools of young children and hence there appeared to be a difference in fermentation pattern between the adult stools and the stools of the young children.

Variability is demonstrated in figures 7f to 7h by the triplicate incubation for each substrate shown in the same colour. The intra-substrate variability was best for pH and then SCFA. The variability of gaseous products increased throughout the time course of the experiment. This suggested leakage of gas from the McCartney bottles. Approaches to this problem are discussed in section 7.3.4.

The cooked and autoclaved freeze-dried preparations underwent fermentation of the same order of magnitude as the raw starch preparation and hence a measurement error in the previous experiment rather than inadequate freeze-drying was suggested.

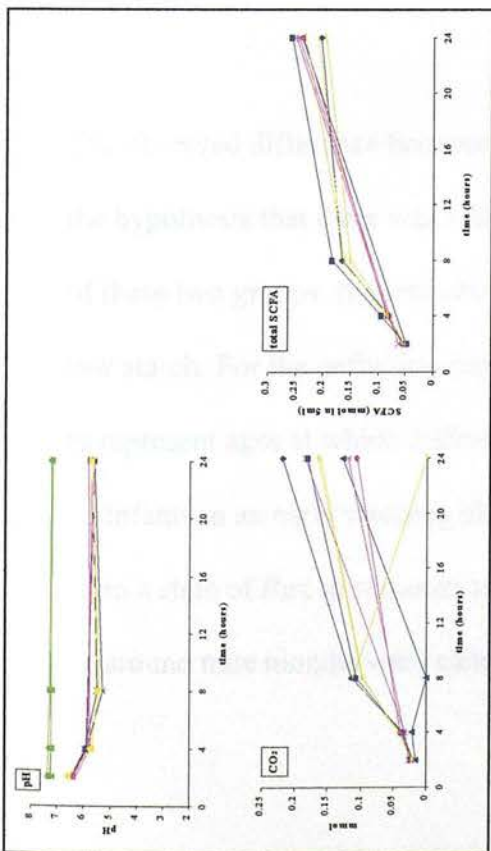


Figure 7f. Subject 1 (16 months, F)

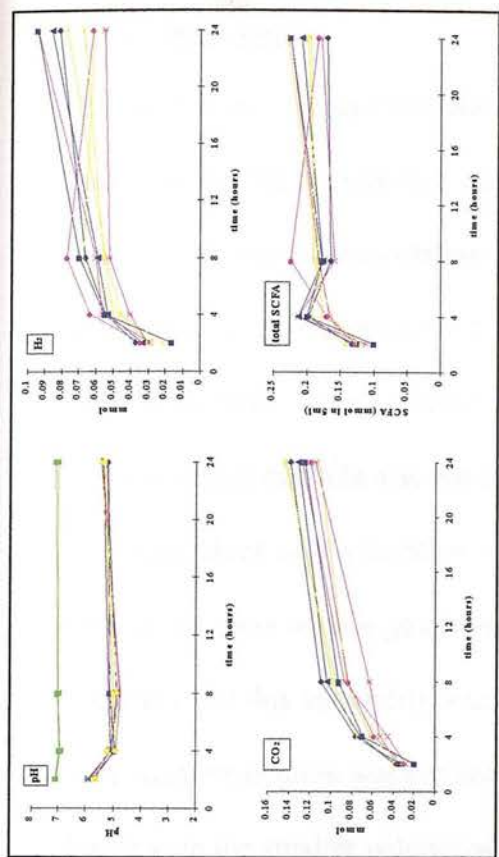


Figure 7g. Subject 2 (16 months, M)

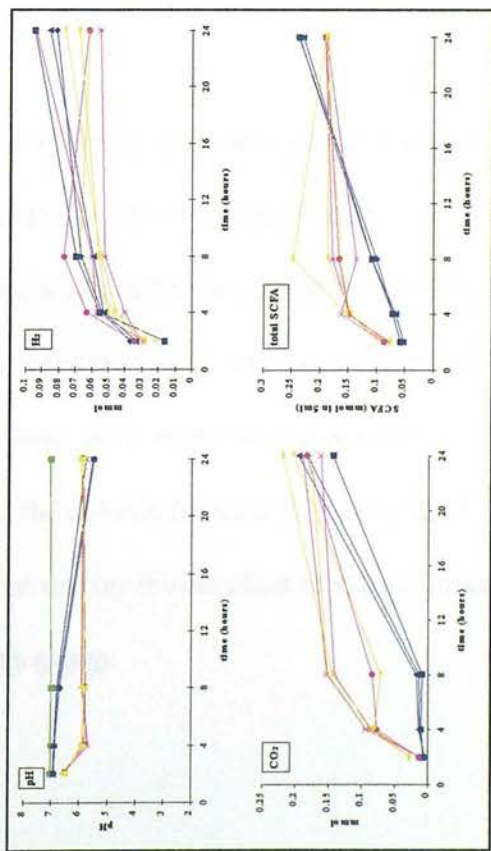


Figure 7h. Subject 3 (29 years, M)

Blank (B)

Raw

Cooked

Autoclaved (A)

7.3.4 Conclusions

Different starch preparations were chosen for their different digestibilities. It was expected that the autoclaving would produce retrograded and relatively resistant starch. However no clear difference was observed between the cooked and the autoclaved preparations and so the latter was dropped from further studies. The intra-individual variability was felt to be acceptable on the whole. However it was acknowledged that this was not the case for measurement of gaseous fermentation products. Much of the problem was perceived to result from gas leakage from the bottles. As there was no guaranteed method of preventing this, a means of reducing the impact of this variability was devised: in the definitive experiments each individual incubation was carried out in duplicate and at each sampling point the bottle with the smaller volume of excess gas was assumed to have leaked and was discarded.

The observed difference between the stools of adults and young children prompted the hypothesis that there was a difference between the stool (and hence colonic) flora of these two groups. In particular, that there was a difference in the fermentation of raw starch. For the definitive experiments, three groups of subjects were thus chosen to represent ages at which difference in colonic flora might be demonstrated:

1. Infants in an early weaning phase when the colonic flora might be expected to be in a state of flux in response to the recent dietary introduction of starch. Infants around nine months were chosen for this group.

2. Toddlers who could be assumed to have a diet established in starchy foods and in whom the colonic flora as a consequence might be expected to be more stable after weaning.
3. Adult colonic flora as a standard against which to measure the two groups above.
4. Sampling at four time points meant intensive work for each subject studied. There was not felt to be additional benefit to reflecting early and late production of fermentation products from sampling at four time points rather than two. Sampling at 4 h and 24 h was felt to achieve this aim.



Figure 24. Fermentation products from the 100 g substrate (100 g) in the 100 g

100 g

This chapter documents the results from the 100 g substrate (100 g) in the 100 g. The results are presented for each fermentation product. The fermentation products are shown together in table 24. Here they are measured as absolute values and are shown for each age group and each substrate. In figure 24, the median fermentation products for each age group and each substrate are shown as RDA's.

PART II: *IN VITRO* MODELLING OF COLONIC STARCH

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CHAPTER 8. RESULTS

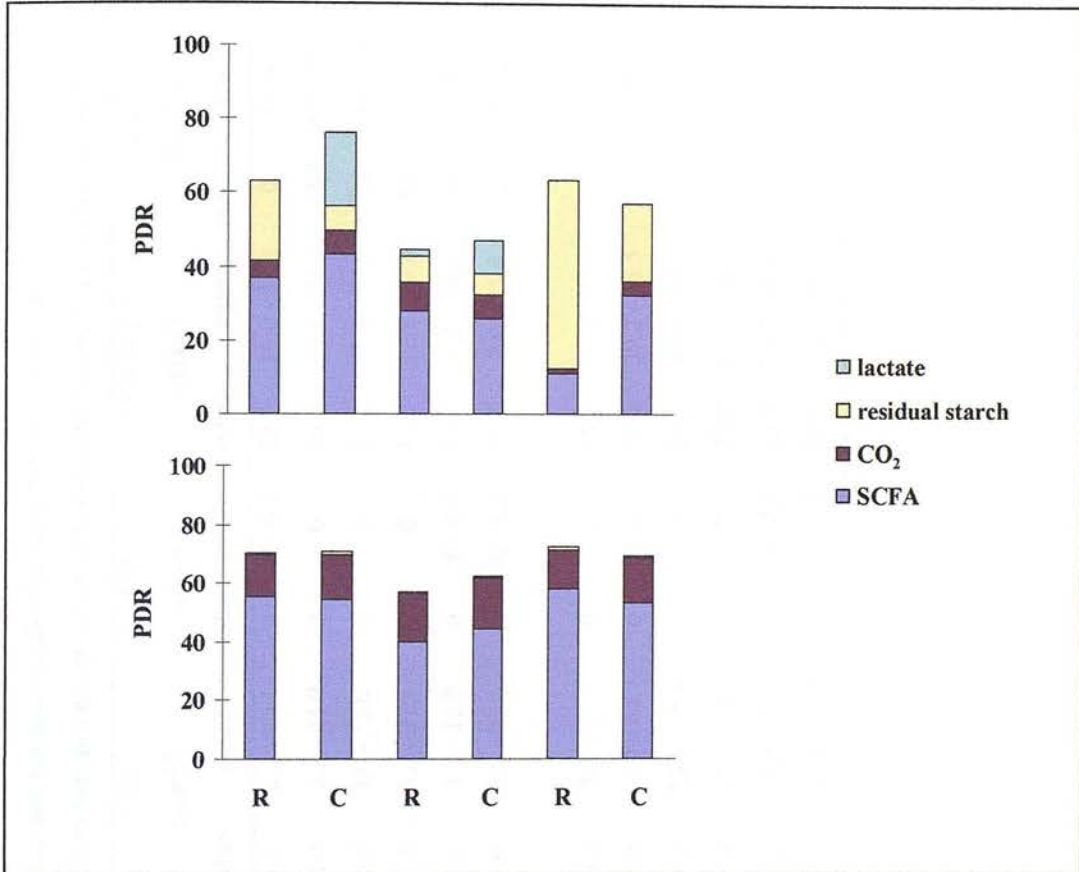


Figure 8a. Fermentation products for raw (R) and cooked (C) starch for each age group expressed as PDR.

This chapter documents the results from the definitive fermentation experiments. The results are presented for each fermentation product. The fermentation products are shown together in table 8a. Here they are measured as absolute values and are shown for each age group and each substrate. In figure 8a, the median fermentation products for each age group and each substrate are shown as PDRs.

Table 8a. Concentrations of CO₂, total SCFA, lactate and residual starch after fermentation of raw and cooked maize starch at 4 h and 24 h by faecal bacteria from infants, toddlers and adults. Significant (5%) differences between groups using Wilcoxon rank sum tests are shown for H₂, CO₂ and SCFA results.

		H ₂		CO ₂		CH ₄		total SCFA		lactate		residual starch		biomass		
		mmol/l	range	median	range	mmol/l	range	median	range	mmol/l	range	mmol/l	range	mg/5ml	range	mmol C/l
4 h	infants	R	6.5	2.1-9.2	8.5	5.1-10.1	0	0-0.1	22.9	15.0-32.8	0	0-4.1	7.3	3.5-10.8	37.6	25.5-63.1
		C	8.6	2.8-17.5	11.4	5.3-24.9	0	0	26.8	23.1-34.4	12.4	5.4-18.9	1.8	0-2.1	11.3	0-29.8
	toddlers	R	12.3 ¹	9.5-21.3	14.0 ²	7.0-30.6	0	0	17.3	12.0-31.5	1.1	0-6.0	1.8	0-10.6	22.1	0-112.8
		C	13.6	10.7-17.8	11.8	7.8-27.1	0	0	16.0	12.8-27.4	5.5	0-10.9	1.4	0.6-9.5	29.0	0-163.3
	adults	R	2.0	0.1-7.3	2.7	1.1-12.9	0	0-0.9	5.4 ³	1.2-24.1	0	0	12.8	2.3-21.4	11.9	0-129.4
		C	11.4	0.4-235	7.0	2.5-25.1	0	0-0.4	19.8 ⁴	2.0-22.0	0	0-6.5	5.3	1.0-12.1	16.6	0-61.4
24 h	infants	R	13.8	1.4-17.5	26.6	8.3-30.7	0	0	34.1	27.8-45.2	0	0	0.1	0.1-0.5	54.2	0-95.5
		C	16.8	3.9-26.0	28.8	14.3-44.4	0	0	33.6	29.2-48.5	0	0	0.8	0-1.3	0	0-104.4
	toddlers	R	17.2	13.4-22.3	30.5	23.4-36.0	0	0	24.5	19.6-33.5	0	0	0.2	0-0.3	26.5	8.9-47.2
		C	16.7	11.2-28.4	33.0	24.1-41.7	0	0	27.2	22.3-38.7	0	0	0.1	0-0.3	29.5	16.0-79.5
	adults	R	3.1	0-30.6	24.9	19.5-37.1	0.8	0-2.3	35.8	19.9-49.8	0	0	0.3	0-2.7	37.2	0-63.0
		C	4.4	0-35.2	29.3	23.3-40.9	0	0-3.7	28.5	23.0-44.4	0	0	0.1	0-3.8	0	0-84.8

R – raw maize starch; C – cooked maize starch.

¹ toddlers significantly greater than infants and adults

² toddlers significantly greater than adults

³ adults significantly less than infants and toddlers

⁴ adults significantly less than infants

8.1 CO₂

At 4 h, the stools of toddlers produced significantly ($p < 0.05$) more CO₂ than the stools of adults and the stools of infants. By 24 h, the same patterns were evident with toddlers producing most CO₂ followed by infants and then adults, but the differences were not statistically significant (figure 8b).

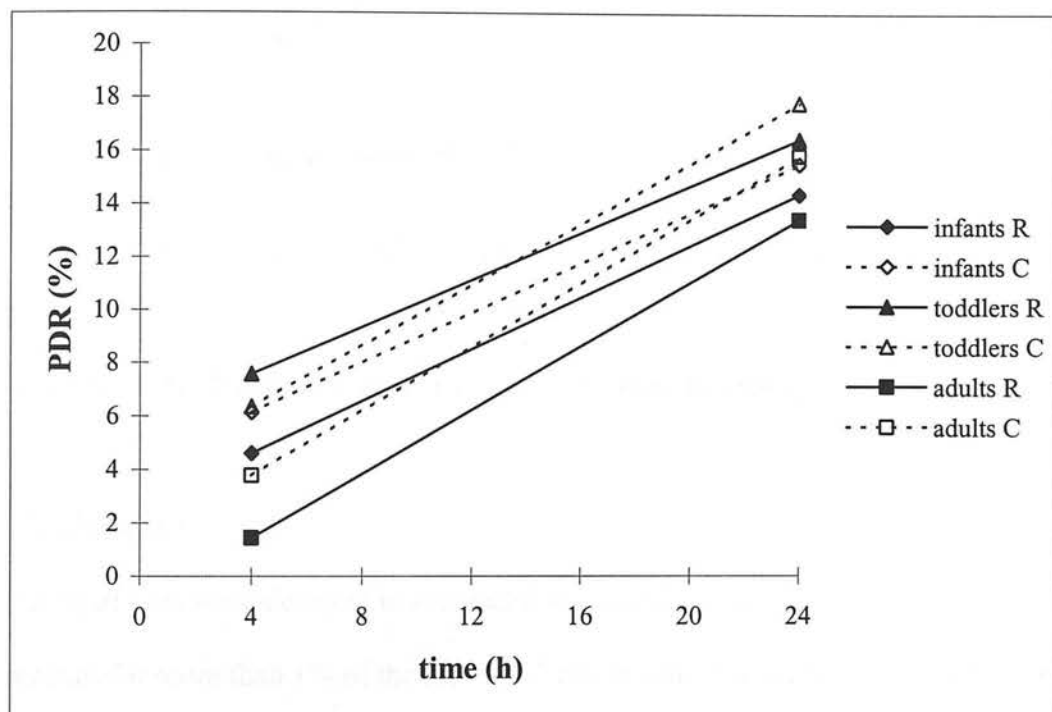


Figure 8b. Median CO₂ results for raw (R) and cooked (C) starch for each age group.

8.2 Other gaseous products

8.2.1 H₂

The median total hydrogen production increased between 4 h and 24 h for the stools of all subjects apart from adults with cooked starch. For raw starch at 4 h, the pattern of H₂ production reflected that of CO₂ production and was significant ($p < 0.01$). At 24 h there were no significant differences (figure 8c).

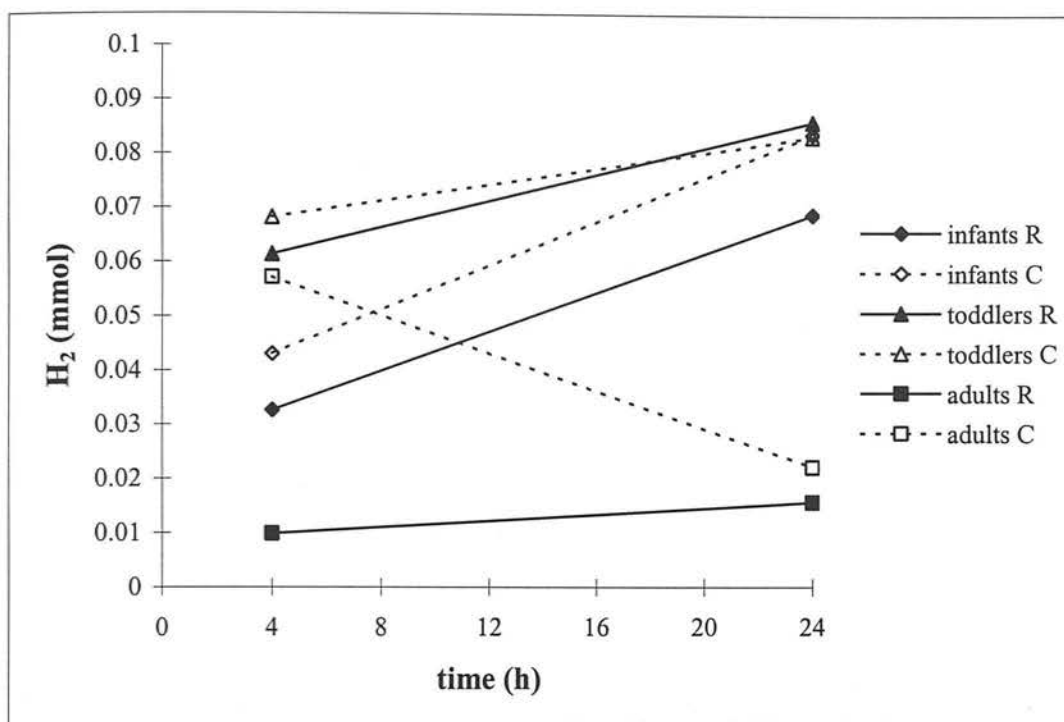


Figure 8c. Median H_2 results for raw (R) and cooked (C) starch for each age group.

8.2.2 Methane

Traces of CH_4 were detected in some subjects, mainly adults. These traces did not account for more than 1% of the recovered starch carbon apart from in the adult stool samples at 24 h.

8.3 pH

The median pH fell between 4 and 24 h for all incubations apart from cooked starch in infants. The lowest median pH was seen for infant stools both at 4 h (median pH 5.44 for cooked starch) and 24 h (median pH 5.71 for raw starch). The results are shown in figure 8d.

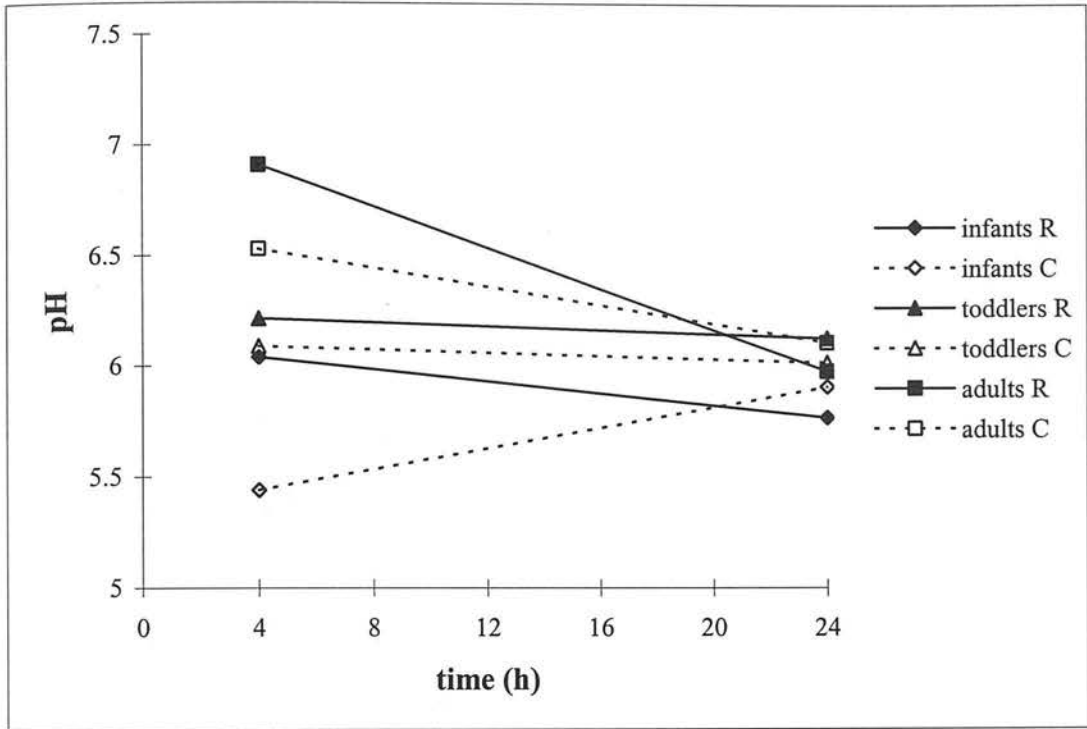


Figure 8d. Median pH results for raw (R) and cooked (C) starch for each age group.

8.4 SCFA

8.4.1 Total SCFA

At 4 h, the highest concentrations of total SCFA were found in infant stools. There was a decreasing trend of SCFA production with increasing age. The difference was significant ($p < 0.05$) between the stools of toddlers and adults for raw starch.

Between the stools of infants and toddlers for raw starch, and for all groups for cooked starch, the differences did not reach statistical significance. At 24 h, the pattern had changed with most SCFA produced by the stools of infants, followed by adults and finally by toddlers. The differences however did not reach statistical significance (figure 8e).

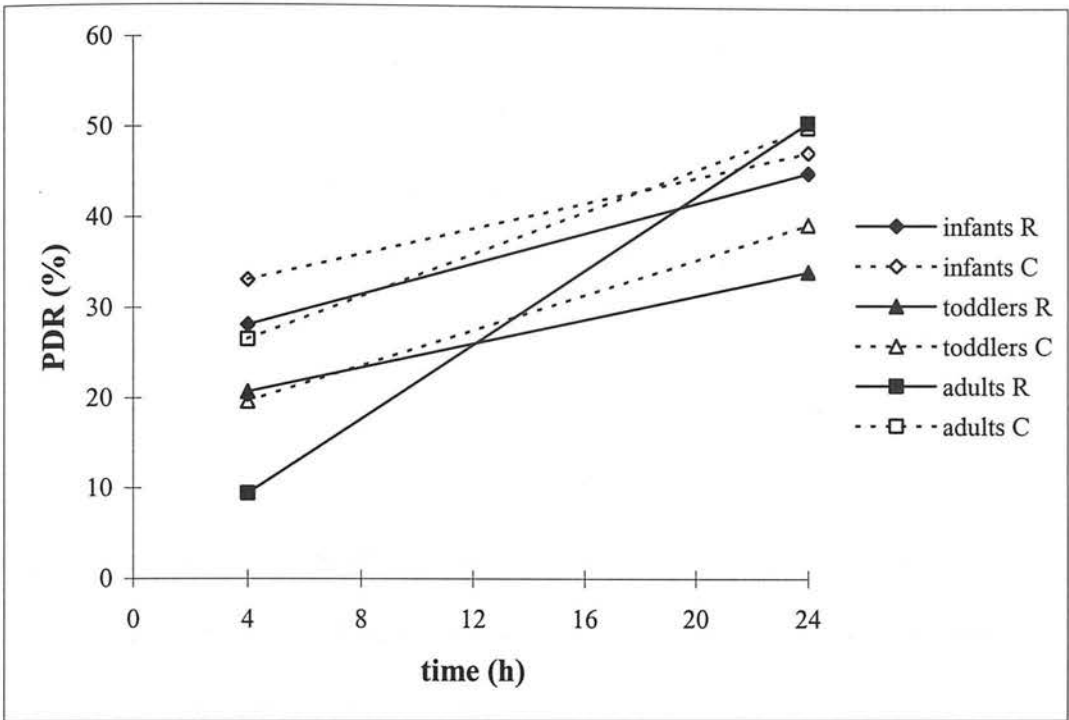


Figure 8e. Median SCFA results for raw (R) and cooked (C) starch for each age group.

8.4.2 Lactate

Lactate was seen mainly at 4 h. There was a significantly ($p < 0.05$) decreasing trend of lactate production with increasing age for raw starch, but no significant differences for cooked starch. The stool of one infant produced unaccountably large concentrations of lactate even in the corresponding blank McCartney tube. The explanation for this was not clear and all her data were omitted from the analysis.

At 4 h, the median lactate PDR for infants' stools fermenting cooked starch was 20%. However, in adults only two out of six subjects produced measurable amounts of lactate. At 24 h it was hardly seen in any age group.

8.4.3 SCFA proportions

The differences in the proportions of SCFA and lactate produced from the fermentation of cooked maize starch are shown in figure 8f. The most striking difference is the large proportion of lactate produced by infants and toddlers at 4 h. At 4 and 24 h, the proportions of acetate were similar for all three age groups. Valerate was seen in measurable amounts only in adult stools. Propionate and butyrate were also seen in similar quantities in all age groups, particularly at 24 h.

Figure 8g shows the differences in SCFA and lactate proportions for raw maize starch. These results differ from the results for cooked starch in that a greater proportion of acetate is produced by infants and toddlers at 4 h. This is offset by a smaller proportion of lactate at 4 h.

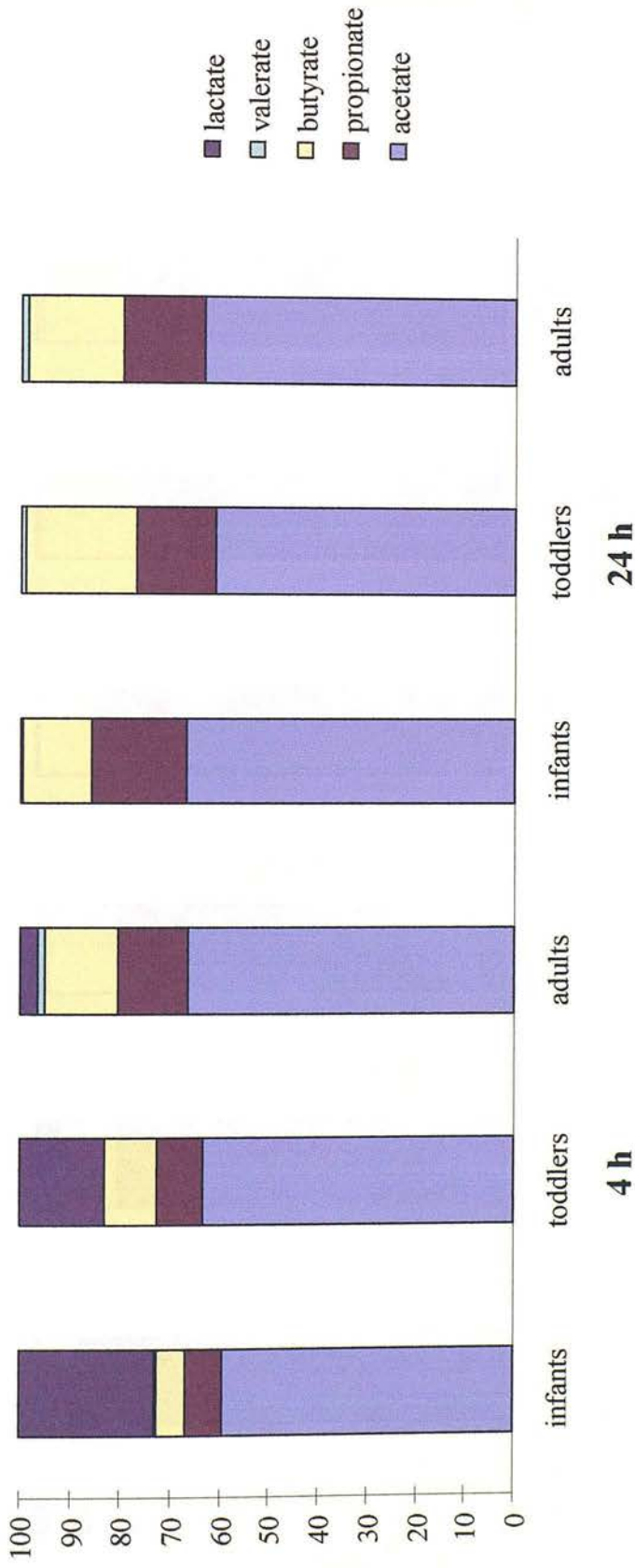


Figure 8f. SCFA proportions for cooked starch at 4 and 24 h.

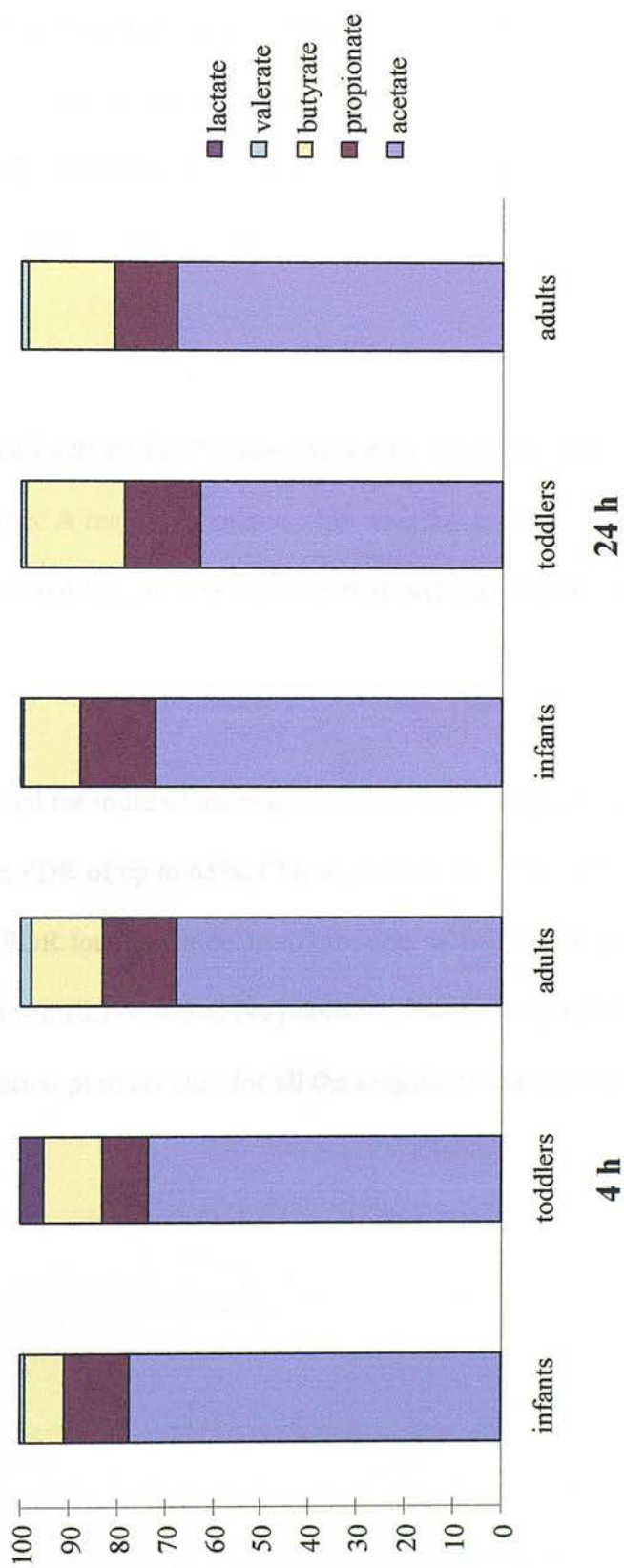


Figure 8g. SCFA proportions for raw starch at 4 and 24 h.

8.5 Residual starch

The residual starch results were variable (table 8a). However, they do support those for SCFA, in that for adults, little fermentation took place in the first four hours. For one infant there was one measurement that was above the analytical range for cooked starch at 4 h. The individual result was left out of the analysis, but the remainder of the data for the subject in question were included.

8.6 Biomass

The confidence intervals for the biomass results (table 8a) were too large to draw any firm conclusions. A reciprocal relationship with the residual starch results might have been expected but this was not seen with analysis of individual results.

8.7 PDR

SCFA accounted for more of the original starch substrate than any other fermentation product with a PDR of up to 65%. CO₂ accounted for up to 18% of the starch carbon. The summed PDR for all fermentation products calculated for each individual subject varied from 25 to 103%. No published data were found with which to compare this attempt to account for all the original starch substrate.

PART II: *IN VITRO* MODELLING OF COLONIC STARCH

FERMENTATION

CHAPTER 9. DISCUSSION

9.1 Summary of main findings

These results have shown that it is possible to carry out incubations of human faeces using raw and cooked starch as a substrate and measure fermentation products in conditions under which the physiology of the proximal colon is simulated. If human faecal flora may be assumed to represent the flora of the proximal colon then this methodology offers an attractive means to study the fate of starch which passes undigested into the colon (resistant starch).

Expressing results as percentage dose recovered (PDR) in the same way as for stable isotope breath tests allows the fermentation product to be referred to the original substrate. The summated PDRs varied between 25 to 103% (median 60%), indicating a need for careful review of the methodology, particularly the starch and biomass assays prior to its routine adoption. The most abundant fermentation products were SCFA but a considerable amount was also in the form of CO₂. Only small concentrations of methane were detected and intermediary production of lactate by the stools of infants and toddlers but not of adults were demonstrated.

There were differences in the fermentation patterns of these three age groups and between the fermentation of cooked and raw starch. These are discussed below.

9.2 Previous *in vitro* models

9.2.1 Faecal analysis

Measurement of SCFA in faecal samples is a useful tool to study fermentation in the human colon and has a long history (266). It can give information about the fermentability of a substrate when faecal SCFA are measured both before and after feeding (112). It gives only indirect information about fermentation in the proximal colon where most fermentation is believed to take place, however if fermentation is largely complete in the proximal colon, there will be very little change in faecal SCFA.

9.2.2 Studies of faecal incubation

Simulated models of fermentation using faecal flora give better information if faecal flora may be assumed to be representative of the flora in the proximal colon. This assumption may only be made if the stool samples are processed quickly to avoid continuing fermentation and the loss of strict anaerobes (112). A second limitation to the technique concerns the ability to mimic conditions *in vivo* since in static cultures fermentation products are not absorbed and may build up to bacteriostatic levels. Such methods are however easy to perform and samples of faeces are readily available. The simplest and cheapest of these approaches is the static batch culture which has been used for the study of colonic fermentation in infants (137,140,267).

Recent systematic evaluations of different laboratory methods have taken place, resulting in a standardised model for dietary fibre (268) and resistant starch (185)

each validated for adult stool samples. The different standardised methodologies are shown in table 9a. There has been no standardisation of infant models. A smaller quantity of faeces is available and there are problems in obtaining a sample on demand making rapid processing difficult.

However, the fermentative ability of faecal flora in infants does vary and is therefore particularly worthy of study. The greater variation of faecal flora fermentation among young children than among adults has been ascribed to diet (188). More specifically, changes related to weaning have been demonstrated. Parrett, Edwards and Lokerse (140) showed that the stools of recently weaned infants were more able to ferment an oligosaccharide (rafitlose) than the stools of pre-weaned infants, but that the ability to ferment guar gum, a complex carbohydrate did not develop until at least 16 weeks after the start of weaning. Whether the type of initial feeding is breast milk or formula is also important: the same investigators showed that the SCFA profile produced from incubations of stool from formula-fed, pre-weaned infants was more similar to an adult pattern than the stool from breast-fed pre-weaned infants (267) and this finding has been endorsed by others (137). Lifschitz, Wolin and Reeds (137) found a higher proportion of propionate in formula-fed infants and a higher proportion of lactate in breast-fed infants suggesting that the colonic flora of formula-fed infants is established earlier than the flora of breast-fed infants. In formula-fed infants they showed a negative correlation with age and stool fermentability and implied that growth and development of flora are independent of the introduction of solids into the diet. Parrett and Edward's work (140,267) and the findings of this thesis show that changes in fermentability also take place during

weaning which may be explained both by changes in the flora and changes in the ability to ferment by individual species.

Table 9a. Comparison of methodologies for stool fermentation.

	Barry et al, 1995 (268)	Edwards et al, 1996 (185)
Medium	Carbonate buffer	Phosphate buffer
	Macrominerals	pH 7.0
	Trace elements	
	Urea	
Inoculum	20 g.L ⁻¹	16 g.L ⁻¹
Substrate	10 g.L ⁻¹	10 g.L ⁻¹
Shaking	Shaking water bath / orbital shaker	Shaking water bath / orbital shaker
		50 strokes/min
Duration	0, 6, 10-24 h	0, 2, 4, 24 h
End points	Residual NSP	Residual starch
	pH	pH
	SCFA	SCFA and lactate

9.2.3 Current data comparison with previous results

The data described in this thesis suggest a greater fermentative capacity in infants and toddlers than in adults, in contrast to Parrett and co-workers (140) who found that the fermentative capacity of the infant's colon even by late weaning was only 75% that of the adult's colon. These results were based on fermentation products at 24 h. The results from the current study show similar results between infants and adults at 24 h, but a greater fermentative capacity at 4 h. The inconsistency between these two sets of results may be explained by differences between subjects and

substrates: the infant group in the current study was very slightly older than Parrett et al's (140) late weaning group (7 - 10 months, median 9 months; and 5 - 11 months, median 7 months respectively). Variations in the timing of the onset of weaning make comparisons difficult, but the stark differences that are observed may be explained by:

- a) the small age difference accounting for much, suggesting a rapid increase in fermentative ability of the colonic flora in late infancy, and
- b) maize starch being present in the diet in greater concentrations than guar gum, which would result in proportionally more maize starch passing undigested into the colon. The subsequent adaptive changes in bacterial flora could then result in greater fermentation of maize starch.

9.3 Stoichiometry

9.3.1 Background

Stoichiometry considers the products of a chemical reaction in relation to its reactants. It is defined as the determination of the relative proportions of the compounds involved in a (single) chemical reaction. In the human colon there are a plethora of complex and inter-dependent reactions and hence a more appropriate term for this "black-box" chemistry might be mass balance. The term stoichiometry is however in general use and will be used for the purposes of this thesis. The importance of deriving such equations lies in the subsequent calculation of available energy.

9.3.2 Previous studies

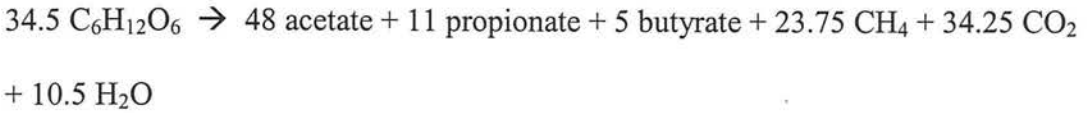
Stoichiometric equations have been derived for specific molecules, for example oligofructose, a non-digestible oligosaccharide, leading to an estimation of its energy value of 1 kcal/g from colonic fermentation (269). Stoichiometric equations have also been derived for specific bacteria to learn more of their nutritional strategies. Marounek and Bartos incubated pure bacterial strains of rumen bacteria with glucose and starch substrates to determine fermentation products and nutritional strategies employed by the various bacterial strains (270).

Overall stoichiometry of the colonic flora has been studied from the perspective of individual organisms. Most bacteria utilise the Embden-Meyerhof-Parnas pathway which has pyruvate as a key intermediate metabolite (194) but bifidobacteria which are known to be a large component of the pre-weaned breast fed infant's colonic flora (105) utilise a unique pathway (271).

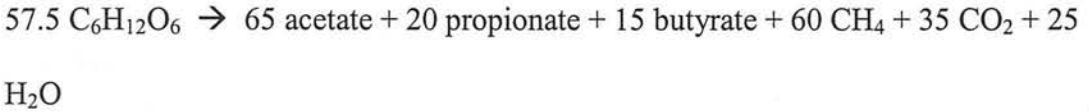
The known fermentation reactions for bacteria of the human large intestine and ruminant forestomach were reviewed by Miller and Wolin (194). Calculating the overall stoichiometry of the colon is more than simply combining various known equations, as different species interact. One such example given is the presence of methanogenic bacteria which are able reduce CO_2 and concomitantly remove H_2 . This may have the effect of favouring oxidising NADH which is a co-factor for the conversion of pyruvate to acetate alone (figure 9a).

By making the assumption that the only products of fermentation were methane, CO_2 and SCFA, and utilising measurements of known concentrations of SCFA in faeces (272) they were able to derive an overall stoichiometric equation for both species and demonstrated that the two reactions were remarkably similar.

Humans:



Rumen:



Assumptions about reactions are made by others studying stoichiometry of the colonic flora. Wolin (273) stated that estimations of fermentation products can be made if four broad assumptions are made:

- (i) SCFA, methane and CO_2 are the only products
- (ii) all products are formed from $\text{C}_6\text{H}_{10}\text{O}_5$ (the major monomeric unit of cellulose)
- (iii) fermentation of 1 mole of hexose generates 4 moles of adenosine triphosphate (ATP), and each mole of ATP provides energy for synthesis of 10.5 g dry weight of bacterial cells
- (iv) SCFA are produced in the same proportions as are found in faeces

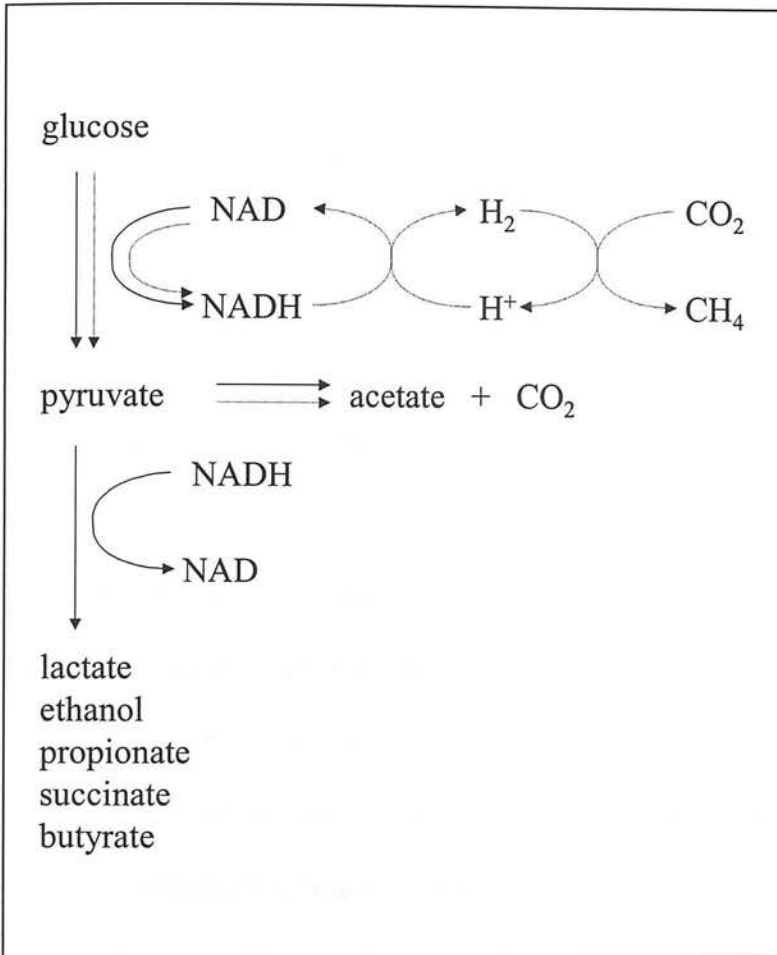


Figure 9a. Pathways of cycling NAD and NADH. Solid lines indicate the pathways without methanogens, broken lines indicate the pathways taken with methanogenic bacterial species showing that acetate and CO₂ are the only products when sufficient methanogens are present to remove NADH and prevent formation of alternative products from pyruvate (194).

These assumptions were made for fermentation in the adult colon. However, the second assumption is still valid for the colon of the young child where resistant starch which is likely to be a more important substrate. Miller and Wolin (194) suggested that further information was necessary to verify their equation for humans and also to consider how it was affected by variables such as age and diet.

Lifschitz and co-workers (137) also made assumptions about known reactions to determine the predominant bacterial species. They assumed three known reactions:

1. 2 hexose \rightarrow 2 lactate + 3 acetate
2. hexose \rightarrow propionate + acetate + CO₂ + H₂
3. hexose \rightarrow 2 lactate

The first reaction is ascribed to bifidobacterial fermentation, so that from the final proportions of acetate, lactate and propionate, the proportion of fermentation via the first reaction can be calculated and hence also the proportion of bifidobacteria. With such a plethora of inter-related reactions, specific conclusions about individual species are difficult to draw with confidence, but it is safe to assume more generally that a difference in fermentation products is related to a change in the composition of the colonic flora.

9.3.3 Findings in this thesis

It has been possible to produce stoichiometric equations from this data by calculating the molar quantities of substrates and fermentation products to compare with Miller and Wolin's derived equations (194). No assumptions about bacterial species and individual reactions have been made, rather this stoichiometry is based on measured amounts of fermentation products. Other important differences between this stoichiometry and that of Miller and Wolin (194) include that these data are based on a single starch as opposed to mixed carbohydrate and that they used data from *in*

vitro modelling rather than measured faecal concentrations of SCFA. Miller and Wolin found that 34.5 moles of carbohydrate produced:

48 acetate + 11 propionate + 5 butyrate + 23.75 CH₄ + 34.25 CO₂ + 10.5 H₂O

Based on the data for cooked starch at 24 h from this thesis, 34.5 moles of carbohydrate produced:

infants: 25.6 acetate + 5.5 propionate + 4.2 butyrate + 0.2 valerate + 0 CH₄ +
 32.2 CO₂ + 18.7 H₂

toddlers: 18.9 acetate + 4.8 propionate + 6.2 butyrate + 0.2 valerate + 0 CH₄ +
 36.9 CO₂ + 18.6 H₂

adults: 21.2 acetate + 4.1 propionate + 5.7 butyrate + 0.4 valerate + 0 CH₄ +
 32.8 CO₂ + 5.0 H₂

In spite of a difference in the derivation of these equations, there is closer similarity between these equations and Miller and Wolin's human data than between their equations for humans and rumen. There are distinct differences however and although these may be due to methodological differences, they are worthy of comment. For all age groups, less methane was found, which may be an artefactual result due to slow development of methanogens or increased partial pressure of hydrogen needed before methane can be produced. In addition, methanogenic bacteria may not have been the predominant organisms in the infant stool cultures. It is known already that methane is the only gas produced by rumen as all hydrogen is used. A different eco-system exists in the colon, but in infancy methanogens are slow to colonise. Also lower amounts of acetate and propionate were found in all age

groups. These findings may also be related to the poorly summed PDRs which are difficult to explain.

Miller and Wolin (194) did not account for lactate in the equation which may be valid when colonic flora from adults is considered. However, it is an important intermediary product in infants and to a lesser extent in toddlers. It is also possible that in adult stool fermentations there is another intermediary product, such as pyruvate or succinate (194) that has not been measured which may in part explain these low summed PDRs.

The concept of PDR is usually associated with stable isotope breath tests, where it expresses the measured amount of expired $^{13}\text{CO}_2$ in relation to the ^{13}C enrichment of the original substrate (168). These results are expressed as PDR because this gives more information than absolute measurements of products by measuring them in reference to the initial fermentation substrate and quantifies the fate of the starch carbon substrate. The concept is subtly different from stoichiometric equations in that with PDR, the fate of an individual carbon atom is considered rather than an individual molecule of carbohydrate. It is also arguably easier to relate any individual product back to the original substrate when considered as percentage dose recovered. Lifschitz and co-workers (137) in similar fashion expressed their data both in terms of overall carbon balance (i.e. PDR) and in terms of the pattern of fermentation or micromole of product per micromole of fermented substrate (i.e. stoichiometry).

9.4 Molar ratios

9.4.1 Previous studies

The relative proportions of individual SCFA resulting from colonic fermentation have been studied previously in different ways. SCFA are the fermentation products with which there is most interest and since they may be measured with a single technique, this approach circumvents many of the potentials for laboratory error resulting from many different and difficult assays. Cummings et al (119) calculated molar ratios for the main SCFA, acetate: propionate: butyrate from colonic SCFA concentrations in the colons, portal vein, hepatic vein and peripheral blood of adult victims of sudden death. Their results are shown in table 9b.

From these results they concluded that others' findings of lower faecal concentrations of SCFA served only to illustrate the limitations of faecal data. They suggested that the decreasing concentrations of all SCFA between the colon and the peripheral blood demonstrated firstly that the lower gut was the source of SCFA and secondly that there was significant uptake of SCFA by the liver. They proposed that lower proportions of butyrate in the portal vein than in the colon suggested butyrate uptake by the colonic mucosa. Although this data is based on only six adult subjects, it is one of the few instances where SCFA concentrations have been measured directly in the colon using a method validated in animals. Such data is not likely to be reproducible in young children and alternative methods have been sought.

Table 9b. Molar ratios of the SCFA, acetate, propionate and butyrate measured from different sites in six adult victims of sudden death. Results are expressed as percentage of total SCFA and are shown as mean (SEM). From Cummings et al (119)

	acetate	propionate	butyrate
right colon	57 (2)	22 (2)	21 (2)
left colon	57 (1)	21 (1)	22 (1)
portal vein	71 (4)	21 (4)	8 (1)
hepatic vein	81 (2)	12 (2)	7 (1)
peripheral vein	91 (1)	5 (2)	4 (1)

Others have calculated molar ratios based on *in vitro* fermentation methods. Parrett and Edwards (267) calculated molar ratios of acetate, propionate, butyrate and lactate from *in vitro* fermentation of breast-fed, formula-fed and adult faeces. The infants were 2 - 10 weeks old. Their results for soybean polysaccharide, the substrate closest to the corn starch substrate of this work, are shown in table 9c, although the results are not strictly comparable as soybean polysaccharide is likely to have a different fermentability in infants than corn starch.

They commented on the lower ratio of butyrate seen in both breast-fed and formula-fed infants, speculating whether the colonic enterocytes of infants have different characteristics to their adult equivalents.

They also looked at molar ratios for older infants at different stages in the weaning process by carrying out *in vitro* fermentations with faeces from pre-weaned infants, in infants during early weaning (within 6 weeks of the onset of weaning) and in

infants during late weaning (at least 16 weeks after the onset of weaning). The substrates used included glucose, rafterose (an oligosaccharide) and soybean polysaccharide as an example of a polysaccharide present in the weaning diet. The results for soybean polysaccharide for these subjects are shown in table 9d.

Table 9c. Molar ratios of acetate, propionate, butyrate and lactate from *in vitro* fermentation of breast-fed, formula-fed and adult faeces at 24 h. Results are expressed as percentage of total SCFA and are shown as median (range). Adapted from Parrett and Edwards (267)

	acetate	propionate	butyrate	lactate
breast-fed	74 (55 - 86)	19 (12 - 36)	1 (0 - 21)	0 (0 - 9)
formula-fed	57 (0 - 73)	29 (0 - 46)	4 (0 - 17)	0 (0 - 1)
adults	55 (42 - 61)	23 (15 - 33)	11 (7 - 19)	4 (0 - 31)

Table 9d. Molar ratios from *in vitro* fermentation from the stools of older infants at different stages in the weaning process. Results are expressed as percentage of total SCFA and are shown as median (range). Adapted from Parrett, Edwards and Lokerse (140)

	acetate	propionate	butyrate	lactate
pre-weaning	76 (55 - 87)	19 (12 - 36)	1 (0 - 21)	0 (0 - 12)
early weaning	67 (57 - 75)	21 (11 - 33)	3 (0 - 28)	0 (0)
late weaning	66 (17 - 77)	15 (8 - 24)	11 (3 - 18)	0 (0 - 3)

9.4.2 Findings in this thesis

The results of this thesis for adult stools at 24 h, graphed in figure 8f and figure 8g, are shown in tabulated form in table 9e. They are broadly in agreement with Parrett and Edward's (267) adult results.

Table 9e. Molar ratios from *in vitro* fermentation of adults' stools. Results are expressed as percentage of total SCFA and are shown as median (range).

	acetate	propionate	butyrate	valerate	lactate
raw starch	71 (52 - 88)	12 (8 - 19)	18 (1 - 34)	1 (0 - 6)	0 (0)
cooked starch	59 (47 - 85)	13 (10 - 29)	18 (2 - 31)	1 (0 - 7)	0 (0)

This group of infants, with a slightly older age range than Parrett et al's (140) late weaning group had molar ratios of SCFA as shown in table 9f for raw and cooked starch at 24 h. The proportions are strikingly similar.

Table 9f. Molar ratios from *in vitro* fermentation of infants' stools. Results are expressed as percentage of total SCFA and are shown as median (range).

	acetate	propionate	butyrate	valerate	lactate
raw starch	68 (64 - 81)	15 (6 - 25)	11 (2 - 24)	1 (0 - 1)	0 (0)
cooked starch	67 (62 - 76)	19 (5 - 24)	14 (6 - 27)	1 (0 - 1)	0 (0)

Molar ratios calculated here for adult stools fermenting cooked starch (59:13:18) were broadly in agreement with the findings of Cummings and co-workers (119) calculated from fresh post-mortem examinations. This finding gives some confidence to the interpretation of molar ratios from *in vitro* fermentation models. There is

consistency with previous studies: between these adult samples and adult samples from Parrett and Edwards (267); and between these samples from infants and those of Parrett, Edwards and Lokerse's (140) older infant group. As a result there is validity in comparing different age groups within this data.

9.5 Interpretation of results

9.5.1 Differences in fermentation patterns between age groups

Rather than a general increase in fermentation with increasing age, there was evidence of increased fermentation with young children. Overall at 4 h there was a trend towards lesser SCFA production with increasing age but these differences were not seen at 24 h, indicating that fermentation may take place more rapidly in the colons of young children than in the colons of adults. Greater proportions of SCFA from incubations of the stools of young children at 4 h, was reflected in a sharper fall in pH at 4 h with the same subjects. This was not reflected with CO₂ and H₂ production and in fact with adults the amount of H₂ measured fell between 4 and 24 h. These results point to CO₂ and H₂ being intermediate products in the formation of either methane or acetate and higher order SCFA.

There were similarities and differences in the SCFA profiles between different age groups. All groups produced acetate, propionate and butyrate but only adults produced measurable amounts of valerate which is consistent with previous results (267). Infants and children produced larger amounts of lactate at 4 h, though this was an intermediate product and was no longer present by 24 h.

What does appear to be the case, rather is a different pattern of fermentation seen in different age groups: a comparison of the SCFA proportions for the three age groups suggests that in infancy there is proportionately more acetate. This is in agreement with the findings of others (267). However, with these data the adult-type pattern of proportionately increased butyrate was established from an early age. This was not seen with Parrett, Edward's and Lokerse's (140) slightly younger "late weaning" group, but the larger variability with that group is noteworthy (table 9e).

Additionally, this may have been influenced by the substrate. Resistant starch is known to be preferentially fermented to butyrate (138,139) and fermentation of corn starch may be of greater nutritional significance than soybean polysaccharide in the diets of newly weaned infants.

If efficiency of fermentation were to be defined in terms of the number of molecules of energy-rich products (SCFA) per molecule of CO₂ in which the energy is lost as heat, it might be speculated that in the colons of infants more efficient early fermentation takes place. The measurement of fermentation products at 4 h and 24 h was arbitrarily chosen; Lifschitz and co-workers (137) measured products after 1 hour. With SCFA rapidly removed from the incubation liquid *in vivo*, closer attention should be paid to those fermentation products that are formed rapidly.

9.5.2 Differences in fermentation patterns between substrates

Raw and cooked starches were chosen as substrates to model what would have been expected to be a resistant starch and a rapidly digestible starch respectively (34).

Since in young children the luminal concentrations of pancreatic α -amylase are

lower than in adults (60), the proportion of starch passing into the colon is likely to be greater and using a model resistant starch might have given more information about the conditions *in vivo*. A difference was observed between the fermentability of the two preparations.

Differences in the rate of fermentation were more marked with raw starch than with cooked starch. At 4 h, adult stools were better fermenters of cooked starch than raw starch with higher concentrations of H₂, CO₂ and SCFA. These results were also mirrored by the measurement of lower residual starch. The same difference between the fermentability of raw and cooked starch was also observed for infants though not for toddlers. These differences between the fermentability of raw and cooked starch were only present at 4 h however.

Greater proportions of lactate were seen with after 4 h fermentation in both infants and toddlers when the substrate was cooked starch. With raw starch the stools of infants and young children produced greater amounts of acetate than adult stools. The greater relative fermentability of raw starch by the faeces of infants and toddlers was surprising. However, raw cereals do form an important component of the weaning diet in the United Kingdom and it is speculated that colonic flora of young children may be more induced to ferment raw starch than the colonic flora of adults. More generally, with better fermentation by toddlers and infants at 4 h, the colonic flora of young children may be more efficient fermenters at a time when energy salvage by the colon is of greater importance than in later life.

9.6 Limitations of methodology

9.6.1 Stool collection

Stool samples were collected and processed within 2 hours of passage. Others (185) have processed within 1 hour, but this was only possible for adult samples, which could be obtained on demand and for samples from children in the hospital nursery. It is possible this may have produced a bias in favour of the survival of greater numbers of anaerobes in the stools of adults. Although no validation studies were carried out comparing fermentation products from samples processed within 60 minutes compared to within 120 minutes, the similar findings to others work (140,267) and good anaerobic bacterial survival in preliminary experiments suggests that processing within 2 hours was equally valid.

9.6.2 Substrate and dose

Corn starch was readily available at the time of the studies. With hindsight, potato starch might have been a better substrate. Potato starch is known to form retrograded starch on cooling after cooking and it might have better differentiated between cooked and raw starch as well as demonstrate differential properties in the autoclaved state.

The substrate dose of 25 mg in combination with 5 ml of a 5% faecal slurry in a 30 ml McCartney bottle differs slightly from the consensus methodology (185). This value was chosen for the experiments in this thesis as it produced the greatest counts of viable anaerobes after 24 h incubation. In spite of this it is not known how

physiological was the ratio of substrate to bacteria. The conclusions and applications *in vivo* are consequently limited.

9.6.3 Reproducibility

9.6.3.1 starch assay

Others have used the rate of disappearance of substrate as a measure of the rate of fermentation (137). The residual starch assay for this thesis required meticulous attention to detail and the wide variation of results limits the conclusions that can be drawn from the residual starch results in isolation. However, the results for both residual starch and biomass broadly mirror those for the gases and SCFA where there is greater confidence in reproducibility.

9.6.3.2 biomass assay

Similarly with the biomass assay, there was large variation in the results limiting interpretation of the findings. Some sample was lost from the tin boats during preparation though with samples combusted in triplicate this does not fully account for the variability. The results have been presented because they represent the final product assay to complete the attempt to account fully for the fate of the fermented starch. Quality assurance work needs to be undertaken before these results are interpreted further. It is also possible to measure nitrogen by this means. This may have provided a checking mechanism when the main product sought would have been bacterial protein.

9.6.4 Modelling assumptions

Many assumptions are made about the accuracy of simulating colonic physiological conditions. Despite limitations to the system, it is currently the best non-invasive method for the large-scale study of colonic fermentation in the human colon. The model has been validated for adults (185) but different colonic conditions in the three different age groups were not accounted for in differences in simulated modelling. Modelling conditions such as the availability of co-factors for age-specific bacterial species could not be taken into account.

To have taken account of changes in pH, which have been previously shown to affect fermentation (137) would have involved removing SCFA products from the incubation. A continuous culture system might have been able to achieve this, but this would not have been practical to study differing fermentation patterns in many different subjects. Since all incubations were subject to the same experimental conditions, referential integrity may have mitigated some of the unphysiological conditions.

9.6.5 Calculations

Many calculations were carried out before arriving at the final percentage dose recovered of fermentation products. Poorly summed PDRs suggest either that there are other fermentation products which were not measured, such as succinate and pyruvate (194), or that large inaccuracies exist in some of the assays. More validation work is required to establish this and refinement of the combustion technique, where all carbon should be accounted for may be one possible approach.

9.6.6 Contamination by product present in faeces

Contamination by endogenous substrate within the faeces was considered by correction for fermentation of a faecal slurry without substrate. The molar ratios of SCFA however were calculated without correction which is generally accepted to be unnecessary when proportions are considered (140,267). When considering fermentation products present in low concentration the capacity for error within this correction method increases. Also, it may not be a valid assumption that fermentation will proceed in a similar pattern without the addition of a substrate.

9.6.7 Dry weight of faeces

Faecal samples were not freeze-dried to determine their dry weight and therefore fermentation products were not referred back to the dry weight of faeces. This would have been able to clarify whether a difference in stool water content and a subsequent difference in bacterial content influenced the outcome of the fermentation reaction. However, Parrett et al (140) found that faecal water did not vary at different ages and Lifschitz and co-workers (137) found that correction for stool dry weight did not change their results.

9.6.8 Sample size

No prior power calculation was carried out to determine the appropriate sample size and in this sense the work represents a pilot study. The significant findings are also limited by the large number of parameters that were compared. The work would be strengthened by focussing on the most important differences that were found and

determining the appropriate sample size to demonstrate a significant difference.

Some parts of the study may be difficult to validate such as the starch and biomass assays and it would be worth consideration in focussing on those areas where there is better validation such as the comparison in SCFA proportion. This is further discussed in the future research section of the next chapter.

9.7 Summary

The main finding that the faecal flora of young children was more efficient at fermenting starch, particularly less fermentable raw starch, fits with existing ideas about the potential for energy salvage by the colon in early life (172) and may indicate that a highly efficient fermentative process takes place in the colons of young children who are well-established onto weaning foods. The next chapter discusses estimation of energy salvage from these data and estimates of stoichiometry for the whole gut by combining the data from stable isotope breath tests from the first part of the thesis.

Chapter 10. General Discussion and Conclusions

10.1 Introduction

The role of the colon in early childhood is not well understood. There is good reason to suppose that the colon plays a part in energy salvage in early life when the digestive capacity of the small intestine is less but this contribution has not been measured or estimated. If the relative roles of small intestine and colon were better quantified, advice about weaning foods could be based on their potential energy yield from small intestinal digestion and colonic fermentation at any given age. If the role of the colon and the consequences of fermentation, both short and longer term, were better understood, nutritionists would have solid evidence upon which to base recommendations about weaning foods that could increase or decrease the amount of resistant starch and dietary fibre and consequently encourage or discourage SCFA production in the colon.

The energy yield from carbohydrate that is not digested and absorbed in the small intestine (termed unavailable carbohydrate) has been calculated for adults (121) but less attention has been focused on the physiology of the large intestine of the young child. This thesis has attempted to address this by defining the site, rate and extent of starch digestion in children around the weaning period.

The stable isotope breath test results described earlier suggested that for children between 8 and 23 months consuming wheat flour, the colon accounts for up to 20% of its total digestion. Since most of the digestible and fermentable material in flour is

starch, it seems reasonable to extrapolate these results to wheat starch. The *in vitro* fermentation studies further suggested that the fermentative capacity of the colon for less digestible, commonly consumed dietary starches may be greatest soon after weaning is established.

This chapter will use data from the *in vitro* starch fermentation studies to interpret more clearly the stable isotope breath tests. Calculations using both data sets will be carried out and implications drawn for whole gut digestion as a result of the combined data. The interpretation and implications of the results will be discussed and how this may impact on current weaning recommendations. There will be some speculation about the metabolic effects and consequences of alterations to the weaning diet. After the conclusions of the thesis, some suggestions of hypotheses generated to be tested in the future and some areas for future research will be discussed.

10.2 Calculations combining both data sets

10.2.1 Bacterially-produced $^{13}\text{CO}_2$

In figure 10a, the derivation of breath CO_2 that is measured in starch stable isotope breath tests is schematically illustrated. It has three potential sources: from oxidised glucose in turn from hydrolysed starch occurring in the small intestine; from the oxidation of SCFA in turn resulting from fermentation of residual starch in the colon; and from CO_2 produced as a bacterial side-product during that same colonic fermentative process. For interpretation of the modelled data, it is assumed that the first deconvoluted curve results solely from oxidation of glucose. The second

deconvoluted curve was assumed to result solely from the oxidation of SCFA, making the additional assumption that the proportions of glucose and SCFA directly oxidised are the same. *In vitro* studies described in the second part of the thesis have shown that in a fermentation model, CO_2 may be derived from two sources, namely oxidation of SCFA and as a direct bacterial product. Only that proportion of CO_2 produced by SCFA oxidation truly represents the contribution of the colon to digestion and by knowing the relative proportions of CO_2 from the two sources the contribution of the colon (and therefore the relative contribution of the small intestine) can be recalculated.

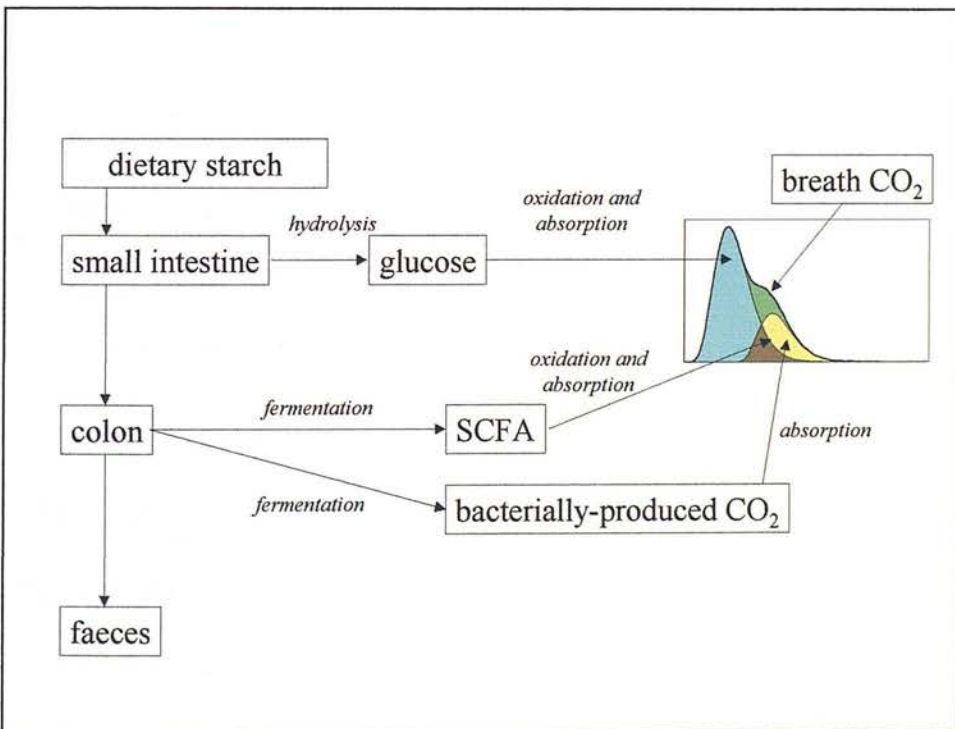


Figure 10a. Different metabolic pathways that have a final common product in breath CO_2 . Before stoichiometric calculations can be carried out, the differing proportions resulting from small intestinal CO_2 , colonic CO_2 produced from oxidation of SCFA and colonic CO_2 as a direct bacterial product of fermentation need to be determined.

Of the carbon-containing fermentation products that were measured at 24 h in the *in vitro* studies, only SCFA and CO₂ itself could be said to account for the colonic CO₂: residual starch and bacterial biomass do not account for the breath CO₂, and at 24 h, there is negligible lactate that was measured. Hence, for the benefit of this calculation the proportion of colonic CO₂ accounted for by directly produced bacterial CO₂, is not the PDR for CO₂, but rather the proportion of CO₂ where the denominator is SCFA and CO₂ (table 10a). These considerations mean that the relative contribution of the colon to whole gut digestion of starch will be less.

Table 10a. PDRs for SCFA and CO₂ for fermentation of raw starch at 24 h from faecal samples from infants, toddlers and adults. Figures shown are median (range).

	total SCFA		CO ₂		CO ₂ as % of SCFA + CO ₂	
infants	45.1	(37.9 - 58.3)	14.4	(4.5 - 16.6)	24.2	(10.5 - 22.1)
toddlers	34.2	(25.8 - 48.8)	16.5	(12.7 - 19.4)	32.5	(32.9 - 22.5)
adults	50.7	(30.9 - 64.6)	13.4	(10.5 - 20.0)	20.9	25.4 - 23.7)

The calculations make the assumption that the cPDR is constant between substrates. Many substrates have been used for ¹³C breath tests and there is great variability in the recovery of ¹³CO₂. The recovery of ¹³CO₂ from bicarbonate studies is high as might be expected. Values range between 73% and 98% in subjects which comprise children and adults (274-277). One study found no significant difference between adults and children (276). Similarly the yield from ¹³C-LU studies has been as high as 80% in some studies (255,257).

In contrast, the yield from other substrates is consistently less. The mixed triglyceride breath test has a reported $^{13}\text{CO}_2$ yield between 21% and 36% in published studies (278,279) and the octanoate breath test to measure gastric emptying has a yield of 31 – 53% (280). These values are in keeping with the $22.7 \pm 9.3\%$ for starch found in the current study. In an attempt to reflect this difference in substrate cPDR from *in vivo* studies, a final corrective factor of 2 has been introduced to correct for the assumed twice higher yield of $^{13}\text{CO}_2$ from bacterially-produced CO_2 than from glucose or SCFA. These adjustments can be seen in table 10b.

10.2.2 Whole gut stoichiometry

Combining the data from stable isotope breath tests and *in vitro* fermentation studies allows an estimation of stoichiometry or mass balance to be made for the whole gut. As before, it is possible to do this either in terms of a stoichiometric equation or expressed as PDR. For Miller and Wolin (194) to derive their equations, certain assumptions had to be made about the products of fermentation, the production of ATP, the requirement of ATP for cell synthesis and that faecal contents of SCFA are a true representation of colonic production of SCFA (273). With this data assumptions have still been made about the validity of faecal SCFA content and the products of fermentation but otherwise this stoichiometry is calculated from measured substrates and products.

Table 10b. Adjustments made to the component cPDRs of small intestine and colon, factoring for CO₂ produced directly from fermentation. Adjustment 1, considered that bacterially-produced CO₂, accounted for some of the colon produced CO₂. The second adjustment factored in that all the CO₂ was produced from either SCFA or directly from bacteria and used “CO₂ as % of SCFA + CO₂” from table 10a rather than CO₂ PDR to calculate more accurately, that contribution of bacterially-produced CO₂. The third adjustment factors for a greater yield from bacterially-produced CO₂ than other tracers. The correction factor of 2 has been used.

		small intestine	colon
median component cPDR (%)		80	20
infants	first adjustment	82	18
	second adjustment	84	16
	third adjustment	82	18
toddlers	first adjustment	83	17
	second adjustment	86	14
	third adjustment	83	17
infants and toddlers	first adjustment	82	18
	second adjustment	84	16
	third adjustment	82	18

Subjects used for the breath test data were not the same as those used for the *in vitro* work. The breath test subjects were not divided into the two age groups corresponding to the infant and toddler groups of the *in vitro* group. The age spreads however for both parts of the experiment were similar: for the stable isotope breath test data, the median (range) age was 11.8 months (7.6 to 22.7), and for the *in vitro* fermentation studies, the median (range) age of the infant and toddler groups

combined was 16.2 months (6.9 to 20.7). Thus, using the combined infant and toddler *in vitro* data (fermentation of raw starch at 24 h) with the stable isotope breath test data, calculations about whole gut stoichiometry of starch have been made for children between 7 and 23 months. The median results for the toddler/infant fermentation studies are shown in table 10c.

Table 10c. Median PDR for infants and toddlers from *in vitro* fermentation of raw starch at 24 h.

CO ₂	CH ₄	acetate	propionate	butyrate	valerate	lactate	residual starch	biomass
14.6	0	21.7	6.2	6.6	0.2	0	0.7	7.3

The stoichiometry may be extended to the whole gut by including the data from the *in vivo* studies. From the three adjustments in table 10b, the contributions to whole gut starch digestion of small intestine and colon respectively for infants and toddlers are 82% and 18% respectively. If it is assumed the implication of this calculation is that 18% (17.7% if this is expressed to one decimal place) of starch is not digested in the small intestine, the corresponding PDRs for the whole gut become as shown in table 10d. Of the remaining 18%, a small amount will pass unfermented through the colon to be detected in the faeces. From table 10c, this amounts to only 0.13% of the original starch, 0.7% of the 18% passing into the colon. The summed median PDR of the products of colonic fermentation is 57.2%. Although it is possible that there are small quantities of other metabolic by-products that were not detected, it is more likely that the true yield was not measured. If it were assumed that a common scaling factor could be used for all fermentation products, the whole gut PDR would look like as shown in the second column.

Table 10d. PDR for whole gut digestion of raw starch in infants and children showing data which is both uncorrected and scaled to assume a 100% yield in the colon.

	normal data	scaled data
glucose produced in small intestine	82.3	82.3
acetate	3.8	6.7
CO ₂	2.6	4.5
biomass	1.3	2.3
butyrate	1.2	2.0
propionate	1.1	1.9
valerate	0.0	0.1

The stoichiometric equation for whole gut digestion of wheat starch can be calculated similarly. The comparison with Miller and Wolin's (194) is no longer valid if the small intestinal component is included and the need for the 34.5 mmol of carbohydrate becomes arbitrary.

Hence for 100 mmol starch entering the gut of the young child, from this data, scaling the summed PDR of products of colonic fermentation to yield 100%, the calculated fate of the starch is as follows:

$100 \text{ C}_6\text{H}_{10}\text{O}_5 \rightarrow 82.3 \text{ glucose} + 20.1 \text{ acetate} + 4.2 \text{ propionate} + 5.1 \text{ butyrate} + 0.2$
 $\text{valerate} + 0 \text{ CH}_4 + 27.0 \text{ CO}_2 + 15.3 \text{ H}_2 + 0.2 \text{ residual starch} + 27.1 \text{ additional}$
 bacterial biomass (expressed as moles of carbon)

10.2.3 Energy calculations

The issue of assigning energy values to carbohydrates which are not completely digested in the small intestine has proved problematic. For carbohydrates which are more or less completely fermented in the colon, it has been recommended an energy value of 1.5 kcal/g be used (281). However, it would seem possible that from the stoichiometric equation, it is possible to calculate the energy yield from a known quantity of starch entering the gut. Based on the data of others, Grossklaus (220), calculated the metabolically utilisable energy value of nutrients in terms of the adenosine triphosphate (ATP) gain per mole of nutrient. By determining the energy content of the nutrient and the kilojoules required per mole of ATP, he determined an energy efficiency with respect to glucose. Factoring in the amount of energy input required for the various metabolic pathways involved, the values for acetate, propionate and butyrate are shown in table 10e.

Table 10e. Energy yield of SCFA. Adapted from Grossklaus (220).

	glucose	acetate	propionate	butyrate
ATP gain: mole ATP/mole	38	10	18	27
Combustion value: kJ/g	15.6	14.6	20.8	24.9
kJ/mole ATP	74.1	87.9	85.4	81.2
Energetic efficiency	100	81.4	84.8	90.4

Experimental evidence has shown that the number of molecules of ATP synthesised per molecule of oxygen (the P/O ratio) for $\text{NADH}+\text{H}^+$ and FADH_2 are not 3 and 2 respectively but rather are non-integral values (2.5 and 1.5 respectively). It is now

accepted that oxidation of one molecule of glucose yields 31 rather than 38 molecules of ATP (282).

Of the SCFA, only acetate is detected in the peripheral blood but propionate and butyrate are used as energy sources in the liver and colonocytes respectively (119). From the stoichiometric equation above, and using Grossklaus's energy yield and calculation of energetic efficiency in table 10e (220), 100 mmol of starch would yield:

- 2551 mmol ATP ($82.3 \times 31 \times 100\%$) from small intestinal digestion and absorption of glucose
- 352 mmol ATP from colonic fermentation of resistant starch to SCFA as follows:
 - acetate: $10 \times 20.1 \times 81.4\%$
 - propionate: $18 \times 4.2 \times 84.8\%$
 - butyrate: $27 \times 5.1 \times 90.4\%$

From a total potential ATP gain of 3100 mmol if starch were to be wholly digested and absorbed in the small intestine, this gives a total energy gain of 2903 mmol per 100 mmol starch ($2551 + 352$), and represents a "loss" of energy of 6.3%. This figure is considerably less than the energy "loss" of approximately 30% empirically derived by Kein and co-workers (124,201), but their study used premature infants aged between 2 and 4 weeks and it is to be expected that greater energy salvage takes place in older infants and children where there is a more established colonic flora.

However, there remain other factors which are impossible to quantify such as the influence of physiological state, that is fed or fasted, which will determine whether energy is used immediately or laid down as glycogen or fat, utilising different amounts of energy. Likewise the energy consumed by the colonic flora in fermentation is difficult to quantify. However the bacterial-produced CO₂ as shown in these *in vitro* experiments gives an indication for bacterial energy consumed and when this is expressed in terms of the ratio of acetate to CO₂ produced a relative efficiency of the colonic flora may be derived (table 10f).

Table 10f. Efficiency of fermentation as calculated by unit acetate produced per unit CO₂ expended. Figures shown are median (range).

	acetate/CO ₂ (mmol/mmol)
infants	0.78 (0.74 - 1.25)
toddlers	0.54 (0.42 - 0.72)
adults	0.88 (0.49 - 1.92)

These figures suggest that the colonic flora of toddlers may be the least efficient at fermenting resistant starch into acetate.

10.3 Interpretations and speculations

10.3.1 Metabolic considerations

10.3.1.1 fed and fasted states

In the stable isotope experiments only the CO₂ production, that is, the end point of oxidation, has been measured. This makes no allowance for different metabolic pathways in the fed and fasted state. During the fed state the proportion of energy

which is directly oxidised (dietary induced thermogenesis) will be greater. Also, during the fed state a greater proportion of the CO₂ will result from normal dietary carbohydrate which is more naturally abundant in ¹³C than body fat stores. All subjects commenced the study in the fasted state but the timing of subsequent meal times varied. Dietary induced thermogenesis varies between individuals (283) so that the balance between direct oxidation of carbohydrate and anabolic pathways also varied between subjects at any time point during the study. These physiological phenomena affect the proportions of the relatively abundant ¹³CO₂ resulting from dietary induced thermogenesis and limit the conclusions to be drawn about the magnitude of the colonic component to digestion.

In addition to the effects of exercise on PDR which was discussed in section 5.5.4, physical activity could also alter the glycolytic, lipogenic or oxidative fate of the hydrolysed tracer starch. Although every reasonable effort was made to minimise the physical activity of the subjects, this remains a limitation to such free-living experiments in young children.

10.3.1.2 effect of gluconeogenic versus glycogenic pathways

In animal and in adult human studies, SCFA have been shown to affect metabolic pathways and in particular the sensitivity of insulin. Holtenius and co-workers (134) demonstrated that increasing the dietary SCFA content of sheep may have a long-term inhibitory effect on basal insulin and glucagon levels. In rats fed a Western diet, Venter (135) demonstrated increased insulin sensitivity when the diets were supplemented with propionate. In adult humans, an increase in insulin sensitivity was

observed in middle-aged men consuming guar gum, a source of NSP (284), while in young adult subjects, acetate and propionate were observed to decrease plasma free fatty acids, which may in turn affect insulin sensitivity (285).

In young children, insulin may have important effects on growth and protein accretion. From data available for preterm infants, Kien (124) speculated that changes in insulin secretion may affect quantity and quality of growth. He suggested that diversion of hexose to SCFA by colonic bacteria may have a deleterious effect on growth. Colonic fermentation occurs also in healthy infants and young children, but such findings and speculations suggest that in addition to being a source of salvageable energy, the proportion of energy that is absorbed as SCFA may have implications for growth. Animal studies of resistant starch created by autoclaving have shown it to be readily fermentable (286) and infant food preparation techniques by large manufacturers may therefore have important metabolic and growth consequences. More work is needed in this area, but quantitation of SCFA production in healthy young children will provide data with which to examine these hypotheses.

10.3.2 Age related changes

The colonic transit time of infants would be expected to be less than older children, and in this physiological setting, fermentation would need to take place more quickly. It also leads to speculation that a more efficient fermentative ecosystem may exist in infants at a time when the digestive capacity of the small intestine is less. It is possible this may relate to differing fermentation patterns and it is speculated that

there may be less early acetogenesis and synthesis of higher order SCFA occurring by the colonic flora of young children than by the colonic flora of adults.

A greater overall cPDR, representing overall starch digestion and absorption may have been expected from the stable isotope breath test data, but with the small number of subjects, such an association was not seen. Neither was there any association found between age and the proportion of starch digested in the small intestine. It is likely to be the case that there is a large inter-individual variation and so large population studies would be necessary to draw conclusions about age-related changes in gut function.

10.4 Applications of results

10.4.1 Introduction: accepted research priorities in complementary feeding

A workshop convened recently by the International Paediatric Association (IPA) and the Committee on Nutrition of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) discussed the current guidelines for the introduction of solid foods to the infant's diet. The research proposals that were suggested by various members of the convention were summarised by the chairman (287). The biggest debate in formulating guidelines still lies in answering the question of what the optimal time to introduce non-milk foods is and what these first non-milk foods should be. Answers to the questions of the development of immunotolerance and atopy have still not been satisfactorily found, but now newer questions are taking centre stage including issues related to metabolic imprinting, the influence on health in later life and how complementary feeding influences the

physiological maturation and metabolic competence of infants to digest, absorb and metabolise weaning foods.

10.4.2 Relative roles of upper and lower gut

This thesis is concerned with the same area of interest in the ontogeny of the young child's gut around the time of weaning. Before these research questions can be answered, the means through which the answers may be found needs to be addressed. Part of the answer lies in a greater understanding of the relative roles of upper and lower gut in digestion and how these roles change from the start of weaning through to the establishment of an adult diet (288). The methods described in this thesis offer an attractive means to investigate the relative roles of small intestine and colon, and in particular to further explore the role of the colon and its role in energy absorption, metabolic imprinting through the colonic flora and in influencing health later in life. The early results which have been found using these methods suggest that this methodology when further validated, will have the potential to make progress in understanding the weaning process and its effects.

A firmer understanding of the quantitative role of the colon in digestion in the young child is the basis upon which to build finer detail knowledge about the changing role of the colonic microflora. Stable isotope breath tests using ^{13}C labelled starches offer a promising means to quantitate the role of the colon.

10.4.3 Metabolic imprinting

In order to understand further the metabolic imprinting of the colonic flora, how diet can influence this and ultimately whether there is a significant effect upon health in later life, the properties and function of the colonic flora needs first to be elucidated. This is no easy task given the changing nature of the bacterial population at this time of life. In rats, weaning diets supplemented with wheat bran resulted in fermentation patterns when the rats reached adulthood (289). Modelling colonic fermentation by incubations of faecal flora still offer the most widely available non-invasive means of investigating the function of the colonic flora. Studies of this kind will determine the absolute and relative quantities of SCFA that are produced in the colons of young children of differing ages, but it will take novel approaches to investigate what the metabolic significance of this is in the short and longer term.

10.4.4 Meeting energy requirements for optimal growth

The question of the timing of weaning from the perspective of meeting energy needs remains an important unanswered question and in particular that time at which milk feeding alone fails to meet energy requirements (287). This has been an issue in its own right, but needs to be considered also in the context of the effects of metabolic imprinting since the outcome measures here are also concerned with anthropometry. Energy requirements are based on growth standards which in turn are based on normal growth in infants perceived to have been fed optimally and the use of anthropometry to define a suitable weaning age is flawed (290). The perceived wisdom has been that nutrition that results in improved height is to be encouraged. A large multi-centre European study has shown that infants fed according to WHO

guidelines of exclusive breast feeding during the first 4 - 6 months (291) tend to be lighter and shorter at the age of 24 months than formula-fed infants, but the differences are small and of uncertain clinical significance (292). The “biggest is best” premise is now being questioned by suggestions that it may lead to an increase in adult morbidity (293) and also on the validity of previous reference standards which have been compiled using data from infants and children whose diets have not followed current guidelines (20).

This issue is of lesser relevance in the developing world where, with widespread growth stunting, optimum growth according to centiles is still to be encouraged. The age at which to introduce non-breast milk feeds is confounded here by the infection risk from contaminated weaning foods and a growth advantage of exclusively breast fed infants is often ascribed to reduced morbidity. In studies where the weaning foods have been nutritious and hygienically prepared there was no significant advantage for infants exclusively breast fed for six months (294,295).

What defines optimal growth needs to be further clarified (296) and functional studies of growth and body composition during the period of complementary feeding have been suggested (297). This area blurs into that of nutritional programming and the ideal definition of optimal growth ought to be that which confers the greatest long term health benefits. A whole range of functional parameters reflecting short and long term health outcomes are called for to interpret infant anthropometric data (20).

10.5 Conclusions

- ^{13}C wheat flour in a dose of 200 mg produces clear peaks over baseline and is therefore a suitable substrate for use in starch digestion studies in young children.
- Limited reproducibility studies suggested good reproducibility when there was better control for external factors such as mealtimes, diet and physical activity.
- 12 h studies are necessary for the whole gut study of starch digestion as in almost all studies the PDR curve reached baseline by 12 h but not before 6 h.
- The mean 12 hour PDR of 21.3% (26.5% for data adjusted for physical activity) compared well with previous findings.
- Mathematical curve fitting of stable isotope breath test data provides an objective and quantitative means of analysing curves which are variable in shape.
- Single curve fitting produces significantly poorer fits than composite curve fitting suggesting the presence of more than one metabolic process.
- Validity for this curve fitting approach to analysis is strengthened by agreement between parameters such as 12 h cPDR and t_{max} where these can also be calculated from raw data.
- The appreciable residual after the small intestinal curve is deconvoluted is most likely to represent $^{13}\text{CO}_2$ production as a result of colonic fermentation, either directly or by oxidation of SCFA and lactate.
- Alternative physiological explanations for the origin of the second curve are much less likely; hepatic glycogen would be expected to “trickle” out over several hours and there was no association between the timing of the second peak and subsequent food ingested.
- Baseline experiments offered no alternative explanation for a second peak.

- Curve fitting of ^{13}C starch breath test data may therefore provide a means of separating the contributions to digestion from the small intestine and colon.
- These experiments have shown that colonic fermentation of starch may account for up to 18% of digested and absorbed starch in young children.
- Not all young children are able to metabolise lactose [^{13}C] ureide implying there is an ontogenetic change in colonic flora which confers this property.
- More validation is required for the use of lactose [^{13}C] ureide as a measure of oro-caecal transit time in young children and in particular the magnitude of the induction response needs further studies.
- Lactose [^{13}C] ureide breath tests offered no additional interpretative information on the starch breath test data.
- The faeces of adults, toddlers and infants are able to ferment starch in a raw and cooked state.
- The fermentative capacity of the colonic flora in young children may be greater than previously thought and in particular may be greater than that in adults indicating the presence of an efficient energy salvage process taking place when the digestive capacity of the small intestine is less than in older individuals.
- Measurement of fermentation products and calculation of PDR allows the creation of stoichiometric equations and also an alternative approach to conceptualising fermentation products. Both approaches offer attractive means to estimate energy production from fermentation.
- An adult type fermentation pattern with increased proportion of butyrate produced may occur from 16 to 19 months.

- The colonic flora of infants may give rise to more efficient fermentation than that of toddlers or adults as measured by the acetate/CO₂ ratios.
- *In vitro* experiments help to interpret the stable isotope breath test data by demonstrating that one important source of colon-produced CO₂ is as a direct fermentation product.
- Combining *in vivo* and *in vitro* aspects offers a means to calculate whole gut stoichiometry for any one substrate and individual, and thereby the energy derived from small intestine or colon.

10.6 Hypotheses generated and areas for further research

10.6.1 Weaning: when and what?

At the start of this dissertation I discussed the two broad questions that define the clinician's interest in weaning: when first to introduce starch into the infant's diet and which starches to use? Through the work described I have sought answers for the questions through an attempt to define the role played the colon in starch digestion in the young child. I have shown that there is a role for the colon in starch digestion and energy salvage which is amenable to quantification. Through the *in vitro* fermentation studies I have further demonstrated that the fermentative capacity of the colonic flora in young children is different from in adults and may indeed be greater than in adults. Through attempts to define the fate of ingested starch, many speculations about the role of the colon in energy salvage and the properties of SCFA in the infant's colon have been discussed.

There is still far to go before there are answers to the question of what and when to wean that say that nutrient X given at Y weeks of age is optimal. Before such weaning advice can be given the outcome measures need to be clearly defined. This is where weaning advice in the developing world can be much clearer because the outcomes are immediate – a weaning food which will be of sufficient energy density and prepared hygienically enough to prevent growth faltering. In the developed world, it is no longer relevant to think purely in terms of growth (293) but rather in terms of long-term health advantage.

Knowledge of the role played by the colon through bacterial fermentation to SCFA during weaning is critical in the search for factors conferring long-term health advantage. The significance of the energy derived from SCFA and their other properties should lead investigators towards metabolic functions that are influenced by the colonic flora.

Stable isotope breath tests and *in vitro* fermentation models may play an important part in this research but many other research disciplines will be needed to provide the many pieces of the jigsaw. Defining questions which are easily answerable will be key to the next steps in further our knowledge of the importance of weaning.

10.6.2 Probiotics and prebiotics

There has been a recent flurry of interest in the exogenous administration of “healthy” bacteria for the colon (probiotics) and substances that promote the growth of healthy colonic bacteria (prebiotics).

10.6.2.1 probiotics

Interest in probiotics has developed rapidly over the last 10 years, but the ability of ingested viable micro-organisms to modify endogenous colonic flora has been known for a century (298). Recent interest in probiotics stems from observed differences between the properties of the flora of breast fed and formula fed infants. By administering species such as *lactobacilli* and *bifidobacteria* the aim is to simulate the healthier breast fed flora by out-competing more pathogenic species which become established in the colonic flora during weaning. Probiotics have been shown to be associated with improvements in the duration of diarrhoea in children (299), with reducing antibiotic associated diarrhoea (300) and the prevention (301) and treatment (302) of atopic disease in children.

10.6.2.2 prebiotics

All nutrients which are not digestible in the small intestine may be used as fuel for colonic flora. Those which target specific bacterial groups to produce a more “healthy” flora are called prebiotics. The term was first coined by Gibson and Roberfroid in 1995 (303). They represent a classical example of a functional food – a nutrient which has beneficial effects beyond adequate nutrition on one or more target

functions in the body in a way which is relevant to either the state of well-being and health or the reduction of the risk of a disease (304).

They act by stimulating the growth of more beneficial species such as *lactobacilli* and *bifidobacteria*, or by suppressing the growth of pathogenic species, or both.

Breast milk contains large amounts of oligosaccharides and attempts have been made to simulate the breast-fed infant's colonic flora by administering oligosaccharides such as fructo-oligosaccharides and inulin. Health claims made about prebiotics include improved lipid metabolism and a reduction in the incidence of type II diabetes, osteoporosis, obesity, and colonic cancer (305).

10.6.2.3 investigation of infant colon physiology via prebiotic and probiotic research

The recent interest in prebiotics and probiotics has been largely stimulated through commercial interests but some of the basic tenets have gone unquestioned. The assumption has been made that colonic flora comprising higher counts of *bifidobacteria* and *lactobacilli* will be as healthy in the adult as it is believed to be in the young infant. As yet it is not clear precisely what are the properties of these bacterial species that confer their health-promoting qualities. Clearly a reduction in urinary tract infections might be expected through outgrowth of more pathogenic species but influence on plasma lipids and the development of colonic cancer is less easy to explain. In infants the health benefits would appear to be the combined result of probiotic and prebiotic (a concept now termed "synbiotic"). As yet, the combined effect has not been studied well in adults.

10.6.3 *In vitro* fermentation models

Such health benefits are likely to be mediated via fermentation products. Models of colonic fermentation hold the key to furthering understanding of changes in fermentation products resulting from manipulation of the colonic flora.

10.6.3.1 developments from this thesis

This thesis has been concerned with the digestion of starch rather than oligosaccharides. However, the methodology might also be used to look at the fermentability of oligosaccharides and combinations of starch and oligosaccharides. Before then further validation studies are warranted. Where the methodology has proved more robust, particularly with the SCFA and CO₂ analyses, a larger sample size of children of a wider age range might be easily batch tested to test the hypothesis that there is a specific age in which the colon is most efficient at salvaging energy. Once these experiments have been validated, further experiments should look at the difference in digestibility between different starches.

The effect of pressurising the system has not been considered before. A newly designed incubation technique which included a pressure valve within a closed system would allow a more physiological simulation of the colon and might be used as a standard against which to validate the traditional McCartney bottle approach.

10.6.4 Metabolic research

The significance of an energy salvaging role by the colon is intriguing. When this is part of a physiological process, it does not seem appropriate to refer to energy “loss”

as some authors have done (124). Others have tried to view weaning in terms of moving from fat to carbohydrate as a main source of energy (237) and following this same line of thought, the significance of a changing balance in energy supply between glucose and SCFA through the weaning process is worthy of study.

Clinical studies show that increasing dietary fibre content over a prolonged period results in beneficial changes in plasma lipids and that this is also associated with increased plasma acetate (306). Metabolic studies of the design used by Kien and co-workers for investigating the effect of infused acetate (92,193) could be developed to gain further insight into the metabolic implications of acetate such as its effect on insulin secretion, anabolism, glycogenesis and lipogenesis. Such studies, at least initially, would need to be carried out in animal models. A better grasp of the metabolic properties of SCFA would provide a context in which to study their properties which might confer long-term health benefits.

Although SCFA are the obvious mediator of the effects of colonic fermentation, through which metabolic processes they act is not clear. Our basic physiology of the use of these energy sources and how they differ from energy supplied as glucose needs a much clearer understanding.

10.6.5 The future for stable isotope breath tests

Stable isotope breath tests have made a contribution to understanding of weaning by defining and quantifying a role for the colon. There is still further validation work to

refine these early ideas but they have other potential uses as this area of research develops.

10.6.5.1 breath tests using ^{13}C labelled starch tracers

The main immediate future direction for this work is repeated experiments using larger tracer doses to eliminate background contamination. There ought to be further reproducibility experiments to ensure that similar external conditions result in similar curves. The curve fitting approach deserves further development and would be strengthened by validating against existing methods of oro-caecal transit time such as with the hydrogen breath test and scintigraphy. The latter would only be possible in adults because of the unacceptability of using radiation in children.

Measuring rather than estimating $\dot{V}\text{CO}_2$, something which would only be possible in adults because existing methods for measurement of energy expenditure by indirect calorimetry require much compliance, would give better point by point accuracy of PDR. As techniques for estimating $\dot{V}\text{CO}_2$ from heart rate measurements become more validated this may become a reality in children.

Alternative hypotheses for the second curve might be tested by performing an equivalent dose glucose and starch breath test in the same subject. Ideally, the wheat flour should be processed to remove the protein and non-starch polysaccharide content, but this would require further and more extensive production of ^{13}C wheat, which has proved costly. If this experiment were performed in subjects with known small intestinal malabsorption, such as children or adults with cystic fibrosis, they

might be used as a surrogate model for the gut of the young child with physiologically small intestinal digestive capacity. To address the glycogenolysis theory specifically, tests might be carried out after a significant period of fasting and after loading the liver with glycogen from a naturally non-enriched source such as pasta.

When the methodology has been sufficiently refined, studies with other labelled starches would allow comparisons to be made in upper and lower gut digestibility. Some initial work has already been done to produce ^{13}C -labelled rice (149). Additionally it may be possible to produce ^{13}C -labelled tracers of oligosaccharides.

Stable isotope breath tests in animal models may help explain the eventual fate of the unaccounted for cPDR which besets all investigators using stable isotopes. In this might come pointers to the metabolic consequences of SCFA.

10.6.5.2 breath tests without tracers

Baseline testing has been useful for validating the tracer experiments. Further baseline testing of children in the free-living state to establish the normal expected background variation would have significant benefits to future researchers conducting free-living experiments in young children. These might be combined with new methods of measuring energy expenditure.

10.6.5.3 breath tests using lactose [^{13}C] ureide

Before this molecule can be of further use in the understanding of the role of the colon, its use in measuring OCTT needs to be validated. Further testing of the effect of induction of enzymes is required for all subjects to determine the optimum dose and duration of induction required. There are concerns about the induction of a potentially pathogenic organism in the colonic flora of young children. Other studies of ^{13}C -LU suggest that all older children and adults are able to metabolise the tracer. It is therefore necessary first to establish the prevalence of *Clostridium innocuum*, believed to be the sole bacterial species responsible for the cleaving of the ureide bond (252) in the colonic flora within a population of children of differing ages.

10.6.5.4 breath tests in combination with other approaches

The use of ^{13}C starch breath tests and faecal incubation studies in the same subjects would be problematic to organise but if carried out, at least in adults first, would add more credence to the approach of combining both sets of data.

10.6.6 Nutritional programming

10.6.6.1 is there evidence that infant nutrition has long-term beneficial effects?

In adults at least, it would appear necessary to continue to consume probiotics and prebiotics to maintain their beneficial effects (307). And this would fit with existing ideas that the adult colonic flora, once established, varies little (112). The mode of early infant feeding (breast or formula) influences the colonic flora (105). Is it possible that early infant nutrition, including the weaning diet, can direct the

development of the adult colonic flora and make lifelong differences to risks of developing chronic and degenerative disease?

This question can only be fully answered through further epidemiological studies of the type that have already established a link between fetal nutrition and health in later life (224). It is unlikely that detailed information about the weaning diet would be easily available retrospectively. However, information about initial mode of milk feeding (breast or formula) is more readily accessible and a recent meta-analysis has shown that exclusive breast feeding in infancy has a small but significant effect in reducing total cholesterol in adults (308).

The authors of the paper raised several properties of breast milk that might be responsible for such a protective programming effect but did not discuss the possible changes in gut flora. The question would be readily addressed through a study which measured the viable faecal bacterial counts of one of the same cohorts of adults who were initially breast fed and have low plasma cholesterol compared to the cohort of adults who were formula fed and now have higher plasma cholesterol.

10.6.6.2 surrogate approaches to nutritional programming hypotheses

Until the results of any prospective cohort studies are available in a generation's time, the question will only be answered through indirect means. One approach might be via animal models. A rat model has already demonstrated that introducing dietary fibre at weaning influences the ability of the adult gut flora to ferment fibre

(289). These studies might be extended to consider the effect of different weaning diets on plasma lipids, insulin resistance and colonic cancer.

A second approach might compare the fermentability of the colonic flora from different adult populations consuming different diets, for example a European population and a rural developing world population. It is well established that the latter population has a lower risk of ischaemic heart disease and colon cancer. Such research would require parallel laboratories different countries but such collaborative work is frequently carried out. If these studies were to provide evidence of differences in colonic flora, further work investigating the significance of any change in colonic flora on increased risk of cardiovascular disease as a result of moving from a rural developing world diet to a Western European diet.

10.6.7 Concluding remarks

The data from this dissertation has shown that the colon has a quantifiable role in starch digestion in young children. This finding dovetails with recent interest in nutrition that has focused on the nutritional benefits of modifying the colonic flora and on the long-term consequences of fetal and early infant nutrition.

Furthering understanding of the human weaning process and its long term effects will require the co-operation of a large number of research disciplines including epidemiology, animal modelling, studies of human colonic flora and metabolic studies. There is a clear role for further research with stable isotope breath tests in defining the site and extent of starch digestion but also in studying the metabolic fate

of macronutrients. *In vitro* fermentation studies will also have an important role as a tool for studying the properties of the colonic flora and its fermentation products.

Nutritional programming ideas provide the framework which contextualises the importance of study of the human weaning process. Further work on prebiotics and probiotics may well provide the financial incentive to drive the work forwards.

Undoubtedly, commercial interests are currently driving the research into functional foods; the high ratio of reviews to original publications one encounters searching the literature for information on prebiotics and probiotics sounds a warning note against too much speculation without a robust innovative research base. Although the incentive exists to further investigation of the colon's role, particularly during weaning, this will need to be harnessed to answer basic questions of physiology. The answers to these questions may have long-term implications for the health of future generations.

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List of Publications arising from this thesis

- 1 Christian MT, Amarri S, Franchini F, Preston T, Edwards CA, Weaver LT.
Contribution of small intestine and large bowel to starch digestion in infancy.
J Pediatr Gastroenterol Nutr 1998;26:588.
- 2 Christian MT, Amarri S, Franchini F, Preston T, Edwards CA, Weaver LT.
Energy salvaging role of the colon in early childhood. Gastroenterol Int
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Patterns of colonic starch fermentation from infancy to adulthood. Proc Nutr
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- 5 Christian MT, Morrison DJ, Dodson B, Preston T, Amarri S, Franchini F,
Edwards CA, Weaver LT. Measurement of oro-caecal transit time in young
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- 6 Christian MT, Amarri S, Franchini F, Preston T, Morrison DJ, Dodson B, Edwards CA, Weaver LT. Modeling ^{13}C breath curves to determine site and extent of starch digestion and fermentation in infants. *J Pediatr Gastroenterol Nutr* 2002;34:158-64.

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- 7 Christian MT, Edwards CA, Preston T, Johnston LA, Varley R, Weaver LT. Colonic starch fermentation in early childhood has importance for energy salvage. *Eur J Clin Nutr* 2003 (in press).

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Invited Review

Starch Digestion in Infancy

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In infancy the diet undergoes a change, from a single food with fat and lactose as major energy sources, to an increasing variety of foods in which starch is the principal source of energy. Many weaning foods are cereal-based, and starch represents a novel nutrient to the digestive system of the hitherto exclusively milk-fed infant. Much of the thrust of recommendations on infant feeding has focused on the benefits of breast-feeding in establishing optimal nutrition in early life (1). The importance of weaning has received less emphasis (2,3). The purpose of this review is to summarise what is known about the digestibility of starch and how well the young infant can make use of dietary starch during weaning.

Weaning may be defined as the period from first introduction of a nonmilk diet to the cessation of breast- (or formula-) feeding. In some rodents there are abrupt and clearly definable changes in the gut's capacity to digest nonmilk carbohydrates at weaning, such as a surge in enterocyte proliferation and the expression of sucrase-isomaltase activities (4). However, in the human infant, the physiology of the digestive system is less well understood, particularly the timing of the processes involved in adaptation to a nonmilk diet.

STARCHES

Complex carbohydrates are polymers of sugar molecules. Those with 10 or fewer sugar residues are called oligosaccharides, and those with 10 or more are called polysaccharides. Polysaccharides can be very large polymers, which are natural or synthetic. Examples of natural polysaccharides include dietary fibre (nonstarch polysaccharide) and starch. Synthetic glucose polymers, made by modifying natural products, can be used to increase the energy content of formulas.

Starches are plant storage complex carbohydrates with molecular weights often exceeding 100 kDa, which comprise glucose polymers with α 1,4 amylose (straight-

chain) and α 1,6 amylopectin (branched-chain) linkage. The proportions of amylose and amylopectin vary between different starches, as does the size and shape of storage granules. Three crystalline forms of starch, A, B, and C, are recognised that differ in their digestibility (5). Type A (e.g., raw wheat or rice starch) has an easily digestible, open-helical structure. The densely packed hexagonal pattern of the double helices of the B type (e.g., raw potato starch) reduces digestibility by denying access to amylases (5,6) (Fig. 1). Type C (e.g., peas and other legumes) is considered to be a mixture of types A and B.

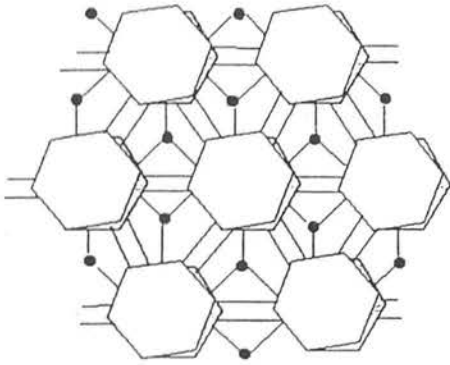
Starches have also been classified empirically by their digestibility *in vitro*, into rapidly digestible, slowly digestible, and resistant forms (6) (Table 1). Rapidly digestible and slowly digestible starches are digested completely in the small intestine, whereas resistant starch is defined as the sum of starch and the products of starch degradation that are not absorbed in the small intestine of healthy adults (7). The digestibility of starch is determined by its structure, the kinetics of oligosaccharic release from the starch after hydrolysis (8), and the degree of inhibition of α -amylase *in vitro* by other nutrients such as leguminous glycoproteins and antinutrients such as tannins (9). All three starch types (A, B, and C) can be rapidly digested in the cooked state. Types B and C are more likely to form retrograded starch on cooling during processing. Retrogradation is recrystallisation of starch to an indigestible product, which may occur during cooking and cooling cycles or under processing conditions of high moisture, pressure, and temperature. Studies of the digestibility of starches *in vivo* have been largely confined to adults (10,11). In infants the low luminal pancreatic α -amylase concentrations give rise to an increased proportion of resistant to digestible starch.

There is a wide range in digestibility of commonly used first weaning foods *in vitro*. Rice starch is rapidly digestible. Freshly cooked potato is also rapidly digestible but may become retrograded and resistant if cooled after cooking. Sterilising techniques in the canning of commercial weaning foods may considerably increase the resistant starch content of the diet of the young child (12), and the consequent effects on energy absorption and growth potential are unknown (13).

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Type A Starch



Type B Starch

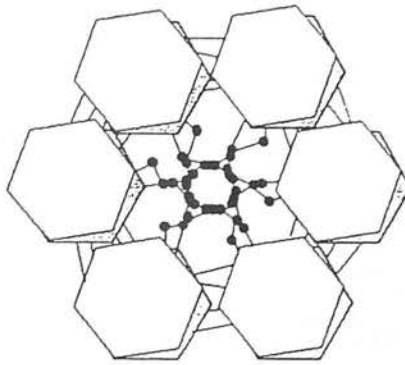


FIG. 1. Types A and B starch. Each hexagon represents a helix of amylose/amylopectin. In type A there is a lattice of starch helices with water spread throughout the molecule structure rendering it easily digestible. An example is wheat starch. In type B there is a super helix with water molecules tightly bound within the centre, which makes it resistant to digestion by human amylase. Examples include raw potato starch and retrograded starch. From Sadler, Caballero, Strain, eds. *Encyclopedia of Human Nutrition*. London: Academic Press Ltd.

Recently, some benefits of resistant starch in infant diets have been identified. In preterm infants, formulas that contain maltodextrins are associated with increased calcium absorption (14), believed to be caused by enhancement of passive absorption (15). In infant pigs, this same effect, attributed to resistant starch, also leads to increased absorption of iron, and possibly zinc (16). Counterbalancing this is a concern that resistant starch, particularly the lower molecular weight portions escaping digestion in the small intestine or after hydrolysis by the colonic microflora, can generate an excessive osmotic load in the large bowel (17), leading to diarrhoea in infancy.

STARCH DIGESTION

Breakdown of starch begins in the mouth under the action of the glycoprotein enzyme α -amylase, which is secreted in saliva and human milk and cleaves the α -1,4 linkages in the starch molecule. α -Amylase is inactivated by gastric acid, but digestion continues in the alkaline duodenal lumen, where the salivary isozyme is reactivated, and further α -amylase is secreted by the exocrine pancreas. The products of this digestive step are maltose, isomaltose, maltotriose, and maltodextrins (branched-chain oligosaccharides) which undergo further digestion

in the brush border of the jejunal mucosa where free glucose is liberated by the action of glucoamylase, maltase, and isomaltase. Glucose is thereafter actively transported across the mucosa.

Breast Milk Amylase

Human milk contains α -amylase, which may help breast-fed infants to digest starches in early weaning. It is structurally identical with salivary amylase but has a broader pH optimum and retains its activity in the stomach (18). There are large interindividual variations in breast milk concentrations, but associations have been found with gestational age at birth (mothers who deliver prematurely have highest activity) (19) and parity (decreased with increasing parity) (20). Human breast milk amylase is found in its highest concentrations in colostrum (19) and decreases thereafter during the course of lactation (20).

Salivary Amylase

Salivary amylase is detectable from 20 weeks' gestation (21). Its activity increases rapidly after birth to reach

TABLE 1. *In vitro* classification of starch based on adult foods and digestive physiology

Type of starch	Example of occurrence	Probable digestion in small intestine
Rapidly digestible starch	Freshly cooked starches	Rapid
Slowly digestible starch	Most raw cereals	Slow but complete
Resistant starch		
Physically inaccessible starch	Partly milled grains and seeds	Resistant
Resistant starch granules	Raw potato and banana	Resistant
Retrograded starch	Cooled, cooked potato, bread and corn flakes	Resistant

Adapted from Englyst HN, Kingman SM. Dietary fiber and resistant starch: A nutritional classification of plant polysaccharides. In: Kritchevsky D, Bonfield C, Anderson JW, Eds. *Dietary fiber*. London: Plenum, 1990:49-65. © 1990 Plenum Press, London, UK.

near adult levels from 6 months to 1 year (22), but there is large interindividual variation (23). It is inactivated by low pH but remains active in the poorly acidified neonatal stomach where the pH may be more than 4 until 3 to 4 weeks (24), and where it is also protected by binding to small-chain glucose polymers (25). Salivary amylase may serve an important role in the young infant in whom there is a physiological deficiency of the pancreatic isozyme, and it has been proposed that the small amounts of amylase detected in the duodenum of young infants (26) may be of salivary origin (27).

Pancreatic α -Amylase

The physiology of the neonatal gastrointestinal tract is different from that of the adult (Fig. 2) with most digestive enzymes found at lower concentrations. Pancreatic α -amylase concentrations in the neonatal duodenum are much lower than in adults (26,27) (Table 2). Serum concentrations at birth are 1.6% of adult levels and do not reach mature levels until 5 to 12 years (28). Explanation for low duodenal concentrations of pancreatic α -amylase may be low synthesis or secretion of the enzyme. Seventy years ago, the existence of α -amylase in the fetal pancreas from 22 weeks' gestation was reported (29), but more recently, none was found in the pancreas of 3-week-old infants (30). Pancreatic α -amylase has been detected in the amniotic fluid from 16 weeks' gestation (30), although in much reduced concentrations compared with salivary amylase (31). Pancreatic fluid and electrolyte secretion increase in response to secretin and cholecystokinin (32), but specific assays for α -amylase show

TABLE 2. Duodenal aspirate volume and pancreatic α -amylase activity in full-term neonates who were fed a standard formula, containing 7 g lactose per 100 ml. Shown for comparison are values from healthy children age 9 months to 13 years

	Volume (μ l)	α -Amylase (IU)
Birth ^a	539 \pm 529 (166-970)	3.2 \pm 3.8 (0.1-9.8)
12 Hours after first feeding	329 \pm 318 (96-800)	0.38 \pm 0.13 (0.2-0.5)
1 Week	434 \pm 330 (96-1040)	1.29 \pm 1.29 (0.1-3.0)
Children	3900 \pm 1500 (1800-8100)	665 \pm 464 (160-2150)

Adapted from Zoppi G, Andreotti F, Pajno-Ferrara F, Njai DM, Gaburro D. Exocrine pancreas function in premature and full term neonates. *Pediatr Res* 1972;6:880-6.

Values expressed per kilogram body weight and represent mean \pm SD (range) of the activities during 50 minutes after injections of pancreozymin and secretin.

^a Before first feeding.

that infants under 1 month are unresponsive to cholecystokinin and have only a minimal response to secretin (27). However, the plasma concentrations of gastrointestinal hormones, including secretin, are themselves low until the sixth day of life (33). The question remains therefore, whether in early infancy, there is low synthesis or secretion of α -amylase, low production of, or response to, secretagogues, or a combination of all these factors.

Small Intestinal Brush Border Enzymes

The small intestinal brush border enzymes catalyse hydrolysis of oligosaccharides and disaccharides to glu-

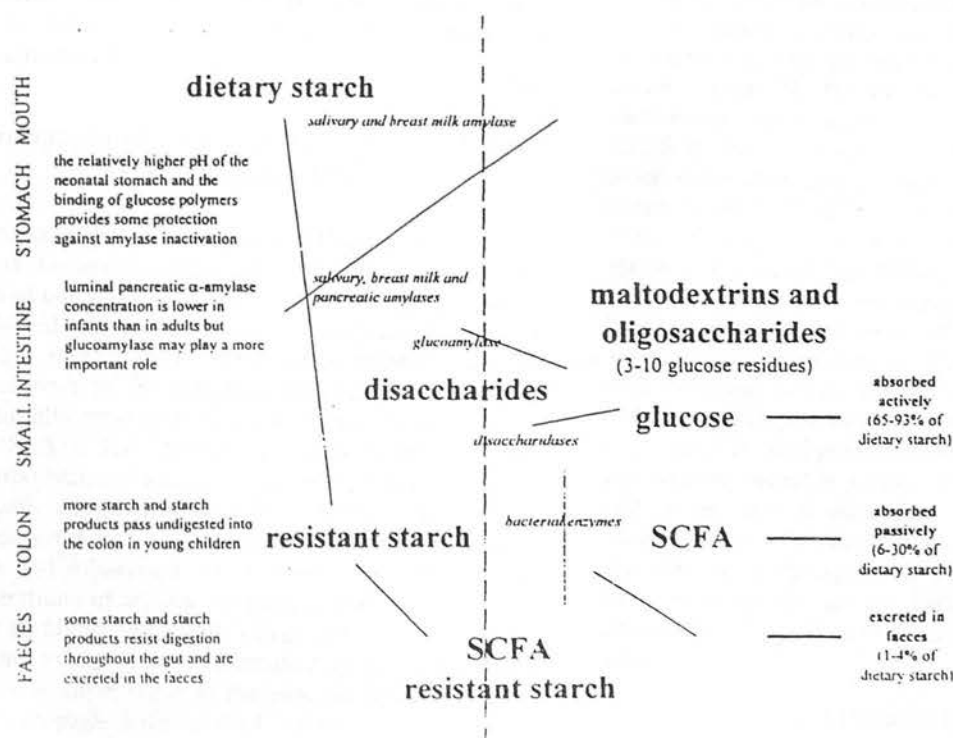


FIG. 2. The fate of starch in an infant's gut. The digestive pathways of starch throughout the whole gastrointestinal tract are shown together with the enzymes at each site. Approximate calculations for the fate of dietary starch are based on unpublished results: calculations of faecal starch in young children (95), faecal short chain fatty acids in young infants (66), and daily stool volumes in young children (112).

A small but significant amount of energy is gained by the colonic absorption of SCFA. In animal studies the partial utilisation of SCFAs has been shown to be 82% from intracaecal infusions, and most of it is retained as fat (68). In adult humans the energy is estimated to be approximately 2 kcal/g of nonabsorbable carbohydrate (69) or a contribution of 5% to 10% of energy requirements (70,71). Kien et al. (72) estimated that 24% to 74% of lactose may be converted into acetate in preterm infants with a potential 30% loss in adenosine triphosphate (73), but there appear to be no data on energy salvage from fermentation of starch in infancy at a time when the limited digestive capacity of the small intestine means that more starch will pass undigested into the colon.

Studies of the effects of SCFAs have been carried out almost exclusively in animals or in adult humans in whom some properties are related to all the SCFAs and some attributed to specific ones (74,75). All SCFAs appear to have trophic effects on the gastrointestinal mucosa, both in the upper and lower gut (64,67,76,77). Butyrate is the preferred fuel for colonocyte metabolism (64,78) and may protect against colonic cancer (79), although this may not happen at physiological concentrations (80). Propionate has been associated with cholesterol-lowering effects (81,82). Basal levels of insulin and glucagon are suppressed by increased loads of SCFA in ruminants (83), and although their metabolism differs considerably from that of humans, similar increases in insulin sensitivity have been demonstrated in humans (84). Insulin has anabolic effects in the human infant, and it is possible that by this means, starch fermentation may indirectly influence growth (73).

Development of the Fermentative Ability of the Infant Colonic Flora

Bacterial populations and diet influence fermentation products. Different components of the diet encourage the growth of particular bacterial species. The faecal flora of formula-fed infants produce more propionate than that of breast-fed infants (85). Fermentation reactions are directly affected by the substrate; for example, butyrate is preferentially produced by the fermentation of resistant starch (86,87). The capacity of infants to ferment complex carbohydrates does not increase significantly until 7 to 9 months (88). In infants before weaning, fermentation products are mainly acetate with small amounts of propionate and *n*-butyrate. By 9 months the mean faecal concentrations of acetate, propionate and *n*-butyrate are as high or higher than adult values (88).

The net effect of the fermentation of polysaccharides to SCFA is an increase in the osmotic load within the colon, although unfermented, resistant starch itself causes an increased osmotic load. If the capacity for SCFA absorption is limited, then diarrhoea results, but in

young piglets the presence of SCFA in the colon enhances water and sodium absorption (89). Absorption of SCFA from the colon of adults is rapid, and more than half of ¹⁴C-labelled SCFA appears in the breath within 6 hours (90). Lifschitz et al. (91) demonstrated that colonic acetate absorption in malnourished infants increases with age, but there are few other human studies of the development of colonic absorption of SCFA.

FUNCTIONAL CAPACITY FOR DIGESTION AND FERMENTATION OF STARCH IN INFANCY

Various methods have been used to measure the digestion and absorption of starches in early life. These include measurement of blood glucose, balance studies, and the use of ¹³C stable isotope breath tests. Studies have been mainly performed in infants aged 1 month or less, often born prematurely, whose formulas contain glucose polymers to increase energy content. There is a glycaemic response after the ingestion of corn starch (92), glucose polymers (93), and lactose in term and preterm (94) infants. By measuring the intake of starch and the amount of residual starch in faeces, investigators have attempted to draw conclusions about whole-gut digestion with conflicting results (17,95). Such studies require meticulous measurements and do not usually take into account losses from methane and CO₂ production. Results of total gut balance studies show that the individual contributions of upper and lower gut to starch digestion cannot be distinguished.

Calculations of intake and stool carbohydrate output are made easier by the use of starches enriched with the stable isotope ¹³C, but inconsistencies in results of balance studies have suggested a potential confounding effect from the excretion of bacterially derived carbohydrate, either contained within the cell walls or as fermentation product (96,97). Concomitant measurement of breath hydrogen allows estimation of the amount of starch that reaches the colon. Hydrogen is a bacterial fermentative product and cannot be produced by mammalian cells. The hydrogen breath test has been used in young infants to investigate the fermentation of lactose (98), although it may be difficult to use quantitatively (99), particularly in young infants (100). Shulman et al. (101) used it semiquantitatively to measure the utilisation of corn cereal in young infants, including information on the site of utilisation. Although young infants clearly possess the functional capacity to digest starch this may be associated with a relatively decreased absorption of energy and nitrogen (102), highlighting the limitations of studying the digestion of a single nutrient alone.

THE FUTURE

There is much still to be learned about the ontogeny of the gastrointestinal tract during the weaning period.

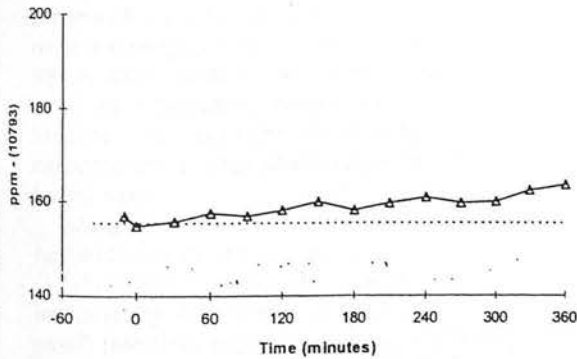
its capacity to make use of dietary starch. Existing work has focused mainly on the site or mechanism of starch digestion, but there is plainly a need to define the contributions of the small and large intestines and to quantify the various fates of different starches in infants of different ages.

The emergence of stable isotope biotechnology (103) to measure nutrient digestion *in vivo* offers a means of studying the fate of ingested starch. Because stable isotopes are nonradioactive, ^{13}C breath tests are safe, and sampling breath rather than blood makes them particularly attractive for the study of infants (104,105). The appearance of $^{13}\text{CO}_2$ in the breath after the ingestion of a particular substrate labelled with ^{13}C depends upon exogenous and endogenous factors that control the digestion, absorption and metabolism of that substrate. The ratio of $^{13}\text{CO}_2$: $^{12}\text{CO}_2$ in breath can be measured by isotope ratio mass spectrometry and, assuming constant or complete oxidation of the products of digestion or fermentation, the site, rate and extent of starch digestion and absorption quantified. ^{13}C breath tests have been used by several investigators to measure starch digestion

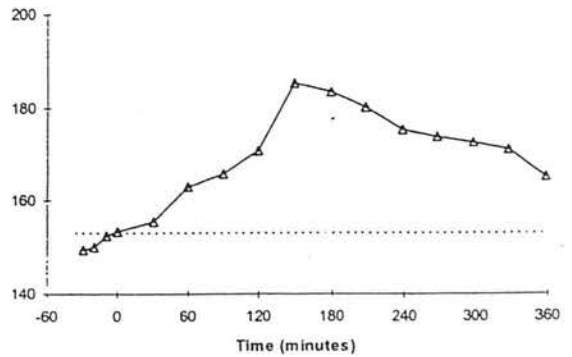
in the small intestine (101) or fermentation in the colon of infants (72,91). Their potential as a tool for the study of the digestion of starch in the whole gut is underrealised. Shulman et al. (101) reported $^{13}\text{CO}_2$ recovery curves over time without remarking on their shape, which showed later maximal abundance of $^{13}\text{CO}_2$ and the suggestion of a more twin-peaked curve with increasing carbohydrate complexity (Fig. 3). Amarrì et al. (106) noted a similar twin-peaked curve in their study of starch digestion in children with cystic fibrosis using ^{13}C -labelled wheat flour biscuits and speculated that the second peak represented $^{13}\text{CO}_2$ derived from oxidised SCFA as a result of colonic fermentation of the starch.

This biotechnology enables the use of mathematical models to analyse whole-gut starch digestion curves. Mathematical models have been used in gastric emptying studies (107), adapting an equation that forms the basis of the χ^2 distribution. Assumptions about curve interpretation would be strengthened if a gold standard for colonic absorption could be found. There is some hope that lactose-ureide, a synthetic condensation product of lactose and ^{13}C -labelled urea, which resists breakdown by

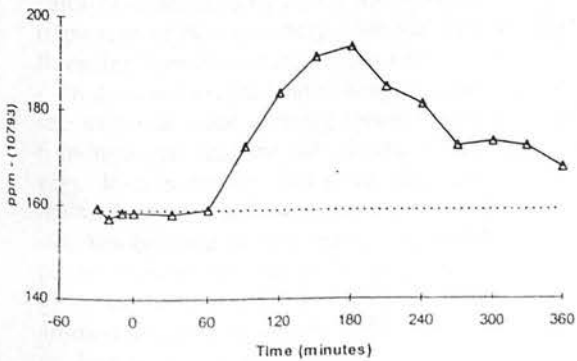
BEET SUCROSE



CORN GLUCOSE



CORN GLUCOSE POLYMERS



CORN CEREAL

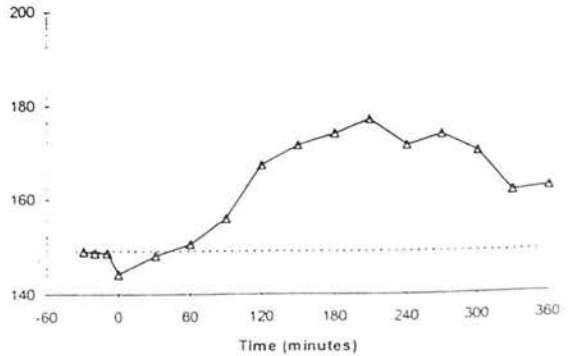
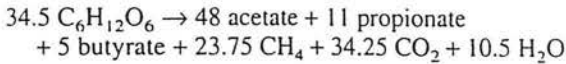


FIG. 3. The utilization of dietary starch by young infants. Adapted from Shulman et al. (101) The enrichment of breath $^{13}\text{CO}_2$ over 6 hours after the ingestion of various carbohydrate preparations by infants aged 1 month. Beet sugar contains little natural ^{13}C , but maize and glucose products that are derived from maize are naturally enriched.

small intestinal enzymes (108), may provide such a standard.

Another way of quantifying the colonic fate of starch is by stoichiometry—calculation of the numerical relationship between the molecular species entering a chemical reaction and the amount and type of molecules produced. Stoichiometric equations for carbohydrate have been produced for adult humans and ruminant animals (69,109), based on known proportions of SCFAs in faeces and known production of carbon dioxide and methane. From these, the energy derived from fermentation reactions can be calculated:



Energy calculations have been made on the whole gut by balance studies, separating the carbohydrate from the fat and protein component of stool and then calculating carbohydrate energy absorption (102,110). An attempt to quantify energy absorption by site of digestion was made for lactose using a stable isotope dilution method to measure the rate of entry of acetate into the peripheral circulation (72), and from the results, energy uptake was estimated from known thermal losses of potential adenosine triphosphate (110). The results show wide variability. A more complex whole-gut model could be developed by combining stoichiometry from fermentation models with data from stable isotope breath tests, and carbohydrate energy source quantified for infants of different ages.

Energy from SCFA produced as a result of colonic fermentation of starches is variously considered as loss (111) or gain, as though the infant small intestine were an inefficiently functioning organ (73,110). However, the small intestinal digestion and colonic fermentation that occur in the infant are interrelated physiological processes that together ensure efficient overall gut function. The need to understand the individual proportions of carbohydrate energy derived from small intestine and colon is underlined by observations that SCFA may have important effects on energy balance and growth by influencing insulin concentrations (73).

In the most recent United Kingdom guidelines (3) it is recommended that weaning should begin between 4 and 6 months and that the diet should contain adequate energy. Recommended first solid foods are nonwheat cereals. Cooked starches are advised in preference to raw starches because of their greater digestibility, and rice in particular is recommended. In comparison with the space given to recommendations for other nutrients, the small amount devoted to starches reflects the relative paucity of data in this field. Yet, the dramatic changes in the source of dietary energy at weaning and its potential metabolic effects suggest that this is an area that requires fuller understanding. A greater insight into the physiology of the infant gut, in particular the small intestinal

digestibility of starch *in vivo*, its fermentability in the colon, the properties of SCFA, and the metabolic fate of starch, is needed to evaluate the overall nutritional value of different starches to define sensible and evidence-based weaning advice.

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Patterns of colonic starch fermentation from infancy to adulthood By MARTIN L. CHRISTIAN¹, CHRISTINE A. EDWARDS², TOM PRESTON¹, LEE ANN JOHNSTON², REBECCA VARLEY², and LAWRENCE T. WEAVER¹, Departments of ¹Child Health and ²Human Nutrition, University of Glasgow, Yorkhill Hospitals, Glasgow G3 8SJ and ¹Scottish Universities' Research and Reactor Centre, East Kilbride G75 0QU

The composition and consequently the fermentative capacity of the colonic flora changes from infancy to adulthood (Guérin-Danan *et al.* 1997). The colon salvages energy by fermenting starch which passes undigested from the small intestine but little is known of the magnitude of this role in young children (McNeil, 1984). Using a simulated colonic environment, the aim of this study was to account for the fate of raw and cooked starch fermented at different ages.

Individual faecal samples of children aged 9 months (*n* 6) (range, 6.7-9.7), children aged 18 months (*n* 6) (range, 18.0-19.4) and adults (*n* 7) were processed within 2 h of passage. A 5% faecal slurry was anaerobically incubated with a phosphate buffer (pH 7) and 25mg of raw or cooked maize starch in McCartney bottles in a shaking water bath (37°, 60 strokes/min). Parallel incubations were stopped at 4 and 24 h. The headspace gas volume was measured and analysed for CO₂ (Servomex 1400, Crowborough, UK) and methane (continuous flow mass spectrometry). The slurry was later analysed for short chain fatty acids (SCFA) and lactate by GLC of acidified ether extracts (Spiller *et al.* 1980), and assayed for residual starch (Englyst *et al.*, 1992). Results are expressed as the percentage dose recovered (PDR) of the starch substrate for each of the fermentation products.

incubation...	PDR of...	4 h						24 h					
		CO ₂		Total SCFA		Residual starch		CO ₂		Total SCFA		Residual starch	
		Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
9 months	R	4.1	3.1-5.1	38.9 ^a	30.9-46.8	22.4	10.8-34.1	11.7	7.8-15.6	59.5	50.5-68.5	1.5	0.2-2.8
	C	5.9	2.7-9.2	46.5	40.7-53.3	20.8	0-47.2	13.8	8.5-19.1	65.0	52.0-77.9	3.5	2.6-4.4
18 months	R	8.8	5.3-12.4	30.5 ^a	22.0-39.1	8.4	2.0-14.8	16.3	14.2-18.3	41.3	34.3-48.4	0.8	0.5-1.1
	C	8.2	4.9-11.5	29.2 ^b	22.5-35.8	6.9	1.3-12.4	17.9	14.5-21.2	46.2	39.1-53.3	0.6	0.1-1.0
Adults	R	2.5	0.8-4.3	12.9 ^c	3.3-22.5	44.3	22.2-66.4	14.3	11.5-17.1	58.3	46.7-69.9	2.7	0.1-5.4
	C	6.1	2.2-9.9	25.7 ^d	14.3-37.1	18.9	10.7-27.1	16.1	13.5-18.7	52.7	44.8-60.7	4.8	0-10.1

R, raw maize starch; C, cooked maize starch. ^a mean values significantly less than R and C for 9-month olds respectively; ^b mean value significantly less than R for 18-month olds and C for adults; ^c mean value significantly less than C for 9-month olds. *p* < 0.05.

Methane was detected in three adults only. At 4 h, lactate was detected, mainly in children, accounting for up to 30.6 PDR. At 24 h, it was detected in only one 9-month old. The production of SCFA at 4 h generally declined with age but the differences at 24 h were less marked, suggesting that fermentation is a more rapid process in young children. At 4 h, cooked starch was better fermented than raw starch, except for at 18 months. Raw starch is less easily fermented than cooked starch, but if the diet contains large proportions of resistant starch, such as in children during weaning, their fermentability may be similar. This study suggests that a highly efficient fermentation process takes place in the colon of young children and the colon has significant potential for energy salvage when the capacity of the small intestine to digest starch is limited in early life.

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male children and decreased sperm count later in life was first suggested in *Lancet* in 1993 (1) but has not been confirmed in animal or human studies. Indeed, cows and ewes fed estrogenic forage showed transiently impaired ovarian function, but males were relatively unaffected (2). More specifically, gestational and lactational exposure to isoflavones had no adverse effects on gametogenic function or reproductive organs in male rats (3,4), nor did they alter the reproductive hormone concentrations and reproductive organ weights in peripubertal rhesus monkeys of both sexes (5). In a recent retrospective cohort study among adults who, as infants, participated in a feeding study with soy- or cow-milk formulas, no adverse reproductive effects were identified in association with previous exposure to soy formula (6). In another pioneering study, isoflavone supplements to the diet of healthy adult volunteers for a period of 2 months had no observable effects on testicular volume or semen parameters of ejaculate volume: sperm concentration or total count, motility, and morphology (7). The deleterious effect of phytoestrogens on male fertility is unfounded based on the current data.

The second concern about the casual relationship between phytoestrogens and early onset of menarche also is unfounded. We have reviewed several studies, all of them in rats, that showed either delay (8) or acceleration of puberty (9) in response to phytoestrogens exposure. As we specifically emphasized, there are no similar studies in girls. Therefore, extrapolating the data from rats to humans, and even further, to delineate conclusions about breast cancer risk is definitely unjustified. Indeed some studies have shown that phytoestrogens have protective effects against breast cancer (10).

Finally, others have not confirmed significant aluminium contamination of soy formulae, and this may be a problem specific to Australia.

We are not advocates of phytoestrogens usage in childhood, but we disagree with Dr. Couper's conclusion of avoiding soy formulae if possible.

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Measurement of oro-cecal transit time in young children using lactose [¹³C] ureide requires further validation

To the Editors: Van Den Driessche and coworkers (1) recently described the use of the lactose [¹³C] ureide (LU) breath test to measure oro-cecal transit time (OCTT) in children. Breath tests in 20 children aged between 3 and 17 years showed a mean (range) OCTT of 255 (165-390) minutes. In 32 children aged 0 to 3 years they performed stool incubations and demonstrated that bacterial enzymatic activity hydrolyzed LU in the stools of children from 8 months and over, but not in the stools of infants aged less than 6 months.

We have performed 11 breath tests on 10 Italian children aged between 7.9 and 21.8 months (median 11.8 mos.). Eight children were exclusively breast fed for at least 4 months, one was bottle-fed and one received mixed feeds. One child was studied at 11.8 months and again at 15.6 months. Median (range) age of weaning was 6 (4.0-6.5) months. The day before the test, each child was pre-dosed with 100 mg of unlabeled LU. Following an overnight fast, each consumed 100 mg ¹³C-LU as part of a starchy breakfast. Duplicate breath samples were obtained 15 minutes and immediately before breakfast and every 30 minutes thereafter for 12 hours using a bag and mask or by slowly aspirating close to the nostrils if the children were sleeping. We have presented this methodology elsewhere to investigate starch digestion in young children (2).

Not all children produced a clear ¹³CO₂ rise above baseline as can be seen from Figure 1. Four had a median (range) age of 12.8 (9.3-21.6) months with a median (range) peak breath ¹³CO₂ enrichment of 111 (90-153) ppme. The remaining children, including both experiments for the child studied twice were non-responders with a similar age spread of 7.9 to 21.8 months (median 11.8 mos). They had peak breath ¹³CO₂ enrichment of 27 (1-34) ppme that was similar to that of four children, median (range) age 12.7 (8.8-16.1) months in whom we performed baseline studies using the same study design. The range of enrichment observed without a ¹³C enriched tracer was -13.4 to 22.5 ppme.

Van Den Driessche et al.'s results suggest that many more children in our study should have responded. Because they did not carry out breath tests in their younger subjects there may be a number of explanations for these seemingly inconsistent observations. A difference in curve parameters is observed if pre-dosing with unlabeled LU is used (3) but no consensus exists about a pre-dosing regimen. Van Den Driessche et al. used three doses of 500 mg unlabeled LU the day before the breath test. For those children undergoing the stool analysis, they used a single dose of 500 mg LU one week before stool

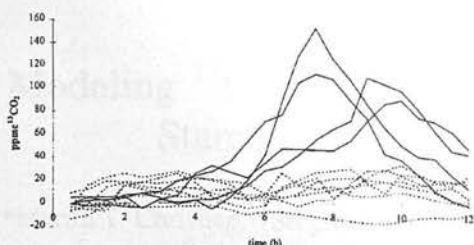


FIG 1. Enrichment of $^{13}\text{CO}_2$ in breath over 12 hours for 11 ^{13}C LU breath tests on infants aged between 7.9 and 21.8 months showing responders in solid lines and non-responders in dashed lines.

collection as an in vivo induction, which resulted in a peak mean $^{13}\text{CO}_2$ production rate at 450 minutes compared to the peak mean $^{13}\text{CO}_2$ production rate for non-induced stool samples of 900 minutes. They also incubated some of the stool slurries with 2.5 mg/ml unlabeled LU one hour before the $^{13}\text{CO}_2$ collection commenced. These results are not reported.

The metabolism of LU to CO_2 involves the action of a specific enzyme that has been proposed to (4) split the glucose from the ureide moieties, followed by a urease that hydrolyses the urea. In addition to enzymatic induction by upregulation of receptors, adaptation may occur over a period of days by selection of bacteria capable of metabolizing LU. The peak $^{13}\text{CO}_2$ production rate seen in Van Den Driessche et al.'s blank stool experiments was seen later than the time to maximal breath $^{13}\text{CO}_2$ enrichment (t_{max}) observed in the breath tests although the converse may have been expected, since there is no OCTT delay factor before the $^{13}\text{CO}_2$ appearance from incubated stool samples. This may have been accounted for by a lag time while enzymatic induction took place from the tracer sample itself rather than simply that the stool samples were cold at the start of the experiment.

The composition of the colonic flora is affected by the diet (5). Differences in the breast-feeding rates and composition of the weaning diet between Belgian and Italian children may account for a differential ability to metabolize LU and there is a need to reproduce the findings of both studies in different population groups.

The ^{13}C -LU breath test is an attractive means to measure OCTT that is more valid than the currently available hydrogen breath test, but it requires further validation in young children. Particular attention must be focused on the role of a pre-dose.

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Reply to Measurement of Oro-Cecal Transit Time in Young Children Using Lactose [^{13}C] Ureide Requires Further Validation

To the Editors: In reply to the letter to the editor by Christian et al. concerning our article entitled "The Lactose- ^{13}C Ureide Breath Test: A New, Non-Invasive Technique to Determine Orocecal Transit Time in Children," we have the following comments.

First of all, we are delighted by their shared interest and meaningful input.

In our article, we demonstrated by incubating stools that infants before complete weaning do not have the appropriate bacterial enzymatic activity (*Clostridium innocuum*) to hydrolyze lactose ureide.

Results of 11 LU breath tests on 10 children performed by Christian et al. showed that although these children were weaned, they did not all produce a clear $^{13}\text{CO}_2$ rise above baseline.

This result can be due to the difference in pre-dose and dose of testmeal. They gave 1×100 mg pre-dose, whereas we administered 3×500 mg the day before the test. Their testmeal contained 100 mg ^{13}C LU; our testmeal 250 mg ^{13}C LU.

Since the literature in adults and our own experience showed that pre-dosing is important (no pre-dose corresponds with no or a low response), the low dosages might be the reason for a lack of response.

In vitro induction with unlabeled LU one hour before the $^{13}\text{CO}_2$ collection showed no difference with no induced stools.

We fully agree with the comment of Christian that in addition to weaning, the type of weaning foods may be important and that further investigation is required.

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Modeling ^{13}C Breath Curves to Determine Site and Extent of Starch Digestion and Fermentation in Infants

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ABSTRACT

Background: The colon salvages energy from starch, especially when the capacity of the small intestine to digest it is limited. The aim of this study was to determine the site and relative extent of starch digestion and fermentation in infants. **Methods:** Thirteen infants (10 male and 3 female infants), median age 11.8 months (range, 7.6–22.7 months), were fed a starchy breakfast containing ^{13}C -labeled wheat flour after an overnight fast. Duplicate breath samples were obtained before breakfast and every 30 minutes for 12 hours. Breath $^{13}\text{CO}_2$ enrichment was measured using isotope ratio mass spectrometry, and results were expressed as percentage dose recovered (PDR) for each 30 minutes. The PDR data were analyzed and mathematically modeled assuming either a constant estimate of CO_2 production rate or adjusted for physical activity.

Results: Mean \pm SD cumulative ^{13}C PDR (cPDR) at 12 hours was $21.3\% \pm 8.4\%$ for unadjusted data and $26.5\% \pm 11.6\%$ for adjusted data. A composite model of two curves fit significantly better than a single curve. Modeling allowed estimation of cPDRs of small intestine ($17.5\% \pm 6.5\%$ and $22.7\% \pm 9.3\%$ for unadjusted and adjusted data, respectively) and colon ($4.6\% \pm 2.9\%$ and $6.3\% \pm 5.4\%$).

Conclusions: Modeling of $^{13}\text{CO}_2$ enrichment curves after ingestion of ^{13}C -enriched wheat flour is an attractive means to estimate the contribution of the upper and lower gut to starch digestion and fermentation. *JPGN* 34:158–164, 2002. **Key Words:** Starch—Carbon isotopes—Intestine, small—Colon; digestion—Mathematical model. © 2002 Lippincott Williams & Wilkins, Inc.

Starches are first introduced into the diet at weaning and thereafter become an increasingly important source of energy to the growing child. However, relatively little is known of the ability of the gut to digest starch in early life. Methods used to measure the digestion and absorption of starches include measuring duodenal enzyme concentrations (1), metabolic balance studies (2), and functional studies of glycemic response (3). However, these methods either call for meticulous and prolonged measurements or are too invasive for repeated use in infants.

Starches are plant-storage complex carbohydrates that comprise glucose polymers with $\alpha 1,4$ (amylose, straight chain) and $\alpha 1,6$ (amylopectin, branched chain) linkages.

They are one of the largest molecules in the diet with a molecular weight often exceeding 100 kd. In the adult, starches are digested mainly by the action of pancreatic α -amylase, but in the young child, this enzyme is found in low concentrations in the duodenum (1,4). Salivary amylase and small intestinal glucoamylase may in part account for the capacity for starch digestion in infants (5).

Starch and the products of starch digestion that escape digestion in the small intestine and that pass into the large bowel are defined as resistant starch (6). In the colon, they are substrates for colonic bacteria and are fermented to gaseous products, including hydrogen and carbon dioxide, and short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate. Short-chain fatty acids have a number of beneficial effects: increasing absorption of water and salt, inhibiting enteropathogens, and promoting colonic mucosal integrity (7). They also may provide a source of otherwise lost energy (8,9). The proportion of dietary starch that is resistant is greater in young children than in adults (2,10), which suggests an important role for the colon in energy salvage in early life, but little is known of the magnitude of its importance.

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¹³C breath tests have been used in infants to measure starch digestion in the small intestine (11) and fermentation in the colon (12,13). Because stable isotopes are nonradioactive, ¹³C breath tests are safe, and sampling breath rather than blood makes them particularly attractive for the study of infants (14,15). We tested the hypothesis that starch is digested in both the small intestine and the colon, and used mathematical interpretation of ¹³C recovery curves to measure the site and relative extent of starch digestion in infants.

METHODS

Thirteen infants (10 male and 3 female infants; mean age, 11.8 months; range, 7.6–22.7 months) were studied. All children lived in the Emilia Romagna region of north Italy. Eleven infants had been breast-fed exclusively for a minimum of 4 months. One child received mixed feeding from birth, and one was exclusively formula-fed from birth. Starch was introduced into the diet of all 13 children between 4 and 6 months, and at the time of the experiments, wheat starch comprised the major starch of their midday and evening meals. No child had received antibiotics in the month preceding the experiment. One child was restudied after an interval of 3 months, and his second set of data has been included as a separate subject. This is justified because we would have expected a change in the digestive capacity of the small intestine during this period.

A stable isotope breath test was used, based on the principle that the appearance of ¹³CO₂ in the breath after ingestion of a substrate labeled with ¹³C depends upon exogenous and endogenous factors that control its digestion, absorption, and metabolism. The ratio of ¹³C to ¹²C in breath CO₂ can be measured by isotope ratio mass spectrometry and by measuring of estimating CO₂ production rate (VCO₂), the percentage of the isotope dose recovered in the breath per unit time (PDR), or the cumulative PDR for the duration of the test (cPDR) can be calculated. Assuming that rates of absorption, distribution, and oxidation of the products of ¹³C-labeled starch digestion and fermentation remain constant, PDR values can be used to describe the rates and relative extent of the processes.

The substrate was wheat that had been cultivated in ¹³CO₂ to produce flour with an enrichment of 4 atom percent (ap) ¹³C, which was composed of 54% starch, 15% protein, and 9% nonstarch polysaccharide (16). After an overnight fast of at least 6 hours, two duplicate baseline breath samples were collected. A small amount of labeled wheat flour was then given (100 mg to the first nine subjects and 200 mg to the last five subjects), mixed with milk or water in a small cup and administered with a syringe or teaspoon. The cup was rinsed several times to ensure that the whole dose had been ingested. The child was then allowed to eat a normal breakfast, which in almost all cases included starchy food. Infants did not eat again for 2 hours, when they were allowed to eat normally but avoided those foods naturally enriched in ¹³C, such as maize products and cane sugar. The major source of sugar in Italy is beet sucrose; therefore, no restrictions on sweet foods were necessary.

Breath was collected using two methods. While the infants were awake, a bag and mask with a one-way valve was used to collect expired air (14). The breath collection took several sec-

onds and caused minimal distress in most children. While they were sleeping, breath was collected using a piece of nasogastric tubing attached to a syringe that was placed close to, but not touching, the nostrils. Every time the child exhaled, the syringe was aspirated by approximately 0.5 mL. Breath samples were collected in duplicate every 30 minutes for 12 hours.

In two children, reproducibility studies were performed. In an infant of 8 months, a second 12-hour breath test was performed 5 days after the first test, under similar conditions of meal timing and composition and of sleep periods. In the second infant, also 8 months old, the tests were performed 11 days apart. For this infant, sleep periods and meal composition varied more between the tests.

The samples were collected in evacuated tubes and analyzed using isotope ratio mass spectrometry (20-20 IRMS and Roboep-G preparation system (Europa Scientific; Crewe, UK) (17). Current isotope ratio mass spectrometry system analysis gives a co-efficient of variation of 3.04% for CO₂ analysis and 0.037% for ¹³C analysis for standard samples. Previous research has shown that ¹³CO₂ is stable for up to 8 weeks if stored in these sampling tubes (18). The ¹³C enrichment of expired CO₂ is expressed in parts per million excess (ppme), that is, the difference of the ¹³CO₂ abundance of a measured breath sample from the reference ¹³CO₂ abundance (the mean baseline sample). Expression as ppme rather than atom percent excess (ape) is conventionally chosen (ppme = 104 ape) to scale the results to a convenient range. The PDR for each 30-minute period was calculated using a value for basal VCO₂ (5 mL · min⁻¹ · m² surface area) derived from a formula described elsewhere (19) and a height-weight estimation of surface area (20). The results were expressed graphically for the 12 hours. The time taken to reach maximal enrichment (t_{max}) was calculated from the first derivative of a three-point smoothed data plot: $\delta PDR/\delta t = (PDR_{t+1} - PDR_{t-1})/2$, as the point at which the curve first crossed the time axis.

A mathematical model was used to analyze the ¹³C PDR plots, whereby the curve could be extrapolated to baseline and then cPDRs for the whole gut, small intestine, and colon could be calculated. The model was based on the following: $y = a \cdot t^b \cdot e^{-ct}$, where y is the PDR; t is time; and a, b, and c are constants. Simplistically, a represents a scaling factor, b controls the up-slope of the curve, and c the down-slope. This equation comes from the χ^2 statistical distribution and has been used previously for modeling gastric emptying curves (21). For our purposes, the curve was modified by introducing a delay factor d to account for intestinal passage. The equation then becomes $y = a \cdot (t + d)^b \cdot e^{-c(t+d)}$ (22). Using the Solver function of the Microsoft Excel (Microsoft, Redmond, WA) spreadsheet program, values were found for the constants a, b, c, and d so that a coefficient of variation (CV) was minimized. This was calculated as the sum of the square of the differences at each time point and normalized by the mean of the modeled data, with degrees of freedom dictated by the number of variables.

Figure 1 illustrates the modeling method. Each data plot was modeled initially to a single curve. Many data plots suggested that a composite of two curves would give a better fit. Data were therefore modeled to a composite curve, $y = y_1 + y_2$, where y₁ and y₂ take the same form as above, each with four constants a, b, c, and d. Early modeling suggested that the downslopes of both curves were similar. Physiologically this was probably indicative of losses from the body's bicarbonate

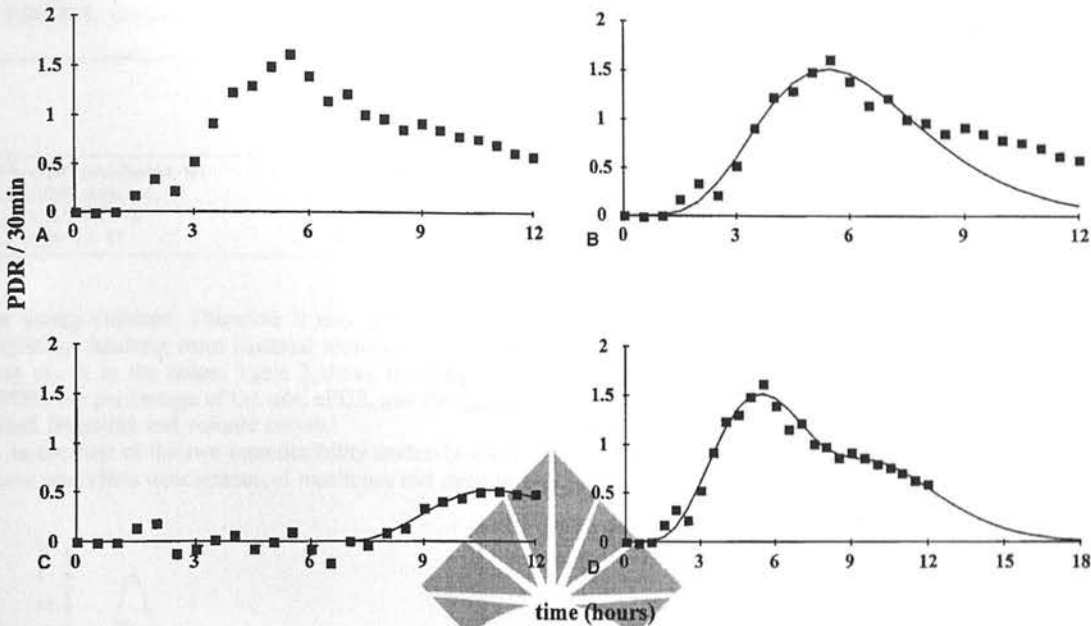


FIG. 1. A schematic illustration of the modeling method. **A:** Percentage dose recovered (PDR) plot. **B:** A best-fit single curve model, assumed to represent small intestinal digestion. If the single model is subtracted from the original PDR plot, the remainder, assumed to represent colonic fermentation, can be similarly modeled (**C**). The Solver function (Excel; Microsoft) was used to find the best fit for two curves simultaneously as is shown in **D**, with the model extrapolated to baseline at 18 hours.

pool at a constant rate; therefore, the same value of c was used for both y_1 and y_2 . From the whole composite curve, an overall CV was calculated. The curve was also extrapolated to baseline so that a cPDR at t_{∞} could be calculated. The parameter t_{\max} could also be calculated from the model, $t_{\max} = d + b/c$.

Frequency diagrams of cPDR results approximated a normal distribution and the two approaches to curve modeling were compared using paired Student t tests. Individual parameters of each curve were compared rather than amassing the individual time point data (23). Estimates of cPDR at t_{∞} , cPDRs for small intestine and colon and time to peak ^{13}C recovery (t_{\max}) were expressed as mean \pm standard deviation (SD).

The University of Modena granted ethical approval for this study in accordance with the Declaration of Helsinki of the World Medical Association. Parents of each child gave informed, written consent.

RESULTS

Following ingestion of 100 mg enriched wheat flour, the mean \pm SD maximal rise of ^{13}C enrichment over baseline was 43.4 ± 19.0 ppme, and after a dose of 200 mg it was 80.9 ± 24.5 ppme. Using a constant value of basal VCO_2 predicted from body surface area (24) throughout the test, the mean \pm SD cPDR over 12 hours from the calculated data was $21.3\% \pm 8.4\%$. Mean \pm SD t_{\max} was 3.9 ± 0.9 hours.

Infants were not at rest throughout the 12-hour test, and an attempt to correct for physical activity was made.

For each 30 minutes that the child was awake, the PDR was increased by a factor of 1.4 (25). The mean \pm SD cPDR of the adjusted data for 12 hours was $26.5\% \pm 11.6\%$.

The CV representing the accuracy of curve fitting for the composite two-curve model was significantly lower than that for a single-curve model when compared using paired Student t tests ($P = 0.002$ for unadjusted and 0.001 for adjusted data). Therefore, the composite model was adopted for further analyses. Validity of the composite curve model was strengthened by comparing cPDRs at 12 hours calculated from the raw data, and cPDRs at 12 hours estimated from that model. Table 1 shows these figures. The models were extrapolated to baseline, which gave estimates of mean \pm SD cPDR at t_{∞} of $22.1\% \pm 8.6\%$ and $29.1\% \pm 11.8\%$ for unadjusted and adjusted data, respectively. Figure 2 shows the modeled curves for both unadjusted and adjusted data.

We assumed that a single modeled curve represented a single metabolic process and therefore assumed that the first and larger of the two modeled curves represented digestion in the small intestine. This curve had a mean \pm SD t_{\max} of 3.4 ± 1.0 hours. The t_{\max} of the second curve was 8.5 ± 1.6 hours. The time to 10% of large intestinal cPDR was defined as orocecal transit time. The mean \pm SD orocecal transit time was 7.3 ± 1.5 hours and compared well with an initial sustained rise in breath hydrogen concentrations observed in our own earlier studies

TABLE 1. Comparison of 12-hour cPDR and small intestinal t_{max} for raw data and modelled data from the single curve and composite curve models

	Raw data		Single curve model		Composite curve model	
	Mean	SD	Mean	SD	Mean	SD
12-h cPDR (unadjusted, %)	21.3	8.4	20.6	8.3	21.2	8.1
12-h cPDR (adjusted, %)	26.5	11.6	27.3	11.6	27.9	11.0
t_{max} (unadjusted, h)	3.9	0.9	—	—	3.4	1.0
t_{max} (adjusted, h)	3.9	0.9	—	—	3.4	1.0

on young children. Therefore it may largely represent digestion resulting from bacterial fermentation of resistant starch in the colon. Table 2 shows the component cPDR, the percentage of the total cPDR, and the t_{max} for small intestinal and colonic curves.

In the first of the two reproducibility studies in which there was close concurrence of mealtimes and sleep pe-

riods, the two curves showed a striking similarity in form of the small intestinal curve but less agreement for the colonic curve. In the second study, there was less concurrence with mealtimes and sleeping and these differences were reflected in the different curve shapes for each day. Table 3 shows the results.

DISCUSSION

We have developed a means of measuring starch digestion that is noninvasive, easy to perform, and well tolerated by infants. Wheat flour intrinsically labeled to 4 ap ¹³C is sufficient to generate clear peaks of ¹³CO₂ enrichment over baseline when used in doses of 100 or 200 mg. The cPDRs were comparable with those obtained from other stable isotope studies of children and adults (16,26) but were quite variable in form. Mathematical models provide an objective means to analyze the data plots, and a composite two-curve model produced significantly closer fits than a single curve. From this, it was possible to separate the individual curves that we have attributed to small-intestinal digestion and colonic bacterial fermentation. This is of greater significance and utility when the two curves are not clearly separable by eye.

Stable isotope breath tests have been used in studies of starch digestion in young children (11). However, without the use of mathematical models, it is not possible to distinguish between ¹³CO₂ produced by oxidation of glucose digested in the small intestine and that produced by oxidation of SCFA derived from colonic fermentation. The initial rise in breath hydrogen has been used to mark the arrival of undigested starch in the colon and therefore measure the contribution of colonic fermentation to starch digestion (11). This provides only limited information about the extent of digestion in the small intestine and no information about the extent of colonic fermentation. Moreover, other limitations of using the hydrogen breath test quantitatively must be borne in mind: different routes of disposal of hydrogen exist in different individuals (27), and therefore the concentration of hydrogen in the breath does not reflect the degree of starch fermentation (28,29).

Qualitative differences in curve shapes have been observed among infants consuming carbohydrates of in-

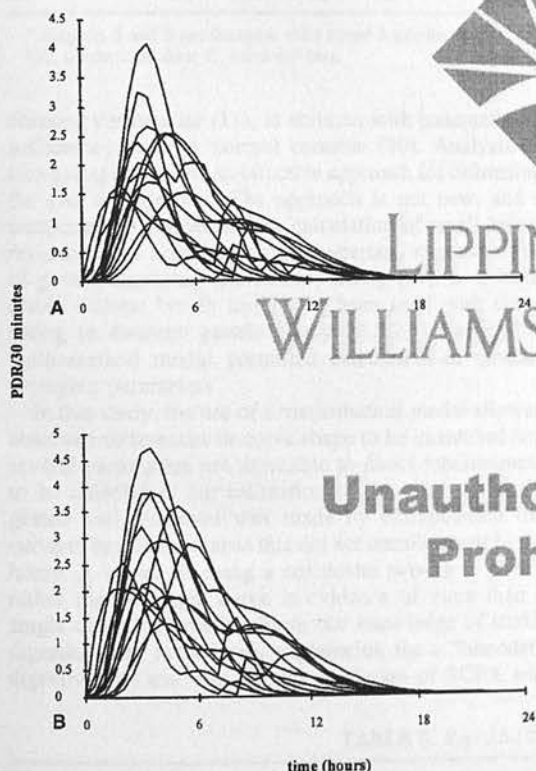


FIG. 2. Modeled percentage dose recovered curves for all subjects shown together. **A:** Unadjusted data, based on a constant value for V_{CO_2} . **B:** Data have been adjusted for periods of wake and sleep. For many subjects, the modeled curve reflected the two curves that could be teased out with the naked eye. For most, however, the form of the two curves was not immediately apparent, but the significantly lower mean coefficient of variation for a composite as opposed to a single-model curve suggests that a second physiologic process is taking place.

TABLE 2. cPDR and t_{max} derived from modelled data for each subject using V_{CO_2} based on predicted BMR and on physical activity adjusted data

Age (mo)	Whole gut				Small intestine				Colon						
	cPDR		cPDR		% of total cPDR		t_{max} (h)		cPDR		% of total cPDR		t_{max} (h)		
	UC	C	UC	C	UC	C	UC	C	UC	C	UC	C	UC	C	
1	7.6	9.8	12.6	9.2	12.0	94	96	4.1	4.3	0.6	0.5	6	4	7.3	7.2
2	7.8	26.9	34.6	18.7	16.6	70	48	3.1	2.5	8.2	18.0	30	52	6.1	6.0
3	7.8	33.7	42.8	25.8	32.1	76	75	4.8	4.9	7.9	10.6	24	25	10.3	9.9
4	8.3	26.5	32.4	19.8	25.6	75	79	3.3	4.2	6.7	6.8	25	21	9.1	9.6
5*	9.1	19.2	25.0	18.0	24.0	94	96	3.2	3.2	1.2	1.0	6	4	6.7	8.4
6	9.6	25.9	39.1	23.8	34.1	92	87	3.5	3.6	2.2	5.0	8	13	10.8	11.0
7	11.4	18.3	21.3	15.9	19.6	87	92	2.9	3.0	2.5	1.6	13	8	8.6	8.5
8*	12.1	21.9	28.4	16.3	16.9	74	59	5.4	4.8	5.6	11.6	26	41	10.8	9.8
9	15.5	30.6	40.1	25.0	37.1	82	92	4.4	4.0	5.6	3.0	18	8	9.5	8.0
10	15.6	10.7	16.4	8.8	14.3	83	87	1.9	1.9	1.8	2.1	17	13	6.1	6.3
11	15.8	19.7	22.7	14.3	17.5	73	77	3.0	3.4	5.3	5.2	27	23	7.9	7.9
12	16.6	18.3	25.4	12.6	17.8	69	70	1.8	1.8	5.6	7.6	31	30	8.3	9.6
13	21.4	37.3	52.5	28.3	38.6	76	74	3.4	3.3	9.0	13.9	24	26	8.9	9.8
14	22.7	10.0	13.5	8.3	11.8	84	87	3.1	3.1	1.6	1.8	16	13	8.3	8.3
Mean	22.1	29.1	17.5	22.7	80	80	3.4	3.4	4.6	6.3	20	20	8.5	8.6	
SD	8.6	11.8	6.5	9.3	9	14	1.0	1.0	2.9	5.4	9	14	1.6	1.4	

* Subjects 5 and 8 are the same child tested 3 months apart.
UC, uncorrected data; C, corrected data.

creasing complexity (11), in children with pancreatic insufficiency, and in normal controls (30). Analysis of curve shapes may be an effective approach for estimating the site of digestion. The approach is not new, and a mathematical model for the calculation of small bowel residence has been made from γ -camera measurements of gastric emptying and colonic filling (31). In infants, stable isotope breath tests have been used with curve fitting to measure gastric emptying (32) in which the mathematical model permitted calculation of gastric-emptying parameters.

In this study, the use of a mathematical model allowed observed differences in curve shape to be quantified and several parameters not amenable to direct measurement to be calculated. An estimation of the total starch digested and absorbed was made by extrapolating the curve to baseline because this did not usually occur by 12 hours. A closer fit using a composite two-curve model rather than a single curve is evidence of more than a single digestive process. From our knowledge of starch digestion, the most likely explanation for a "bimodal" digestive process was through oxidation of SCFA and

bacterially produced CO_2 , produced and absorbed as a result of colonic fermentation (in addition to small intestinal digestion). Alternative explanations are less likely. Ingestion of naturally enriched carbohydrate was controlled, and we demonstrated no relationship between the time of the second peak and midday mealtime. The short duration of the experiment makes enrichment of body fat and protein unlikely (33) and in the minimally exercising state, muscle glycogen will not be mobilized to any extent. Subsequent breakdown of labeled glycogen from liver stores trickling out over several hours may in part account for the second curve (34). However, it probably does not wholly explain the second curve because such breakdown would not be expected to cause the discrete second peak that we have observed. The main strength of this modeling approach lies in the possibility it offers to separate the contributions of small intestine and colon to the digestive process in a more comprehensive way than does use of a simple time of rise in breath hydrogen concentration. We have estimated that, for children of this age, the colon could be the site of up to 20% of total starch digested.

TABLE 3. Reproducibility studies on subjects 3 and 2

Subject	Uncorrected data					Corrected data				
	Whole gut cPDR	% of whole gut cPDR		t_{max}		Whole gut cPDR	% of whole gut cPDR		t_{max}	
		Small intestine	Colon	Small intestine	Colon		Small intestine	Colon	Small intestine	Colon
2	33.7	76.5	23.5	4.8	10.3	42.8	75.2	24.8	4.9	9.9
	37.8	70.0	30.0	4.2	10.5	52.5	68.5	31.5	4.3	11.1
3	26.9	69.5	30.5	3.1	6.1	34.6	47.9	52.1	2.5	6.0
	23.1	44.1	55.9	1.7	3.8	31.5	90.7	9.3	2.3	3.2

In early experiments, the dose of labeled wheat flour used was 100 mg. Reappraisal of the technique after several experiments suggested that in some cases the curves were close to baseline ¹³CO₂ variability, and the higher dose of 200 mg was used from then on. There was no significant difference in cPDR between the two doses. The wheat flour contained 9.4% nonstarch polysaccharide and this may have contributed to the colonic curve. Bran comprises most of the nonstarch polysaccharide and its enrichment, measured on the first of the batches of ¹³C wheat was 2.9 ap as compared with 2.6 ap for the flour (M. Harding, personal communication). However, this is unlikely to have contributed greatly to the CO₂ enrichment because unpublished *in vitro* experiments with fecal cultures in our laboratory have suggested that wheat bran may not be readily fermentable *in vivo*.

Of the two reproducibility studies, the one more closely matched for periods of sleep and mealtimes had better agreement. However, in neither study was the agreement improved by correcting for physical activity. This suggests that before this approach can be used for interindividual comparison, consideration needs to be given to controlling mealtimes and sleep periods. Furthermore, until better reproducibility can be achieved, the use of these experiments is appropriate for population measures but not for diagnostic purposes. This finding supports those of others who have attempted to use starch breath tests diagnostically (26).

Stable isotope breath tests are usually performed in controlled conditions, with regulation of diet and activity so that a basal estimate of VCO₂ can be considered valid. This was not possible with infants for 12 hours; therefore, the PDR was adjusted for physical activity by multiplying the VCO₂ by a factor of 1.4 (25) during wake periods. In many cases, smooth data set curves were transformed to look less physiologic, and for this reason both the adjusted and unadjusted data were presented, although use of the former may have offered a closer reflection of the free-living situation. Ideally, VCO₂ should have been measured for each 30 minutes, but with existing techniques of directly measuring VCO₂, this was not practical in children so young.

The source of ¹³CO₂ from the colon includes CO₂ produced directly from bacterial fermentation and from SCFA, which may have many different metabolic fates. Our own *in vitro* experiments with fecal bacterial cultures (35) suggest that CO₂ produced directly by bacterial fermentation, and therefore not contributing to energy salvage, may account for up to one third of the total CO₂ measured in the breath. We have also measured only the end product of oxidation. This makes no allowance for different metabolic pathways in the fed and fasted state. During the fed state, the proportion of energy that is directly oxidized will be greater. Also, during the fed state, a greater proportion of the CO₂ will result from normal dietary carbohydrate, which is more naturally abundant in ¹³C than in body fat stores. Such physi-

ologic considerations limit the conclusions that can be drawn about the magnitude of the colonic component for digestion.

In our experience, ¹³C natural background variation in breath CO₂ in children occurs toward the upper end of the range quoted for adults (36). We performed 12-hour baseline studies in four subjects and found that breath CO₂ enrichment varied between -13.4 and 22.5 ppme with respect to the first sample of the day. For the small intestinal component, the CO₂ enrichment was clearly above this baseline variability. The second peaks often fell within this variability. However, in four subjects, 12-hour baseline studies performed under the same physiologic conditions showed a clear difference in ¹³CO₂ breath enrichment between baseline and tracer. Also the general homogeneity of the curves (Fig. 2) suggests that they are more likely to represent a physiologic phenomenon than to represent artefact.

The role of the colon in early childhood is not well understood. The colon probably plays a part in energy salvage in early life, when the digestive capacity of the small intestine is limited. If this role of the colon were better quantified, advice about weaning foods could be based on their potential energy yield at any given age, and the extent to which they might promote SCFA production in the colon. The energy yield from unavailable carbohydrate has been calculated for adults (9), but less attention has been focused on the physiology of the small intestine of the young child. We estimate that for children between 8 and 23 months, up to 20% of total wheat flour digestion may take place in the colon. Because most of the digestible and fermentable material in wheat flour is starch, it seems reasonable to extrapolate and apply these results to starch. This suggests that the colon has a large reserve for energy absorption that may be used in conditions of limited small intestinal digestive capacity, such as pancreatic exocrine insufficiency.

In summary, we have demonstrated how ¹³CO₂ recovery data from stable isotope breath-test curves can be modeled. A number of limitations to our method exist, some of which will be resolved with further studies controlling for activity levels and mealtimes, and considering the effect of liver glycogen enrichment. Other approaches to separate the role of small and large intestines in digestion have at best provided only a semiquantitative analysis. This approach offers an attractive and quantitative means to measure the contributions of the small and large intestine to starch digestion in young children, giving information about the digestibility of various starches at different stages of weaning and the capacity of the colon to use undigested starch in the production of SCFA with their potential benefits of providing "unavailable" energy and in promoting colonic health.

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ORIGINAL COMMUNICATION

Starch fermentation by faecal bacteria of infants, toddlers and adults: importance for energy salvage

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Objective: Little is known of the degree to which the colon salvages energy through starch fermentation in young children. Using a simulated colonic environment, we aimed to account for the fate of fermented raw and cooked starch in two groups of young children and in adults.

Design: A slurry was prepared from faecal samples from six infants (7–10 months), six toddlers (16–21 months) and seven adults (24–56 y). Each slurry was anaerobically incubated with raw or cooked maize starch in MacCartney bottles in a shaking water bath. Parallel incubations were stopped at 4 and 24 h. The headspace gas volume was analysed for CO₂ and methane. The culture supernatant was analysed for short-chain fatty acids (SCFA), lactate and residual starch.

Results: Different patterns of fermentation were seen at 4 and 24 h. For raw starch, the production of SCFA decreased with subject age at 4 h but not at 24 h. With both substrates at 4 h, toddler stools produced significantly more CO₂ than infants or adults, but there were no statistical differences at 24 h. Methane was detected in three adults only. Lactate was detected mainly at 4 h in children.

Conclusions: The results suggest that fermentation, particularly of raw starch, is a more rapid process in young children than in adults. A highly efficient energy salvage process may occur in the colon of young children.

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Keywords: fermentation; colon; child; short-chain fatty acid; starch; stoichiometry; energy salvage

Introduction

The composition of the bacterial flora of the colon changes through infancy and childhood. Early colonisation is influenced by the type of milk feeds, with higher proportions of *Bacteroides* spp and facultative organisms found in the faeces of formula-fed infants and higher proportions of *Bifidobacterium* spp in the faeces of breast-fed infants (Bullen *et al*, 1977; Long & Swenson, 1977; Balmer & Wharton, 1989). During weaning, in particular in the breast-fed infant (Stark & Lee, 1982), there is a change in the colonic flora in association with the introduction of dietary complex carbohydrate. Higher proportions of *Bacteroides* spp are seen in older infants but they are still less prevalent than bifidobacteria, as distinct from the proportions seen in the

adult colon (Guérin-Danan *et al*, 1997). By adulthood there is a stable population of over 400 bacterial species (Moore *et al*, 1978).

Colonic bacteria ferment nutrients which pass undigested from the small intestine into the large bowel. Fermentation products include the volatile short-chain fatty acids (SCFA), lactate and gases, including carbon dioxide, methane and hydrogen. The patterns of SCFA are attributed to the bacterial species present, but are also because of the composition of diet. Numerous dietary factors influence bacterial colonisation (Edwards, 1994) and thereby fermentation products. The faeces of breast-fed infants contain higher concentrations of lactic acid and lower concentrations of propionic and *n*-butyric acid than those of formula-fed infants (Edwards *et al*, 1994). *Bacteroides* spp and bifidobacteria have both been shown to be capable of fermenting some polysaccharides (Salyers & Leedle, 1983; Edwards & Rowland, 1992). Starch is preferentially fermented to butyrate (Scheppach *et al*, 1988). Hence with a changing diet throughout childhood, the fermentation patterns of the colonic flora also change. In young children,

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the capacity of the upper gut to digest and absorb starch has been shown to be less than that in adults by luminal measurement of pancreatic enzymes (Zoppi *et al*, 1972), functional studies (Anderson *et al*, 1972) and stable isotope breath tests (Shulman *et al*, 1983). Such studies are too invasive for routine use in children (Anderson *et al*, 1972; Zoppi *et al*, 1972) or rely on breath hydrogen measurements which have fundamental flaws (Modler *et al*, 1988; Khin-Maung-U *et al*, 1992). It would be very difficult to separate hydrogen produced from starch fermentation from that produced from nonabsorbed lactose or oligosaccharides in milk or from dietary fibre in weaning foods. It is possible that in young children the colon receives significant amounts of undigested or resistant starch and this may make a contribution to energy supply through oxidation of SCFA produced in the colon (Bond *et al*, 1980; Kien *et al*, 1987).

Models based on stool incubation have been used previously to simulate fermentation in the colon (Edwards *et al*, 1996). *In vitro* studies circumvent some of the problems encountered with *in vivo* studies: they are cheap, the collection of faeces is simple (if unpleasant) and retention of fermentation products within the system makes their measurement easier. However, the validity of *in vitro* models has been questioned on several counts: the degree to which faeces are representative of colonic flora has been debated (Fernandez *et al*, 1985; Drasar, 1988); the inaccessibility of the colon makes precise mimicking of its conditions very difficult (Edwards & Rowland, 1992), but more importantly, removal of faeces from the colon inevitably changes bacterial composition and metabolic activity; because fermentation products are not absorbed, and their accumulation in the incubation chamber can itself be bacteriostatic.

Continuous and semicontinuous culture models ameliorate some of these limitations (Edwards & Parrett, 1999), but at the cost of complex and expensive systems.

In this study, using stool as a model for colonic composition, we aimed to quantify the fermentation patterns and products of a raw and cooked starch at different ages and to account for the fate of the starch carbon within the fermentation products.

Subjects

In all, 19 healthy subjects were studied: six infants (two male) median age 9 months (range 7–10 months), six toddlers (three male), median age 19 months (range 16–21 months) and seven adults (three male), median age 29 y (range 24–56 y). Adults were recruited from the staff of the Yorkhill hospitals. The children and infants were recruited from friends and relatives of the staff of Yorkhill Hospitals. Ethical approval was granted by the Yorkhill Ethics Committee. An information sheet was given to all parents and prior written consent was obtained. All infants and children had been exclusively breast fed for at least 3 months. No subject had taken antibiotics for at least 2 months prior to the study.

Stool samples were collected and processed within 2 h of passage.

In vitro model

A 5% faecal slurry was prepared by homogenising a weighed faecal sample with phosphate buffer at pH 7. A 5 ml volume of this slurry was pipetted in parallel into 30 ml McCartney bottles which contained 25 mg of cooked or raw maize starch, or no carbohydrate to allow for measurement of fermentation of endogenous carbohydrate. The raw maize starch was a standard laboratory substrate (Corn starch, Sigma). The cooked starch was prepared by boiling a solution of raw starch for 10 min and then freeze-dried to constant weight. The incubation method was based on previously validated methods (Edwards *et al*, 1996). The relative amounts of slurry and starch were determined from pilot studies in which different proportions of slurry and maize starch were incubated for 24 h and then plated out under aerobic and anaerobic conditions. The plate which contained the greatest ratio of anaerobes to aerobes was considered to be that which most closely mimicked conditions *in vivo*.

Before incubation, each bottle was flushed with oxygen-free nitrogen to initiate anaerobic conditions. These were maintained by the addition of a cysteine hydrochloride-sodium sulphide reducing solution to the slurry (cysteine hydrochloride 0.3 mg/ml, sodium sulphide 0.3 mg/ml, sodium hydroxide 1.9 µmol/ml). The samples were incubated in a shaking water bath at 37° and 60 strokes/min, aiming to simulate the conditions in the colonic lumen. At 4 and 24 h, parallel incubations were stopped and the contents were analysed.

Measurement of fermentation products

The volume of the headspace gas was measured using a syringe as a manometer. Samples of gas were diluted with oxygen-free nitrogen and then analysed for concentrations of carbon dioxide (Servomex, Crowborough, UK) and methane (continuous flow isotope ratio mass spectrometry). The slurry was frozen and at a later date thawed and sampled to measure SCFA and lactate content by gas liquid chromatography (GLC) of acidified ether extracts (Spiller *et al*, 1980). The remainder of the slurry was then used to measure the residual starch content using a method based on that first described by Englyst *et al* (Englyst & Cummings, 1987; Edwards *et al*, 1996).

After dispersion of the starch, a sample was taken for combustion analysis to measure the bacterial cell mass. This was first neutralised with concentrated sulphuric acid, then pipetted into 100 µl aliquots in tin boats. After drying, the samples were combusted and the carbon content was determined by continuous flow isotope ratio mass spectrometry (CF-IRMS) (Prosser *et al*, 1991).

Expression of results and statistical methods

The amount of each fermentation product was corrected for fermentation of endogenous substrate by subtraction of the values from the no carbohydrate control cultures. The results were then expressed both as absolute concentrations and as percentage dose recovered (PDR). This is the percentage of carbon within the original starch substrate that was recovered in each fermentation product. The data did not conform to a normal distribution and therefore statistically significant differences between groups were determined nonparametrically, using the Wilcoxon rank sum test in its paired and nonpaired forms as appropriate to the data and results were expressed as median and range.

Results

The concentrations of the main fermentation products for each age group are shown in Table 1. In Figure 1, means of the fermentation products for each age group and each substrate are shown as PDRs.

At 4 h, the stools of toddlers produced significantly ($P < 0.05$) more CO_2 than the stools of adults and the stools of infants. By 24 h, the differences were not statistically significant.

At 4 h, the highest concentrations of SCFA were found in infant stools. There was a pattern of decreasing SCFA production with increasing age. The difference was significant ($P < 0.05$) between the stools of toddlers and adults for raw starch. Between the stools of infants and toddlers for raw starch, and for all groups for cooked starch the differences did not reach statistical significance. At 24 h, the pattern had changed with most SCFA produced by the stools of infants, followed by adults and finally by toddlers. The differences however did not reach statistical significance.

Lactate was seen mainly at 4 h. There was significantly ($P < 0.05$) less lactate production with increasing age of subject for raw starch, but no significant differences for cooked starch. The stool of one infant produced unaccountably large concentrations of lactate even in the corresponding no carbohydrate MacCartney tube. All her data were omitted from the analysis.

The residual starch results were more variable (Table 1). However, they do support those for SCFA, in that for adults, little fermentation took place in the first 4 h. For one infant there was one measurement that was above the analytical range for cooked starch at 4 h. This made the value obtained unreliable and so this individual result was left out of the analysis.

The confidence intervals for the biomass results were too large to permit the drawing of any conclusions. We would have expected a reciprocal relation with the residual starch results, and this was broadly seen for the infants' stool results. At 4 h, methane was detected from one adult stool only, accounting for <1% PDR. At 24 h it was detectable from three adult stool cultures with a maximum PDR of 2%.

Table 1 Concentrations of CO_2 , total SCFA, lactate and residual starch after fermentation of raw and cooked maize starch at 4 and 24 h by faecal bacteria from infants, toddlers and adults

		H_2 (mmol/l)		CO_2 (mmol/l)		CH_4 (mmol/l)		Total SCFA (mmol/l)		Lactate (mmol/l)		Residual starch (mg/5 ml)		Biomass (mmol C/l)		
		median	range	median	range	median	range	median	range	median	range	median	range	median	range	
4 h	Infants	R	6.5	2.1-9.2	8.5	5.1-10.1	0	0-0.1	22.9	15.0-32.8	0	0-4.1	7.3	3.5-10.8	37.6	25.5-63.1
		C	8.6	2.8-17.5	11.4	5.3-24.9	0	0	26.8	23.1-34.4	12.4	5.4-18.9	1.8	0-2.1	11.3	0-29.8
	Toddlers	R	12.3 ^a	9.5-21.3	14.0 ^b	7.0-30.6	0	0	17.3	12.0-31.5	1.1	0-6.0	1.8	0-10.6	22.1	0-112.8
24 h	Infants	R	13.6	10.7-17.8	11.8	7.8-27.1	0	0	16.0	12.8-27.4	5.5	0-10.9	1.4	0.6-9.5	29.0	0-163.3
		C	2.0	0.1-7.3	2.7	1.1-12.9	0	0-0.9	5.4 ^c	1.2-24.1	0	0	12.8	2.3-21.4	11.9	0-129.4
	Toddlers	R	11.4	0.4-23.5	7.0	2.5-25.1	0	0-0.4	19.8 ^d	2.0-22.0	0	0-6.5	5.3	1.0-12.1	16.6	0-61.4
Adults	R	13.8	1.4-17.5	26.6	8.3-30.7	0	0	34.1	27.8-45.2	0	0	0.1	0.1-0.5	54.2	0-95.5	
	C	16.8	3.9-26.0	28.8	14.3-44.4	0	0	33.6	29.2-48.5	0	0	0.8	0-1.3	0	0-104.4	
	Toddlers	R	17.2	13.4-22.3	30.5	23.4-36.0	0	0	24.5	19.6-33.5	0	0	0.2	0-0.3	26.5	8.9-47.2
Adults	R	16.7	11.2-28.4	33.0	24.1-41.7	0	0	27.2	22.3-38.7	0	0	0.1	0-0.3	29.5	16.0-79.5	
	C	3.1	0-30.6	24.9	19.5-37.1	0.8	0-2.3	35.8	19.9-49.8	0	0	0.3	0-2.7	37.2	0-63.0	
	C	4.4	0-35.2	29.3	23.3-40.9	0	0-3.7	28.5	23.0-44.4	0	0	0.1	0-3.8	0	0-84.8	

Significant (5%) differences between groups using Wilcoxon rank sum tests are shown for H_2 , CO_2 and SCFA results.

R—raw maize starch; C—cooked maize starch.

^aToddlers significantly greater than infants and adults.

^bToddlers significantly greater than adults.

^cAdults significantly less than infants and toddlers.

^dAdults significantly less than infants.

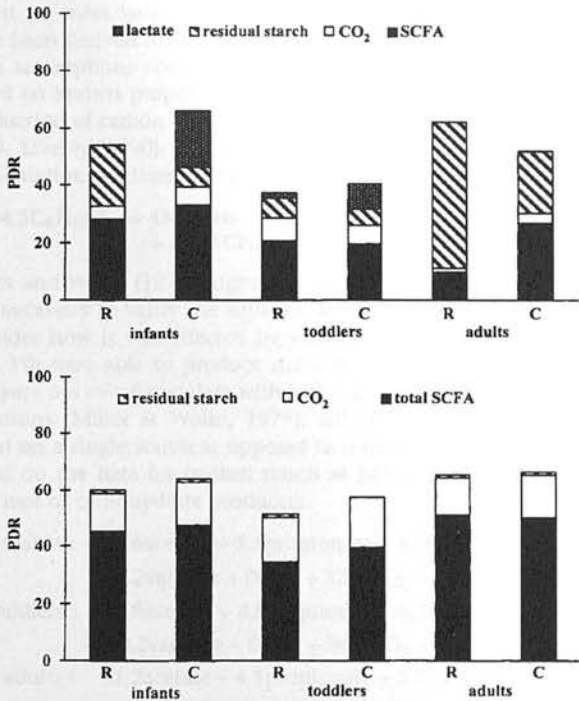


Figure 1 Median percentage dose recovered (PDR) of starch carbon converted to total SCFA, CO₂ and residual starch at 4 and 24 h after fermentation by stool of infants, toddlers and adults. Median PDR for methane and bacterial cell mass were <1% and are not shown. R—raw maize starch. C—cooked maize starch.

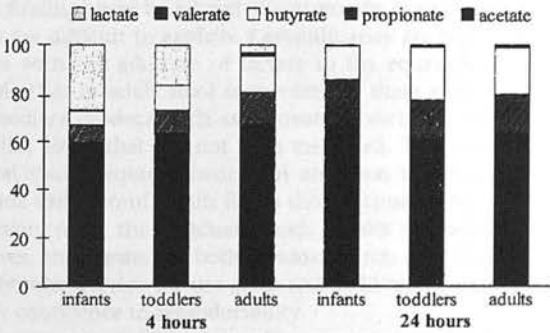


Figure 2 Individual SCFA and lactate as a percentage of the total SCFA and lactate after fermentation of cooked maize starch by stool of infants, toddlers and adults at 4 and 24 h. Values shown are means for each age group.

SCFA accounted for more of the original starch substrate than any other fermentation product with a PDR ranging up to 65%. CO₂ accounted for up to 18% of the starch carbon. The summed PDR for all fermentation products calculated for each individual subject varied from 25 to 103%.

The differences in the proportions of SCFA and lactate produced from the fermentation of cooked maize starch are shown in Figure 2. At 4 h, the highest proportions of acetate were found in the fermentations by the stools of infants and the lowest proportions of acetate were produced by adult stools. By 24 h, the proportions were similar. Butyrate and valerate were seen in measurable amounts only in adult stools. In contrast at 4 h, lactate accounted for up to 22% of the total PDR in infants, but little in adults. At 24 h it was hardly seen in any age group.

Despite expectations that cooked starch would be better fermented than raw starch, there were few significant differences. Adult stools produced significantly ($P < 0.05$) more H₂ at 4 h with cooked starch as a substrate, while at 24 h they in fact produced significantly less SCFA and CO₂.

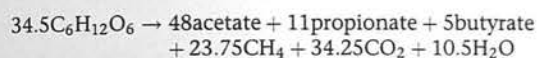
Discussion

We have shown that human faeces from infants, toddlers and adults can ferment raw and cooked maize starch. We have measured each of the main fermentation end products and expressed the results as a percentage dose recovered (PDR) of the original starch substrate. There were differences in the fermentation patterns of these three age groups: the most abundant fermentation products were SCFA but a considerable amount was also in the form of CO₂. We detected only small concentrations of methane and demonstrated intermediary production of lactate by the stools of infants and toddlers but not of adults.

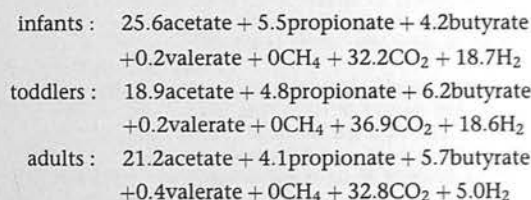
Others have studied carbohydrate fermentation by the colonic flora of infants and young children. Parrett *et al* (1997) studied the development of fermentation of a complex carbohydrate, guar gum, through weaning and demonstrated that it was still slow compared to glucose in late weaning (>16 weeks after the start of weaning). Compared to values of SCFA production for adult fermentation of complex carbohydrate, the capacity of children in late weaning was still less. In comparison to adults, our data show a more rapid fermentation of starch in infants and toddlers at 4 h. This suggests that fermentation is a more rapid process in the colons of young children than of adults. Our group of infants was of similar age to Parrett *et al*'s (1997) late weaning group. Differences may be explained by the presence of maize starch in the diet in greater concentrations than guar gum, which would result in proportionally more maize starch passing undigested into the colon. The subsequent adaptive changes in bacterial flora could then result in greater fermentation of maize starch.

Stoichiometry considers the products of a metabolic process in relation to the starting substrate. It is the calculation of the numerical relation between the molecular species entering a chemical reaction and the amount and type of molecules produced. Where such a plethora of complex and interdependent reactions are taking place, it is based on several assumptions and a more appropriate term

might be mass balance. Such equations for carbohydrate have been derived for adult humans and ruminant animals from assumptions about the products of fermentation and based on known proportions of SCFA in faeces and known production of carbon dioxide and methane (Miller & Wolin, 1979; Livesey, 1990). From these, the energy derived from fermentation reactions can be calculated, as for example:



Miller and Wolin (1979) suggested that further information was necessary to verify the equation for humans and also to consider how it was affected by variables such as age and diet. We were able to produce stoichiometric equations to compare our calculated data with Miller and Wolin's derived equations (Miller & Wolin, 1979), although our data are based on a single starch as opposed to mixed carbohydrate. Based on the data for cooked starch at 24 h, a comparable 34.5 mol of carbohydrate produced:



We found less methane, which may be an artifactual result because of slow development of methanogens or increased partial pressure of hydrogen needed before methane can be produced. In addition, methanogenic bacteria may not have been predominant in the infant stool cultures. We also found lower amounts of acetate and propionate in all age groups. These findings may be related to our poorly summed PDRs which are difficult to explain. Certainly, they are higher in infants with the addition of lactate to the equation. It is possible that in adult stool fermentations there is another intermediary product, such as pyruvate or succinate (Miller & Wolin, 1979) that has not been measured. The assay for residual starch requires meticulous attention to detail and the wide variation of results limits the conclusions that can be drawn from the residual starch results in isolation. However, the results for both residual starch and biomass broadly mirror those for the gases and SCFA where there is greater confidence in reproducibility.

The concept of PDR is usually associated with stable isotope breath tests, where it expresses the yield of expired $^{13}CO_2$ in relation to the ^{13}C enrichment of the original substrate (Weaver *et al*, 1993). We have chosen to express our results as PDR because this provides more information than absolute measurements of products by measuring them in reference to the initial fermentation substrate and quantifies the fate of the starch carbon substrate.

Cummings *et al* (1987) calculated molar ratios for the main SCFA, acetate:propionate:butyrate from colonic SCFA concentrations in the colons of victims of sudden death. They

calculated the molar ratio for the whole colon as 57:22:21. While our methodology was different, our own molar ratios for infants (73:15:12), toddlers (63:16:21) and adults (68:13:18) calculated from the data for cooked starch at 24 h were broadly in agreement with their findings. Comparing the figures for the three age groups suggests that in infancy there is proportionately more acetate and that the adult-type pattern of proportionately increased butyrate is established from an early age. Lactate is an intermediary product and is not therefore seen in the infant molar ratios at 24 h.

We chose raw and cooked starch as substrates in this model of a rapidly digestible and resistant starch (Englyst & Kingman, 1990). Since in young children the luminal concentrations of pancreatic α -amylase are lower than in adults (Zoppi *et al*, 1972), the proportion of starch passing into the colon is likely to be greater and using a model, resistant starch may give more information about the conditions *in vivo*. It was surprising to note a lack of significant differences between raw and cooked starch as substrates and an occasional finding of significantly better fermentation from a raw starch substrate. Alternative starches in raw and cooked forms such as potato starch might have demonstrated a difference. It is also important to note that the power of the significant results is limited by the number of parameters for a small sample size.

Fermentation products were measured at 4 and 24 h to determine the rate of fermentation. In general, the stools of infants and toddlers produced more SCFA at 4 h but by 24 h the difference was less evident, suggesting that the stools of young children were able to ferment starch more quickly than the stools of adults. These differences were not mirrored by differences in CO_2 production. It is possible that this may relate to differing fermentation patterns, and we speculate that less early acetogenesis and synthesis of higher order SCFA occurs by the colonic flora of young children than those adults.

Calculation of human colonic stoichiometric equations paves the way to calculate recovery of carbohydrate energy by the colon. In adult humans, the colonic salvage of energy is estimated to be around 2 kcal/g of nonabsorbable carbohydrate (Livesey, 1990) or a contribution of 5–10% of energy requirements (Cummings, 1981; McNeil, 1984). Kien *et al* (1992) estimated that 24–74% of lactose may be converted into acetate in preterm infants with a potential 30% loss in ATP (Kien, 1996). However, there are sparse data to quantify the colonic salvage of starch-derived energy in young children. Our data used in combination with data for upper-gut starch digestion might give useful information about colonic salvage of energy in young children.

The finding that the faecal flora of young children was more efficient at fermenting starch, particularly less fermentable raw starch, fits with existing ideas about the potential for energy salvage by the colon in early life (Christian *et al*, 1999). We speculate that a highly efficient fermentative process takes place in the colons of young children who are

well-established on weaning foods, and that this leads to a significant potential for energy salvage, when the digestive capacity of the small intestine is less fully developed.

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