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**Measurement of *in vivo* fermentation  
of resistant starch**

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**A Thesis submitted for the degree of Doctor of Philosophy**

**to**

**The Faculty of Medicine, University of Glasgow (1998)**

**from research conducted at the**

**Department of Human Nutrition, University of Glasgow**

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## **DEDICATION**

To my dear parents whom I owe my life to them and dear brother.

To my dear husband of whom I am proud for accompanying me for our PhD in the Department of Human Nutrition in Glasgow University, and also to our sweetheart daughter, Yasaman.

To the Ministry of Health and Medical Education of I.R. Iran for awarding scholarship for PhD in Human Nutrition.

To my supervisor Dr Christine Edwards, Department of Human Nutrition, University of Glasgow, Yorkhill Hospital.

## SUMMARY

It is now well established that some starch escapes digestion in the human small intestine. This resistant starch (RS) can be one of three types; either protected by plant structure, in resistant starch granules of B-type starch or retrograded during processing. Resistant starch is fermented by bacteria in the colon increasing butyrate production. Butyrate may prevent colon cancer, promoting cell differentiation and apoptosis. Butyrate enemas have also been used to heal ulcerative colitis. It is important to know whether all types of RS are fermented at the same rate and to the same extent. It is very difficult to measure fermentation *in vivo* due to the inaccessibility of the proximal colon where most fermentation takes place.

The aim of this thesis is to establish better methods for *in vivo* measurement of RS fermentation. There is no direct non-invasive way to measure fermentation *in vivo* in human subjects which is usually measured indirectly by measuring the products of fermentation in blood, breath or faeces.

Two approaches were used to assess resistant starch fermentation:

- 1) Measurement of plasma acetate and breath hydrogen. The major source of acetate in blood in the fed state is from colonic fermentation of carbohydrate. Plasma acetate has been used previously for studying fermentation of dietary

fibre. Breath hydrogen is also commonly used but there are several disposal routes for hydrogen making it a poor marker for slowly fermented carbohydrates like RS.

2) Use of stable isotope tracers ( $^{13}\text{C}$ ). Although  $^{13}\text{C}$  enriched starch has been used to measure digestion, this is the first study to use  $^{13}\text{C}$ -labelled pea flour to measure fermentation in human adults.

Fermentation of raw potato starch was monitored by serial plasma acetate and breath hydrogen. Five subjects were fed 100g raw potato starch (34%; RS<sub>2</sub>) in the evening and breath hydrogen and plasma acetate were measured throughout the next day. Guar gum was used as a fermentable standard. Breath hydrogen and plasma acetate increased within approximately 11 hours, peaking within 14 hours compared with 4 and 6 hours after guar gum.

There was much variation in breath hydrogen and plasma acetate responses. The rise in plasma acetate occurred at a very different time to breath hydrogen. In most, but not all subjects, the rise and peak of plasma acetate happened earlier than for breath hydrogen. This makes it difficult to use these measurements for studies of slowly fermented carbohydrates.

An alternative approach using stable isotopes was therefore explored. Starchy

food had to be enriched with  $^{13}\text{C}$  during their starch deposition phase. Peas and potatoes were chosen because of their potential high RS and faster rate of growth. A high  $^{13}\text{C}$  enrichment of pea flour was gained by photosynthetic incorporation of  $^{13}\text{CO}_2$ . Pea plants (Baccara variety) were grown and when pods began to form, placed in a  $^{13}\text{CO}_2$  enriched environment in polypropylene bags sealed air-tight. 250 ml of  $^{13}\text{CO}_2$  were added and the bags filled to capacity with room air. The plants were incubated for 6 days on two occasions separated by 1 week. Peas were allowed to ripen under normal conditions and were harvested and dried to form flour. The mean atom % excess of  $^{13}\text{C}$  in once labelled pea flour was 2.4% and for twice labelled peas was 8.64%. Chemical and enzymatic attempts to separate the components of the pea flour was not totally successful but it was clear that the label was distributed throughout the pea flour. Potatoes were not successfully labelled (mean atom % excess of  $^{13}\text{C}$  0.71%) because the plants could not tolerate a long time in the polypropylene bags.

The digestibility of starch in the pea flour, measured using the Englyst method (*in vitro* model), was 14.4% rapidly digestible starch, 63.7% slowly digestible starch and 21.9% RS.

300 mg labelled-pea flour incorporated into biscuits was fed to six subjects and breath samples taken every 30 mins for up to 34 hours (with a short gap

when subjects were asleep) and analysed for hydrogen and  $^{13}\text{CO}_2$  enrichment. The appearance of  $^{13}\text{CO}_2$  in breath showed a complex of three peaks. The first peak occurred over the first 6 hours and should correspond to digestion and absorption of rapidly digestible and slowly digestible starch fractions in the small intestine.

The second peak occurred at 7-11 hours, in most subjects, and coincided with the rise in breath hydrogen. Confirmation that this represented colonic fermentation, was obtained with lactose- $^{13}\text{C}$ ureide. The second peak may result from fermentation of fibre. The third peak of  $^{13}\text{CO}_2$  in breath happened much later at 12-20 hours. Unfortunately in some subjects complete data for this peak was not obtained. This third peak may reflect fermentation of RS which occurs at this time after ingestion.

The three peaks were quantified in two ways. First the area under the curve (AUC) of breath  $^{13}\text{CO}_2$  was calculated for each peak. The third peak was extrapolated from the data points available. Second, estimated total PDR was calculated using a mathematical method:  $y = a \cdot (t-d)^b \cdot e^{-c(t-d)}$ . Both methods suggested that over 40% of the  $^{13}\text{CO}_2$  was produced from fermentation or fermentation products.

When compared with the Englyst *in vitro* assay, the first peak should



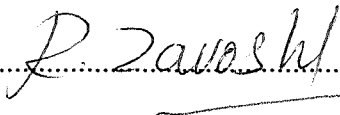
correspond to the RDS and SDS fractions in the pea flour. The second and the third peaks should be equivalent to the amount of RS. The *in vitro* model produced values of RDS and SDS equal to 78.1%, whereas the first  $^{13}\text{CO}_2$  peak accounted for only 57.9% of the total PDR. Conversely, the second and third peaks corresponded to 42.1% and RS *in vitro* was only 21.9%.

In conclusion, the  $^{13}\text{CO}_2$  breath test is a novel and more practical method for measuring digestion and fermentation of RS than breath hydrogen or plasma acetate alone or in combination. The Englyst *in vitro* method may underestimate physiological resistant starch.

## DECLARATION AND EXTENT OF COLLABORATION


The present thesis has used several different experiment designs and was carried out by myself in the laboratory of the University Department of Human Nutrition, Yorkhill Hospital, under the principal supervision of Dr Christine Edwards. I personally designed all the experiments and conducted all the ordering and preparing of chemicals and solutions for experiments and analysed them myself, except where acknowledged. The extent of collaborations and my personal input to the research are indicated in each Chapter. Measurement of enrichment with  $^{13}\text{C}$  in peas, potatoes and in breath  $^{13}\text{CO}_2$  in Chapters 4 and 5 were all conducted by Dr Tom Preston, Scottish Universities Research and Reactor Centre, East Kilbride, Scotland. Mathematical calculation of cumulative percentage of administered dose of  $^{13}\text{C}$  recovered in breath was conducted by Douglas J. Morrison, Bell College of Technology, Hamilton, Scotland.

Roza Zavoshy

.....

I certify that the work reported in this thesis has been performed by ROZA ZAVOSHY, and that during the period of study she has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy.

Dr CHRISTINE EDWARDS

.....

## **ABBREVIATIONS**

NSP = Non-starch polysaccharides

SCFA = Short chain fatty acids

RDS = Rapidly digestible starch

SDS = Slowly digestible starch

RS = Resistant starch

DIT = Diet induced thermogenesis

EE = Energy expenditure

TC = Total cholesterol

TAG = Triacylglycerol

HDL = High density lipoprotein

LDL = Low density lipoprotein

MTT = Mean transit time

AUC = Area under the curve

PDR = Percentage dose recovered

MCTT = Mouth to caecum transit time

OCTT = Oro-caecal transit time

GLC = Gas liquid chromatograph

G<sub>20</sub> = Glucose released after 20 minutes

G<sub>120</sub> = Glucose released after 120 minutes

TG = Total glucose

RPS = Raw potato starch

TT = Transit time

RMR = Resting metabolic rate

PAL = Physical activity level

# CHAPTER ONE

## Literature review and background

## **1.1. Carbohydrates**

Carbohydrates are the major source of energy in human diets, providing 45-60% of total daily needs. Although in the U.K., fat can contribute up to 50% energy, on a global basis up to 85% of energy in the diet in poorer regions comes from carbohydrates (Englyst & Kingman, 1993).

Most carbohydrates in the diet are ingested as plant material. Plants, by the process of photosynthesis, use the energy of sunlight, with chlorophyll in their green leaves as a chemical catalyst, to convert the carbon dioxide in air to sugar, which is readily soluble in water and can be transported rapidly through tissues of both plants and animals. To avoid the osmotic load of sugars, plants convert this to storage polysaccharides mainly starch, but in some plants non-starch polysaccharides for example galactomannan in the cluster bean.

## **1.2. Classification of carbohydrates**

According to the degree of polymerization (number of sugar units), carbohydrates are classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides. Glucose, fructose and galactose are the most important monosaccharides in human diet. Disaccharides consist of two monosaccharides linked together. Sucrose, maltose and lactose are the most important disaccharides in diet. The degree of polymerization (DP) in

oligosaccharides is 3-10. All oligosaccharides are water soluble compounds which exhibit some sweetness and most are resistant to digestion in the upper gastrointestinal tract, but are readily fermented in the colon. Raffinose and stachyose, for example, are oligosaccharides found in legumes such as beans and peas (Williams, 1990, Norton, 1993).

Polysaccharides are complex carbohydrates with a DP of more than 10. The most important polysaccharides in human nutrition are starch and non-starch polysaccharides (NSP or dietary fibre). Animal tissue does not contain much carbohydrate; mainly glycogen in muscle and liver, and mucopolysaccharides secreted in the intestine and lungs (Williams, 1990, Englyst & Kingman, 1993).

### **1.3. Non-starch polysaccharides**

Although the bulk of this thesis concerns actions and digestibility of starch, resistant starch potentially shares physiological actions with dietary fibre. I will therefore briefly consider dietary fibre. The original definition of dietary fibre was plant cell wall material which escaped digestion in the small intestine (Trowell et al., 1976), then entered the colon and after exposure to enzymes of bacteria, the residue was excreted in the faeces. By 1978 it was suggested by Cummings & Englyst that dietary fibre should be measured as

non-starch polysaccharides (James & Theander, 1981) and in 1987, Englyst et al, (1987c) proposed that dietary fibre should be defined as non-starch polysaccharides (NSP), since this gives the best index of plant cell-wall polysaccharides and is keeping with the original concept of dietary fibre. It is now clear, however, that other dietary materials escape small intestinal digestion including starch, protein and mucopolysaccharides (Eastwood, 1988).

NSP make up the structure of the plant cell wall, are produced as storage polysaccharides, such as the galactomannan guar gum in the cluster beans, or as exudates eg gum arabic from acacia tree, or mucilages like ispaghula from *Plantago ovata*. NSP have a wide range of structures and physiological properties but can be roughly divided into soluble and insoluble NSP. They are not digested in the upper intestine of man and, depending on the type of fibre, may affect small bowel function and promote colonic health, preventing some colonic disease.

#### **1.3.1. Soluble non-starch polysaccharides**

30% of the total dietary fibre intake in the Western diet is soluble fibre and the average percentages of soluble polysaccharides in plant foods are: vegetables 32%, cereals 32%, bean 25% and fruits 38% (Anderson et al., 1990).

Most soluble polysaccharides, such as guar gum and pectin, produce viscous

solutions in the gut. This increased viscosity can affect the digestion and absorption of other nutrients by various mechanisms including a reduction in gastric emptying rate (Eastwood & Brydon, 1985; Edwards et al., 1987). However, viscous polysaccharides may also decrease post prandial glycemia after carbohydrate meals by reducing the rate of absorption in the small intestine as a direct result of their viscosity (Jenkins et al., 1977; Blackburn et al., 1984; Leclerc et al., 1994). Viscous polysaccharides probably act as anti-motility agents inhibiting the effect of intestinal contractions which will reduce the mixing of enzymes and substrates and the movement of nutrients from the bulk phase to the epithelial surface. High viscosity may also reduce plasma lipids and cholesterol by trapping bile acids and fat in the lumen of the gut.

Some fibres, such as pectin, bind bile acid to charged sites in their structure and thus faecal loss of bile acid is increased. Increased loss of bile acids from the enterohepatic circulation will reduce the enterohepatic pool which must be renewed by increased synthesis of bile acids from cholesterol, reducing body cholesterol (Eastwood & Hamilton, 1968; Edwards et al., 1988; Anderson et al., 1990; Higham & Read, 1992; Mee & Gee, 1997). Serum cholesterol decreases 10-20% when soluble fibres such as oat bran, beans, guar gum and pectin are ingested (Eastwood et al., 1986; Anderson et al., 1990; Higham et al., 1992).



Polysaccharides which enter the colon are fermented by colonic microflora to short-chain fatty acids (SCFA) such as acetate, propionate and butyrate and gases (hydrogen, methane, carbon dioxide). SCFA are almost completely absorbed from the colon and promote absorption of water and electrolytes. SCFA have many possible beneficial actions which are summarised in **Table 1.1**, and will be discussed in more detail later in this thesis (Spiller et al., 1980, Fleming et al., 1983, Chen et al., 1984, Edwards 1990, Edwards & Parrett, 1996a). Soluble NSP are more easily fermented with less effect on stool output than insoluble fibres.

**Table 1.1. Possible actions of short-chain fatty acids**

---

Provide energy for the body and colonocytes
Promote absorption of water
Protect colonic mucosa against cancer
Promote growth of colonic bacteria
Reduce blood cholesterol
Increase faecal excretion of nitrogen
Increase mineral absorption in the colon

---

### **1.3.2. Insoluble non-starch polysaccharides**

Insoluble polysaccharides, like cellulose and wheat bran, speed up transit through the digestive tract and increase stool output. They are more resistant to colonic bacterial degradation than soluble fibre (Spiller et al., 1980; Fleming et al., 1983; Edwards, 1990; Williams, 1990).

Low faecal weight and slow transit time to be thought are associated with increased risk of bowel cancer. Faecal output is increased by consumption of NSP (Eastwood et al., 1984). Using data collected from adults in Edinburgh and Bristol, Cummings et al. (1992a) observed a significant inverse relationship between stool weight and incidence of colon cancer and a negative correlation ( $r = -0.52$ ) between stool weight and transit time (Burkitt, 1971; Burkitt et al., 1972).

Constipation is a frequent problem in the UK and USA. Stool weight in many Westernized population is low (80 -120 g/day; Cummings et al., 1992a). The daily dietary fibre intake in UK is 13 g/d. Increased dietary intake of NSP from 13 g/d to 18 g/d, associated with a stool weight of 150 g/d, should reduce the risk of bowel cancer (Cummings et al., 1992a). Recommendations for NSP intakes from the World Health Organization (WHO) and the UK Department of Health are: 16-24 g/d (WHO) and 18 g/d (UK, range = 12-24 g/d; WHO, 1990, UK Department of Health, 1991).

The physiological effects of soluble and insoluble non-starch polysaccharides, in the small and large intestine, are summarised in **Table 1.2**.

**Table 1.2. Physiological effects of non-starch polysaccharides**

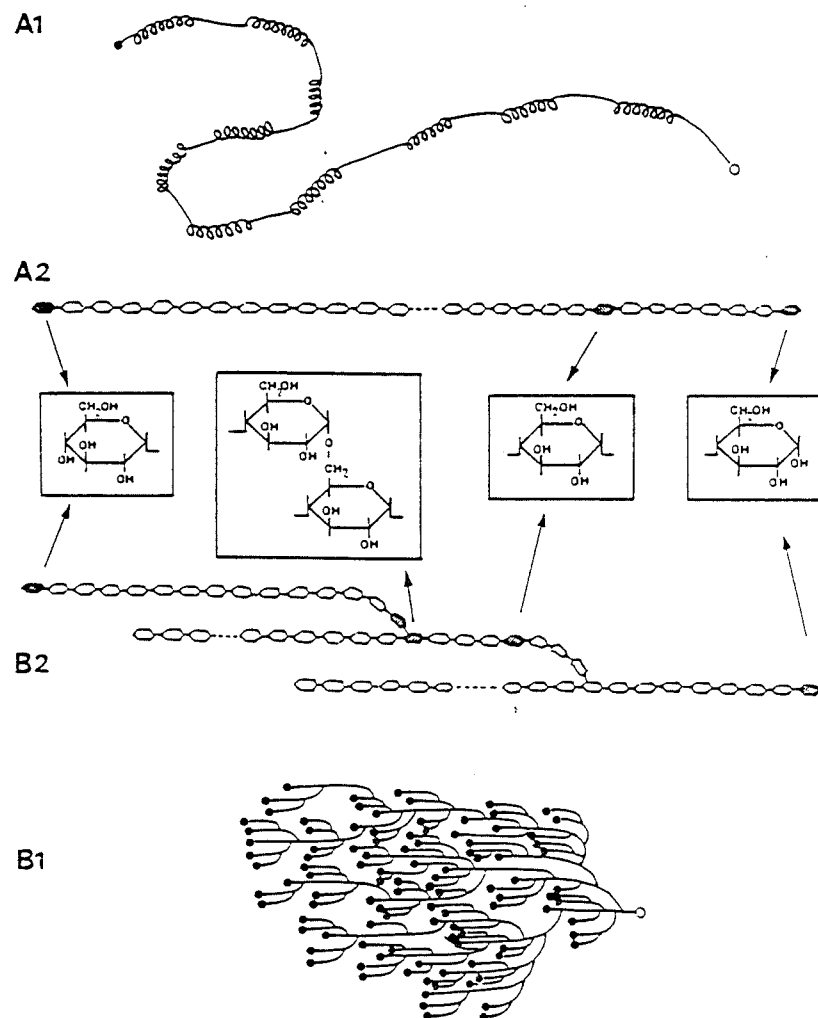
Non-Starch Polysaccharides	
Soluble	Insoluble
<u>Small intestine:</u>	<u>Small intestine:</u>
Delays gastric emptying	Decreases transit time
Increases satiety	<u>Large intestine:</u>
Lowers plasma glucose	Increases stool frequency
Reduces plasma lipids	Increases stool weight
and cholesterol	Are resist to colonic bacteria-
<u>Large intestine:</u>	degradation
Fermented in the colon	Reduces risk of colon cancer
Increases bacterial growth	Reduces constipation
Increases production of SCFA	
Increases colonic gas formation	

#### 1.4. Starch

Starch is derived from plant storage organs, such as seeds, fruits, roots and tubers, and consists of two main macromolecules, amylopectin and amylose (Cummings & Englyst, 1995), associated with small amounts of protein, below 0.5%, and lipid, usually around 1% in cereal starch. As the plant ages, the content of amylose and lipid increases (Eliasson & Gudmundsson, 1996).

70-80% of total starch is amylopectin and 20-30% is amylose. Amylopectin is the major form in most starchy foods but the amount of amylose and amylopectin varies between different plant sources. For example, high amylose maize and wrinkled pea contain up to 80% of amylose, but waxy varieties of maize, rice, barley contain only up to 2% amylose. The majority of starchy plants contain between 15% and 35% amylose (Englyst & Kingman, 1993).

Amylose is a long, unbranched chain of glucose units with  $\alpha(1-4)$  glucosidic linkages (**Figure 1.1**). Amylopectin is one of the largest natural macromolecules, and is a highly branched polymer consisting of  $\alpha(1-4)$  linked glucose units with branches formed by  $\alpha(1-6)$  linkages (**Figure 1.1**).



**Figure 1.1.** Schemes of the linear, helicoidal amylose macromolecule (A1 and A2) and of the grape-like clustered amylopectin macromolecule (B1 and B2) (From Gallant & Bouchet, 1986).

The natural twist of the amylose macromolecules results in a helical conformation with six anhydroglucose units per turn and hydrogen bonding between the glucose chains making amylose a compact structure. This is physically less accessible to amylolytic attack than the more open and branched amylopectin (Gallant et al., 1992).

An *in vivo* study by Jenkins et al., (1981) showed that whole legumes (30 - 40% amylose) produce lower glycemic responses than cereals (25 -30% amylose) and a study by Behall et al., (1988) reported lower glucose and insulin responses after a meal containing cornstarch with 70% of the starch in the form of amylose than after a meal containing cornstarch with 70% of the starch in the form of amylopectin.

#### **1.4.1. Digestion and absorption of starch**

The digestion of starch is started in the mouth by salivary  $\alpha$ -amylase (ptyalin) secreted from the parotid glands. Chewing also breaks food into small pieces thus increasing the surface area and hence enzyme accessibility (Read et al., 1986). It was thought that salivary  $\alpha$ -amylase did not have a significant role in starch digestion because of the acidic condition of stomach, but recent evidence has shown salivary amylase may be active for up to 30 minutes in the stomach due to the buffering capacity of food (Norton, 1993). 15% of total activity of amylase in the human gut is contributed by salivary amylase (Bjork,

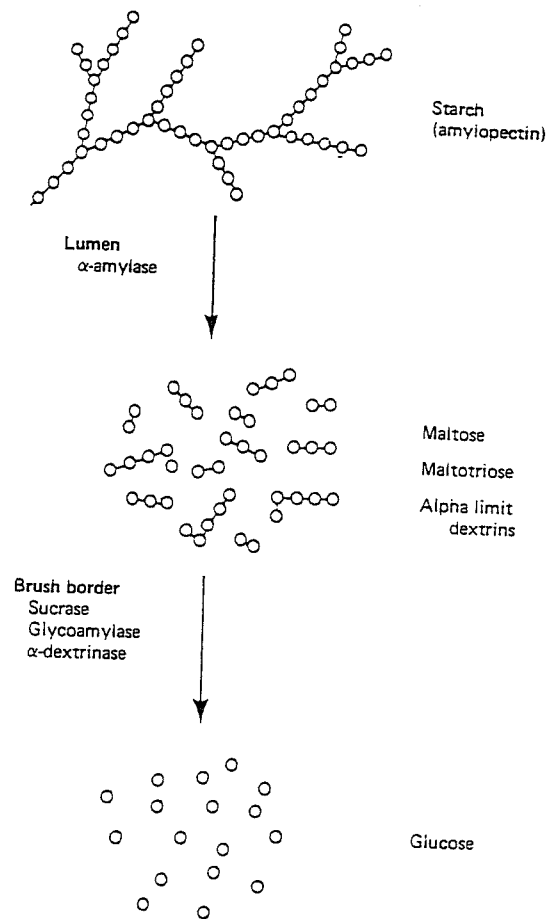
1996). However, pancreatic  $\alpha$ -amylase secreted into the small intestinal lumen is responsible for most starch digestion. Human amylase digests only  $\alpha(1-4)$  bonds.

Maltose and maltotriose are the end products of amylose digestion and maltose, maltotriose, and  $\alpha$ -limit dextrins, containing the  $\alpha(1-6)$  branch linkage, are produced from amylopectin (Bjorck, 1996). All products of amylase digestion must then travel to the brush border, where they are further digested to glucose by disaccharidases and oligosaccharidases such as isomaltase, maltase, glucoamylase and  $\alpha$ -limit dextrinase on the mucosal brush border (Bjorck, 1996) (**Figure 1.2**).

The glucose is then absorbed using a co-transporter with  $\text{Na}^+$  by secondary active transport using the  $\text{K}^+/\text{Na}^+$  pump on the basolateral membrane (Emslie-Smith et al., 1988). This produces a peak level of glucose in blood 30 minutes after a meal which returns slowly to the fasting level after 90-180 minutes (Englyst & Kingman, 1993).

#### **1.4.2. Physical characteristics of starch granules**

In the plant cell, the starch is contained within starch granules. The shapes of the starch granules are characteristic of each species and the size is different,



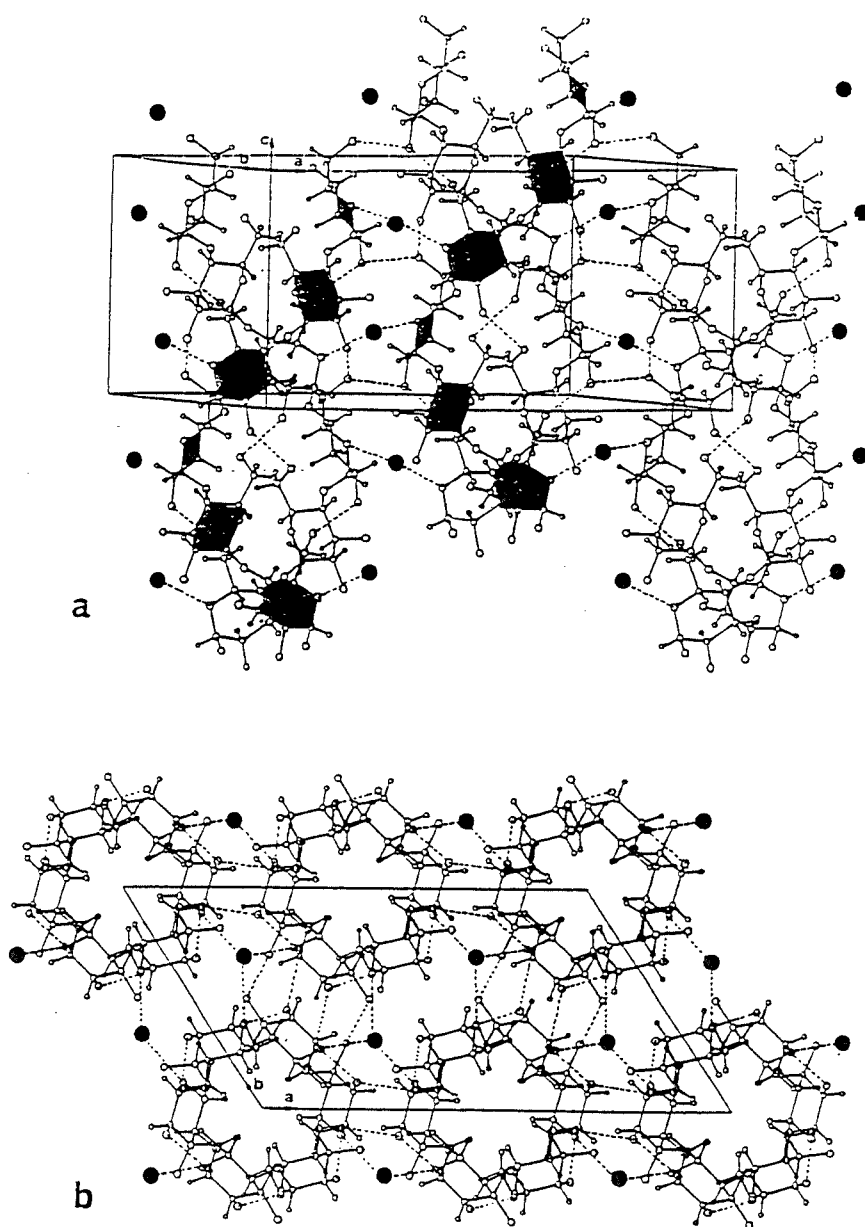
**Figure 1.2.** The digestion of starch (Emslie-Smith et al., 1988).



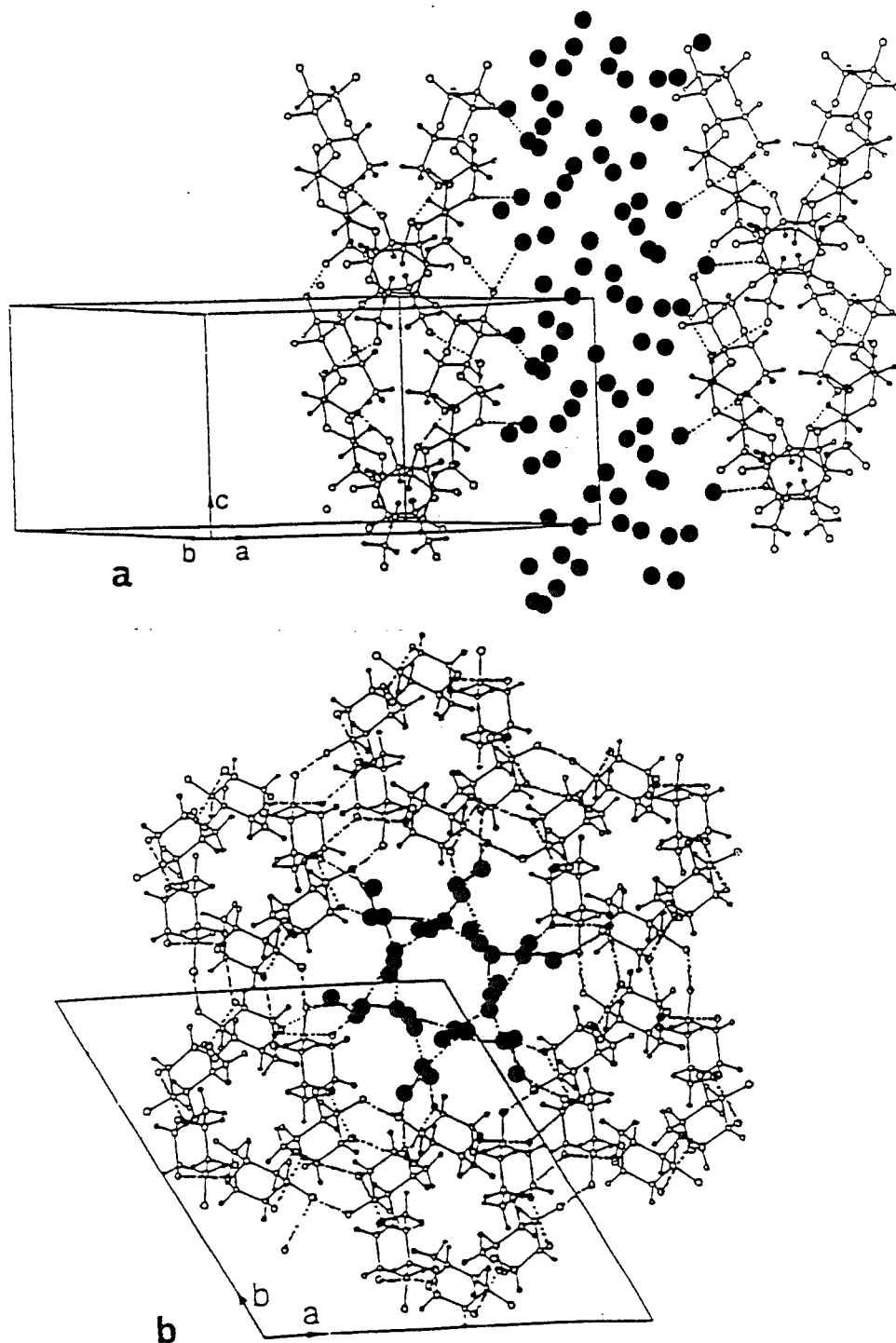
from the tiny granules in rice and oat to the large ones in potato and banana starch (Gallant et al., 1992; see later). Within the granules, the amylose and amylopectin chains are arranged in a semi-crystalline structure, which may affect their digestion by pancreatic enzymes (Katz , 1934).

Four crystalline forms A, B, C and V, have been found in starch granules by x-ray diffraction studies. Cereal starches give the A-pattern (except high - amylose varieties) (**Figure 1.3**). The B-pattern is characteristic of root and tuber starches (and for high amylose varieties) and for retrograded starch (**Figure 1.4**). The C-pattern is obtained from most legume starches such as beans and peas . However the C-pattern is also considered to be a mixture of A and B-patterns. The V-pattern is produced after gelatinization of the starch granules, due to a complex between amylose fraction with fatty acids, phospholipids or other polar molecules (Gallant et al., 1992; Eliasson & Gudmundsson, 1996).

In normal and waxy starches, the branched molecule (amylopectin) makes up the crystallites. The branches of the amylopectin molecules form double helices in crystalline structure (Sarko & Wu, 1978) and the crystallinity increases with the ratio of amylopectin in the starch (Gernat et al., 1993).



**Figure 1.3.** Structure of type-A starch. The helices are arranged in a lattice pattern and the water molecules (closed circles) are located between the helices. (a) is a longitudinal and (b) a top view (Imberty et al., 1988).



**Figure 1.4.** Structure of type-B starch. The helices are arranged in a super-helical structure and the water molecules (closed circles) firmly bound inside the super helices. (a) is longitudinal and (b) a top view (Imberty & Perez, 1988).

### 1.4.3. Starch granules

The larger or A-type granules are lenticular in shape (20 -25  $\mu\text{m}$  diameter) while the smaller or B-type granules are spherical in shape (approximately 4  $\mu\text{m}$  diameter). Potato and banana have ovoid-spherical shaped granules 10 -80  $\mu\text{m}$  in diameter with smooth surfaces (Norton, 1993).

B-pattern starches are formed in plant organs at high humidity and low temperature. At low humidity and high temperature, B-pattern starch can be converted irreversibly to A-pattern (Gallant et al., 1992).

Gallant et al., (1992) reported the structure of A-pattern is more dense than that of the B-pattern so a higher resistance to enzymic attack could be expected of the A-starch granules. Observation by scanning electron microscope (SEM) has provided a different picture, however, most of the starch granules are eroded superficially by the enzymes. Some granules have specific susceptible zones which when digested form pits. These become bigger and canals are formed as the starch is digested.

Each starch granule is made up of hard and soft material. During enzymic hydrolysis, the hard (most crystalline) part of the starch granule is less digestible than the soft parts. In studies under the SEM, Duprat et al., (1980)

reported that during *in vitro* hydrolysis, the starch forms small more or less spherical blocklets. Blocklets are 100 nm in diameter, in the hard parts, but they are only 25 nm in the soft parts. In granules showing B-pattern, much larger blocklets (4 - 500 nm diameter) exist at the peripheral level to a depth of about 10  $\mu$ m. Blocklets as large as this are also seen in starch granules with the C-pattern. This could explain the resistance of these starches granules to hydrolysis.

#### **1.4.4. Gelatinization of starch granules**

It is well known that starch digestion is affected by the physical state of the granules . Gelatinization is a collective term which describes a range of irreversible events occurring when starch is heated in water. Although the polymers from which starch is built up, are hydrophilic, the starch granule itself is not soluble in water. This is due to the semicrystalline nature of starch granule structure (Eliasson & Gudmundsson, 1996).

On heating at a temperature  $>50^{\circ}\text{C}$  (the gelatinization temperature) and in excess water, the granules swell and the structure of granules disrupts irreversibly. The amylose leaches out. This is called gelatinization and at this stage accessibility of enzymes to the starch is increased (Colonna et al.,1992; Englyst & Kingman, 1993; Norton, 1993; Cummings & Englyst, 1995). During heating, absorption of water and the leaching of material from starch

occur at the same time. This material is mainly amylose, although amylopectin might also be leached out. Lipid has not been found in the leaching material (Tester & Morrison, 1990). Amylose leaching leads to formation of an unstable gel, which is used in the food industry as a thickener and in custard (Norton, 1993).

Ellis et al., (1988) reported that during heating, not all the amylose leaches out. After heating at 90°C, 16% of amylose in pea starch, 8.3% in wheat and 8.0% in maize was found remaining inside the starch granule.

Amylopectin is the main constituent remaining in the granules after gelatinization, without any crystalline order (Prentice & Stark, 1992). Loss of crystalline order is observed in the X-ray diffraction pattern. In cereal starches the V-pattern (which is related to the amylose-lipid complex) is seen after gelatinization (Zobel, 1988).

Gelatinization generally happens in excess water, (more than 300% added water on dry matter basis). In this condition gelatinization usually takes place between 50- 70°C (Colonna et al., 1992). Svensson & Eliasson, (1995) observed that the temperature range during which loss of crystallinity occurs, and the rate of this procedure, depends on the water content and the type of starch. If water content is decreased, the temperature range would be

increased. For example, with a water content below 50%, the temperature for complete loss of crystallinity approaches 100°C. Svensson & Eliasson, (1995) described how the loss of crystallinity occurred with two steps: the rate of procedure is slow at first but then increases at a temperature characteristic of the starch. Tester & Morrison, (1990) reported that swelling curves of wheat and normal and waxy barley and maize starches, were characterised by an initial phase of slight swelling, a second phase of rapid swelling, and a final stage of maximum swelling.

In low-moisture conditions, gelatinization is considered more as a melting of the crystallites, which happens at higher temperature (100- 150°C). In this low moisture situation, the melting temperature could be mistaken for the gelatinization temperature (Colonna & Mercier, 1985). Melting temperature increases up to 150°C when the water content decreases . The melting temperature of the most perfect crystallinities, without water, is between 150°C and 220°C. Adding 10% water to cereal starches reduces the melting temperature to 115- 130°C and addition of 20% water reduces it further to 100- 120°C (Colonna et al., 1992).

In some foods, such as bread and biscuits, the amount of water is limited. In others, the structure of the foods, as in pasta, rice and potato, means the granules can not swell completely, so no disruption is observed in the granule

structure and a limited solubilization of starch occurs.

Although water content is the most important factor, other factors such as the presence of monosaccharides and oligosaccharides (sucrose, glucose, fructose) or salt can change gelatinization temperatures to higher values, and prevent the gelatinization of starch in some foods (Colonna et al., 1992).

#### **1.4.5. Retrogradation of starch**

During gelatinization, amylose is solubilised but amylopectin remains in the swollen starch granule. On cooling, both the soluble and the partially soluble polysaccharides (gelatinized starch) recrystallize. This process is known as retrogradation. This takes place very rapidly for linear amylose, for example after only few hours in bread, while the retrogradation of amylopectin occurs over several days in bread and causes staling. Retrogradation of amylopectin is limited by its branching structure and the retrograded starch is more easily reconverted to non-retrograded starch than retrograded amylose (Colonna et al., 1992; Cummings & Englyst, 1995; Eliasson & Gudmundsson, 1996).

X-ray diffraction analysis gives both the type and the degree of crystallinity of retrograded starch (Brennan et al., 1973). The A-pattern is lost during gelatinization in the cereal starches and only the V-pattern is seen due to the formation of amylose-lipid complexes. On cooling and ageing the B-pattern



will develop which is attributed to the double helical conformation, characteristic of retrogradation. The proportion of B-pattern increases with time. Both of these reorganisations of structure spontaneously happen on cooling (Zobel, 1973; Colonna et al., 1992).

In particular baked goods, such as bread, retrogradation of starch is the first consequence of ageing. Sometimes the retrogradation is desirable, such as in the production of breakfast cereals or parboiled rice, due to hardening of starch and a lower stickiness. Repeating thermal treatments causes increasing retrogradation by increasing inter-chain interaction of amylose (Sievert et al., 1991).

Longton & LeGrys, (1981) reported that crystallization occurred only in gels with 10-80% content of starch during ageing, and maximum crystallization was observed in gels with 50-55% starch.

Retrogradation of amylose and amylopectin could be reversed by heating to high temperatures, 120°C and 60°C, respectively (Norton, 1993). It was shown, in some studies, (Teo & Seow, 1992) that the rate and the extent of retrogradation could be increased with increasing amount of amylose.

However longer term retrogradation is observed with amylopectin. In waxy starch with only 2% amylose retrogradation occurs slowly, but in peas and

potatoes, which have high amount of amylose, retrogradation happens to a greater extent (Teo & Seow, 1992). Retrograded starches are not digested in the human small intestine and enter the colon (Englyst & Kingman, 1993; Norton, 1993; Cummings & Englyst, 1995). They are classified as resistant starch type RS<sub>3</sub> (see later).

#### **1.4.6. Starch and food structure influences on starch digestion**

The physical form of food can cause inaccessibility of pancreatic amylase to starch, slowing starch digestion. This happens if starch granules are trapped in the food matrix, eg intact cell wall, which prevents the starch from swelling completely (Wursch et al., 1986). This type of starch is classified as type RS<sub>2</sub> (see later).

A decreased rate of starch digestion has the advantage of reducing or delaying postprandial glucose and insulin responses. O'Dea et al., (1980, 1981) have demonstrated that ground rice meals produce much higher peak responses of glucose and insulin than unground samples. This appears to be due to the more rapid absorption of the ground samples, in which the physical barriers of the food matrix to enzymes have been breached. Heaton et al., (1988) showed that when finely ground flour of wheat, maize or oat were given to human subjects, the glucose and insulin responses were greater than after whole or coarsely ground grains were ingested. Studies in patients with ileostomies

showed that only  $2 \pm 1\%$  of starch remained undigested after finely milled barley was eaten, but after flaked barley was eaten  $17 \pm 1\%$  resisted digestion (Livesey et al., 1995). In addition, if starch is very densely packed in a food such as spaghetti, made from durum, the postprandial blood glucose response was lower than after ingestion of cooked potato or cooked parboiled rice (Hermansen et al., 1986).

Food processing also affects starch hydrolysis. Cooking made starch much more accessible to enzymic hydrolysis by gelatinizing it (Snow & O'Dea, 1981; Thorne et al., 1983) and grinding food samples (legumes) finely prior to cooking increased the rate of starch hydrolysis (Wong et al., 1985) to a greater extent than blending them after cooking (Wong & O'Dea, 1983). Englyst & Cummings, (1987a) showed, in ileostomy subjects, that starch from freshly cooked potato was well digested with only 3% being recovered from the stoma, however, 12% from cooked and cooled potato escaped digestion in the small intestine. This was possibly due to food processing that led to retrogradation of the starch making it resistant to enzymic digestion.

The cooking method is also important. The degree of starch gelatinization is strongly related to plasma glucose and insulin responses as well as to the rate of *in vitro* hydrolysis of starch with  $\alpha$ -amylase (Holm et al., 1988). Increases

in temperature, pressure and duration of pressure cooking, in baked beans, resulted in increased starch digestibility (Traianedes & O'Dea, 1986). Jenkins et al., (1982) found flatter glycaemic responses with lentils boiled for 20 minutes than after lentils which were dried and blended for 12 hours at 250°F, after boiling. There are inhibitors of amylase activity in raw beans and lentils. These are destroyed by heat and the 12 hours of dry heat may explain these results.

The composition of starch can also influence its digestibility. Goddard et al., (1984) and Heijnen et al., (1995a) found that increasing the amount of amylose in starchy foods lowered postprandial glucose and insulin responses, probably due to delay in digestion or absorption of these foods. Amylopectin is a much larger molecule than amylose, therefore, the surface area per molecule is much larger than in amylose. The glucose chains in amylose are also more bound to each other by hydrogen bonds, making amylose less available for enzymic attack than amylopectin (Leach, 1965).

The type of starch crystalline is also important for the digestibility of starch. As was described above, the B-pattern of starch is more resistant to enzymic attack due to its structure (Duprat et al., 1980) although the degree of resistance depends on the plant source. This resistance to hydrolysis affects the digestibility of starchy foods normally eaten raw, such as banana. The

starch content of banana depends on the ripeness. It changes from 37% of dry weight in the least ripe banana to 3% in the most ripe, most carbohydrate now being sugar. A study by Englyst & Cummings, (1986a) showed that feeding ileostomy subjects banana of different ripeness, gave a range of 4-19 g/d carbohydrate in the ileostomy effluent. This was directly related to the starch content in banana.

Holm et al., (1983), showed that amylose-lipid complexes reduce starch digestibility *in vitro* and *in vivo*, by reducing susceptibility to  $\alpha$ -amylase (see above section of physical characteristics of starch granules).

NSP may also affect amylase activity. Hamberg et al., (1989) noted a number of ways in which NSP may have such an effect, by decreasing transit time through the small intestine, by increasing viscosity, which reduces enzyme substrate contact and by presenting a physical barrier, which limits the access of enzymes to substrate. Dunaif & Schneeman, (1981) incubated human pancreatic juice with several dietary fibres such as oat bran, wheat bran, cellulose and hemicellulose. The results showed that these dietary fibre substantially decreased amylase activity.

Starch-protein interactions are other factors intrinsic to starchy foods which influence the digestibility (Levitt et al., 1984) and the glycemic response to the

starch. It was shown by Jenkins et al., (1987) that a test meal of white bread made from gluten-free flour, resulted in significantly greater blood glucose than a test meal of white bread made from regular flour. This could be due to disrupted starch granules in the gluten-free flour. However when the gluten was added to the gluten-free flour no decrease in glycaemic response was seen.

In addition to the above factors which are intrinsic to starchy foods, polyphenols in plants, such as phytate, can also interfere with starch digestion. Yoon et al., (1983) and Thompson et al., (1987) have shown the presence of phytate was inversely correlated with the glycemic response in man. *In vitro* the rate of starch digestion, in the presence of sodium phytate, was reduced significantly, this was reversed by the addition of calcium which is known to complex phytic acid.

This may be because phytic acid combines directly with starch (Badenhuizen, 1959) or phytate could act through combination with proteins which are structurally associated with starch, or by combination with digestive enzymes which are themselves proteins. Furthermore, phytic acid could bind minerals such as calcium which is known to catalyse amylase activity (Afonsky, 1966). Rea et al., (1985) have studied the effect of lectin on starch digestibility. Lectins or haemagglutinins are proteins or glycoproteins which bind with intestinal mucosa cells, leading to disruption of intestinal microvilli and

malfunction in the small intestine. This interferes with the absorption of nutrients (Pustzai et al., 1982). Rea et al., (1985) found an inverse relation between lectin content of starchy foods and glycemic responses. Similar relationships, however, were seen between the *in vitro* rate of digestion and lectin content. This may explain the delayed appearance of glucose in the blood. In this case lectin could act by binding to the digestive enzyme or directly to the starch.

#### **1.4.7. Physiological factors influencing on starch digestion**

The digestibility of starch in the small intestine is also affected by a variety of factors that may be independent of crystallinity and the physical form of starchy food.

Physiological parameters can affect starch digestibility, these include the extent of chewing, which increases the physical accessibility of starch in rigid structures to enzymic hydrolysis (Read et al., 1986; Englyst & Kingman, 1990; Englyst et al., 1992). The concentration of amylase in the gut, the amount of starch present in the diet, the presence of other food components that might retard enzymic hydrolysis (see above), and the transit time of the food from mouth to terminal ileum are all factors that might affect starch digestion (Englyst et al., 1992).

The amount of unabsorbed starch appears to be directly related to the quantity ingested and to the small intestinal transit time. Stephen et al., (1983) found 9.3% of unabsorbed carbohydrate after a test meal containing 20g starch, and 6% after a meal containing 60g of starch, similar results were found by Chapman et al., (1985). Thus it is difficult to predict the relative extent to which each of these factors may influence the digestion of starchy foods .

#### **1.4.8. Nutritional classification of starch**

Englyst's group has classified starch in to three major fractions which are based on the determination of digestibility *in vitro* believed to reflect the rate of starch digestion *in vivo* (Englyst & Kingman, 1993; Cummings & Englyst, 1995). The three types of nutritional classification of starch are: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst et al., 1992).

The *in vitro* methodology consists of incubating a starchy food with pancreatic amylase and amyloglucosidase under controlled conditions (**Figure 2.4**). The glucose released from the food within 20 minutes represents RDS, and that released in the following 100 minutes represents SDS. Any starch unhydrolysed after 120 minutes is classified as RS (Englyst & Kingman, 1993; Cummings & Englyst, 1995). Other groups with a different range of digestive enzymes use a digestion period of 16 hours (Berry, 1986; Champ, 1992).



However, the classification of starch is similar in both types of method.

RDS is found in large amounts in freshly cooked starchy foods such as bread and potatoes. These are rapidly digested in small intestine of man (Englyst & Kingman, 1993). SDS is also expected to be completely digested in the small intestine, but much more slowly than RDS. This type of starch is not as easily accessible to enzyme and includes seeds and grains which are partly milled and in highly dense foods such as pasta. SDS is also found in most raw cereal foods. This type of starch consists mainly of A and C-pattern in X-ray diffraction but includes a small proportion of B-pattern (Englyst & Kingman, 1990; Englyst et al., 1993; Cummings & Englyst, 1995).

The third type of starch in this classification is resistant starch (RS), which will be described in detail in next section.

RDS, SDS and RS can exist in the same food, but the amount of each type of starch varies and depends on the source of starch and type of cooking food processing it has been subjected to (Englyst et al., 1992). The highest amount of RDS was found in corn flakes. The products containing most RS were raw potato starch and banana flour (Cummings & Englyst, 1995).

### 1.5. Resistant starch

About 15 years ago it was assumed that starch was completely hydrolysed and absorbed in the upper gastrointestinal tract. It is now clear that the amount of starch which escapes digestion in the small intestine is variable and depends on physical form (Englyst et al., 1992).

Englyst et al., (1982) introduced the term of "resistant starch" (RS) for starch which was not hydrolysed by  $\alpha$ -amylase and pullulanase (an enzyme that can break  $\alpha$ 1-6 glycosidic bonds).

Starch resistant to digestion has been defined by EURESTA (European Food-Linked Agro-Industrial Research)-Concerted Action on Resistant starch) as:

*"The sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals"* (Asp, 1992). RS enters the colon and there it may be fermented by the colonic microflora.

During the Eureka study, several participants estimated the resistant starch intake (Dysseler & Hoffem, 1995) of their countries. The mean intake of RS in Europe was estimated to be 4.1g dry matter/day. This value was calculated from food intake data from published national food surveys of 10 European countries during the period 1992 to 1994. Two different methods were used

for determination of the resistant starch values: the Englyst method (Englyst et al., 1992) and the Berry method as modified by Champ (Champ et al., 1992). The ranges of RS intake varied between European countries, from 3.2 g/d in Norway to 5.7 g/d in Spain. In England the mean of RS intake was estimated at 3.97 g/d. The major source of RS in the most European countries is bread and potatoes which could provide together between 60% to 90% of the total intake of RS. These values of resistant starch intake were surprising low and did not compare well with dietary fibre intake (even in Scotland with 12g/d dietary fibre).

It should be mentioned that, because of the importance of resistant starch has on human health, reliable data for the nature and amount of resistant starch in diet is needed. The data available so far does not provide reliable evaluation for the intake of resistant starch from different food products for each individual European country for several reasons but most importantly due to the use of historical data on food intake. This may not have indicated the method of cooking, brand names for cereals (eg Kellogg's corn flakes 3g/100g resistant starch, other brands none) and whether the food was eaten hot or cold. A prospective study using detailed description and weighed intakes is needed.

### **1.5.1. Classification of resistant starch**

Resistant starch consists of three subcategories: RS<sub>1</sub>, RS<sub>2</sub> and RS<sub>3</sub> (Englyst et al., 1992). The first category (RS<sub>1</sub>) is based on the physically inaccessible starch to  $\alpha$ -amylase due to intact plant cell wall, found in whole or partly grains and legumes and in some of very dense type of processed starchy foods.

RS<sub>2</sub> refers to raw B-type starch granules such as raw potato and banana starches and raw high-amylose maize starch. RS<sub>2</sub> escapes digestion in the small intestine because of its structure and conformation of the granules. This type of resistant starch shows B-pattern in crystalline structure of the granule, the centre of the super-hexagonal array is occupied by tightly bound water, making it resistant to hydrolysis by pancreatic amylase.

The third category of resistant starch (RS<sub>3</sub>) is retrograded starch, mainly amylose, formed during the cooling of gelatinized starch, eg cooked potato after cooling, bread, cornflakes or pasta, although only a small proportion eg 3-10% of the total starch in these food products may be RS<sub>3</sub>.

## **1.6. Physiological effects of resistant starch**

Several beneficial effects of resistant starch on human health have been reported which will be explained in more detail in this section.

### **1.6.1. Effects on glycaemic response, satiety, and thermogenesis**

Since resistant starch is not absorbed in the small intestine, foods that have a large proportion of resistant starch will produce lower postprandial glucose than foods containing the same amount of digestible starch. This may therefore influence satiety which is believed to be related to glucose absorption and metabolism (Grossman, 1986) and, in the longterm, to insulin sensitivity.

Achour and co-workers (1996) compared the metabolic consequences of highly digestible maize starch with the same maize starch after retrogradation in healthy volunteers. Blood samples were collected hourly for 8 hours after ingestion of test meals. The areas under the curves of blood glucose and insulin were significantly higher after the ingestion of digestible starch compared with retrograded maize starch (RS<sub>3</sub>).

The effect of RS on postprandial plasma concentration of glucose, insulin and on subjective satiety was investigated in healthy human subjects (Raben et al., 1994). The test meals consisted of 50g pregelatinized starch (0% RS) or 50g

raw potato starch (RS<sub>2</sub>; 54% RS). After the test meal with raw potato starch, postprandial plasma concentrations of glucose and insulin were significantly lower compared with those after the test meal with pregelatinized starch. Moreover, subjective scores for satiety and fullness were significantly lower after the raw potato starch than after the pregelatinized starch.

However, in this study the texture of the test meals was different. For the raw potato starch, a drinkable mixture was used whereas, the pregelatinized starch was a porridge-like gel to be eaten by spoon. This more solid meal may have caused a reduction in gastric emptying rate (McMahon et al., 1989) and increased feelings of satiety and fullness after this meal compared with the liquid raw potato starch meal.

Another study investigated whether RS<sub>2</sub> and RS<sub>3</sub> were more satiating than glucose in man. Healthy volunteers consumed a daily supplement of either glucose, high-amylose maize starch (RS<sub>2</sub>) or extruded and retrograded high-amylose maize starch (RS<sub>3</sub>). Supplement with RS<sub>2</sub> caused significantly lower appetite scores than supplement with RS<sub>3</sub> and glucose, however subjects felt less full while consuming RS<sub>2</sub>. When consuming resistant starch, the energetic compensation tended to be smaller than when consuming glucose, so resistant starch produced less satiation. Presumably, no effect on appetite was found because, in this study, subjects were allowed to eat and drink *ad libitum*.

Consumption of 30g/d RS<sub>2</sub> and RS<sub>3</sub> had little influence on appetite and food intake (de Roos et al., 1995).

It was shown by Byrnes et al., (1995) that in rats, the prolonged consumption of high-amylose maize starch, instead of a readily digested low-amylose maize starch, resulted in reduction in both glucose and insulin responses. In the rats fed the high amylopectin diet, glucose tolerance was significantly lower and the insulin response was twice as high as in rats fed the high amylose diet. The results suggested that quickly digested starch may promote the development of insulin resistance in rats.

Because resistant starch is not digested but may be fermented, ingestion of large quantities may influence diet induced thermogenesis (DIT). Heijnen et al., (1995b) compared DIT and the postprandial glucose and insulin concentration in blood, in healthy men after ingestion of RS<sub>2</sub> from uncooked potato starch or digestible starch from pregelatinized potato starch. Postprandial level of glucose and insulin were increased proportional to the amount of digestible carbohydrate in the meal. The replacement of digestible starch by resistant starch in a single meal lowered DIT because resistant starch does not contribute to DIT. In agreement with the results of this study, Tagliabue et al., (1995) also, noted that intake of resistant starch was followed by lower thermogenesis in man than pregelatinized starch ingestion . One of

the possibly useful effects of increasing the amount of resistant starch in foods is the reduced energy density that may be obtained. This could be useful in the prevention and treatment of obesity and diabetes (Ranganathan et al., 1994).

A comparative study of the acute effects of resistant starch (lintner: i.e. acid extracted high amylose corn starch; RS<sub>3</sub>) and dietary fibres (cellulose, pectin) on energy expenditure (EE) and blood glucose and insulin concentration in man has shown no significant differences between lintner and cellulose on measured indexes. There was no thermogenic effect with either lintner or cellulose. The metabolic characteristics of lintner were closer to cellulose than to pectin. The result of this study showed the acute effect of the ingestion of a resistant starch (lintner) on the measured metabolic indexes is similar to that of insoluble fibre-cellulose (Ranganathan et al., 1994). However, RS is thought to have physiological effects comparable with soluble dietary fibres (Faulks et al., 1989; Stephen, 1991; de Deckere et al., 1992; de Roos et al., 1995). Some resistant starch (RS<sub>3</sub>) is more like insoluble fibre in that it is poorly fermented and has little effect in the small intestine but more fermentable resistant starch, such as RS<sub>1</sub> and RS<sub>2</sub> may be more comparable to soluble fibre as they are fermented to SCFA and will have similar actions in the colon. All RS, however, will result in lower blood glucose than digestible starch and may therefore appear like viscous soluble fibre but are unlikely to influence the absorption of other carbohydrates.



### 1.6.2. Effects on plasma lipids

Soluble dietary fibre has been shown to decrease plasma cholesterol in man (Topping, 1991). This may be by trapping bile acids and fat in viscous gut contents or by binding of bile acids. Resistant starch is not viscous and would not at first appear to be likely to affect lipids. However, the effects of RS have been studied in animal and human experiments. de Deckere et al., (1992) in meal-fed (two *ad libitum* meals of 30 min per day) male Wistar rats investigated the effects of a diet with large amounts of RS<sub>3</sub> compared with a diet with a low resistant starch (gelatinized amylose-rich maize starch), on serum fasting TC and TAG. After 4 weeks of feeding the diets, serum fasting TC was significantly lower after the high RS diet, but there was no effect on TAG concentration.

In a more recent study de Deckere et al., (1995) investigated whether the cholesterol lowering action of RS could be explained by an effect on the faecal excretion of neutral sterols and bile acids in rats. Two groups of rats were meal-fed semipurified diets containing either a large amount (14%) or a small amount of RS (1%) supplement. After 2 weeks on the diets, the total amount of bile acid in the caecal contents and the faecal bile acids excretion were higher in the high-RS groups than the low-RS groups. Bile acids are synthesized from cholesterol in the liver, when daily faecal output of bile acids increased, more cholesterol is used to synthesize bile acids reducing serum

cholesterol.

In a human ileostomy study (Langkilde & Andersson, 1992) the ileal excretion of cholesterol and bile acids from a low-fibre diet including 100g of either ordinary gelatinized corn starch (RS: 0.7g/100g) or autoclaved amylomaize; RS<sub>3</sub>: 11.6g/100g was studied in seven ileostomy subjects. The study consisted of one run-in day and two study days, one with each type of starch. They found no significant changes in excretion of cholesterol or chenodeoxycholic acid. However, a significantly lower excretion of cholic acid was seen during the amylomaize period (mean 243 mg/day) than during the ordinary corn starch period (mean 419 mg/day;  $P < 0.02$ ). The concentration of bile acids in ileostomy effluents was also significantly lower during the amylomaize period (mean 4.6 mg/g dry weight ) than during the corn starch period (mean 11.2 mg/g dry weight  $P < 0.02$  ). This seems to contradict other studies but may be due to shorter exposure to diet and different resistant starch.

Olesen et al., (1995) added 50g/d of raw potato starch; RS<sub>2</sub> or common maize starch to the usual daily diet of human volunteers for three weeks. No significant effect on blood cholesterol concentration was seen, however, baseline values varied considerably, which could mask any minor effects of the resistant starch.

Raben et al., (1995) studied the consumption of two test meals, containing of 50g pregelatinized starch or 50g raw potato starch (RS<sub>2</sub>: 54%) gently mixed in 500g artificially sweetened syrup, on plasma glycerol and triglyceride in healthy volunteers. The meals were served on two separate days. Fasting and 5-hour postprandial blood samples were collected and analysed for plasma glycerol and triglycerides. Both parameters decreased with no differences between the meals.

In a recent study in healthy volunteers (27 males and 30 females; Heijnen et al., 1996a) the effect of consumption of supplements containing glucose, or RS from raw high-amylose maize starch; RS<sub>2</sub> or from retrograded high-amylose maize starch; RS<sub>3</sub> on fasting serum cholesterol and triacylglycerol concentration was investigated. Both supplements provided 30g RS/d and were taken in addition to the habitual diet for 3 weeks. There were no significant differences in the fasting concentrations of serum total high density lipoprotein cholesterol (HDL), and low density lipoprotein cholesterol (LDL) or triacylglycerol after glucose, RS<sub>2</sub> or RS<sub>3</sub>. Neither the RS<sub>2</sub> nor RS<sub>3</sub> supplements lowered serum lipid concentrations in healthy men. This lack of effect of resistant starch (RS<sub>2</sub> or RS<sub>3</sub>) consumption on serum lipid concentration in human, and the lowering effect found in rats studies, may be related either to species effect or to the higher amount of resistant starch used in rats studies.

In conclusion, it does not seem that RS has any major effects on levels of plasma lipids in healthy humans.

### **1.6.3. Effects on mineral absorption**

Although most mineral absorption occurs in the small intestine and, unlike viscous fibre, RS would not be expected to affect small intestinal absorption, a significant proportion of mineral absorption does occur in the large intestine and fermentation of resistant starch may influence mineral absorption here. Fermentation could affect mineral absorption by 1) reducing pH making the mineral soluble and more absorbable and 2) the SCFA produced will increase the size and mucosal surface area of the proximal colon increasing absorption capacity.

In a rat study by Schulz et al., (1993) calcium concentrations in the liquid phase of the ileum and liquid cecal contents tended to be elevated with RS<sub>2</sub> but not with retrograded resistant starch, due to increased solubility of the mineral in ileal and caecal digesta in the decreased luminal pH during fermentation of the RS<sub>2</sub>.

The effect digestible wheat starch or resistant starch, i.e. raw potato starch; RS<sub>2</sub>, on the colonic absorption of Ca and Mg was investigated in rats by Younes et al., (1996). The microbial fermentation of RS, caused a more acidic

pH with a higher percentage of soluble Ca in the caecum. The hypertrophy of the caecal wall induced by SCFA and the elevated level of soluble Ca increased the absorption of Ca 5-6 fold in the resistant starch group. Feeding RS<sub>2</sub> also increased caecal absorption of Mg . Thus, the large intestine may represent a major site of Ca and Mg absorption when acidic fermentation occurs.

To study the possible effect of resistant starch on mineral absorption in man, Heijnen et al., (1995*c*) gave 32g/d of either RS<sub>2</sub> or RS<sub>3</sub> as a supplement to the usual diet of 23 healthy men for 1 week. This had no effect on absorption of magnesium, calcium or phosphorus in contrast to the rat studies. The possible reasons for this difference include: 1) the amount of resistant starch that man is able to consume (0.4g RS per kg body weight daily) is much lower than the amount used in experiments in rats (17g RS per kg body weight daily), 2) the ability of man and rat to ferment different type of resistant starch may differ.

It is very difficult to separate true and apparent absorption of minerals. One approach is to use radiolabelled tracers to follow dietary sources and endogenous secretion into the gut from the blood. Heijnen et al., (1996*b*) showed that dietary RS<sub>2</sub> but not RS<sub>3</sub> increased magnesium absorption in rats, due to increasing ileal solubility of magnesium, caused by reduction in pH as a

result of fermentation. Dietary RS<sub>2</sub> might raise true magnesium absorption and stimulate reabsorption of endogenous magnesium, leading to a lower faecal excretion of endogenous magnesium. They measured the true magnesium absorption in rats ingesting RS<sub>2</sub> or RS<sub>3</sub> after orally and intraperitoneally administered radiotracer <sup>28</sup>Mg. In rats fed with RS<sub>2</sub> instead of RS<sub>3</sub>, there was significantly increased apparent but not true magnesium absorption, because RS<sub>2</sub> lowered faecal excretion of endogenous magnesium. When dietary lactulose and glucose were used, lactulose significantly increased both apparent and true magnesium absorption, but did not affect faecal excretion of endogenous magnesium. It was suggested that the hypothesised mechanism by which both RS<sub>2</sub> and lactulose enhanced magnesium absorption i.e. by increasing ileal solubility of magnesium due to a reduction in pH of ileal contents from fermentation, now appeared to be incorrect. According to that mechanism, RS<sub>2</sub> feeding should raise true absorption of magnesium, and lactulose feeding should lower the faecal excretion of endogenous magnesium, but these effects were not seen. In other words, after the orally and intraperitoneally administered radiotracer <sup>28</sup>Mg, fermentation of RS<sub>2</sub> and lactulose caused reduction in pH of ileal content. This should increase ileal solubility of both endogenous and exogenous radiotracer <sup>28</sup>Mg, therefore, increasing absorption of both radiotracer <sup>28</sup>Mg and also decreasing faecal excretion of magnesium, but this did not happen.

#### **1.6.4. Effects on bowel habit**

Resistant starch could affect stool output by remaining unfermented and increasing excretion of water. However, unlike dietary fibres, such as ispaghula which has a water holding capacity of approximately 7g/g, the water holding capacity of RS is low (about 2g/g) and therefore large amounts would have to be eaten to have a noticeable effect on stool output.

##### **1.6.4.1. Effects on bowel habit in human studies**

The effect of resistant starch on stool output in human studies is summarised in **Table 1.3**. Phillips et al., (1995) showed the effect on faecal bulking of two diets of different RS content, low RS:  $5.0 \pm 0.4$  and high RS:  $39.0 \pm 3.0$ g/d, fed for 3 weeks to healthy volunteers. The high-RS diet significantly increased faecal output. Similar results were reported by Shetty & Kurpad, (1986; Cummings & Englyst, 1991a; Van Munster et al., 1994a and Muir et al., 1995a) after ingestion of a high resistant starch diet in man (**Table 1.3**). However, this effect was not reported by Tomlin & Read, (1990; 1992) who used cornflakes as the dietary source of resistant starch in comparison with rice krispies, providing 10.33g and 0.86g RS/d, respectively. It is probable that the relative low amount of resistant starch used in their study explains the lack of increase in faecal output (**Table 1.3**).

The effect of the different classes of RS on faecal bulking in man was

demonstrated by Cummings et al., (1996) and Heijnen et al., (1997a) (**Table 1.3**). There were no significant differences between the resistant starch categories, when RS<sub>2</sub> and RS<sub>3</sub> stool output were compared, although RS<sub>3</sub> sources produced greater increase in stool weight (Cummings et al., 1996; Heijnen et al., 1997a). All faecal increases with RS were significantly less than that seen with wheat bran. An increase in mean transit time (MTT) was demonstrated with RS<sub>2</sub> (Cummings et al., 1996).

Scheppach et al., (1988a) showed that starch malabsorption, induced by the glucosidase inhibitor acarbose, resulted in a significantly prolonged transit time in man. The starch entering the colon was not true resistant starch, however and may be handled differently in the colon. Cummings & Englyst, (1991a) found that RS<sub>2</sub> and RS<sub>3</sub> decreased the transit time compared with a control. However, they more recently reported (Cummings et al., 1996) that RS<sub>2</sub>; potato and banana granules tended to prolong transit time more than RS<sub>3</sub>; retrograded wheat and maize starch. Cummings et al., (1992b) observed laxative effects of resistant starch in humans after ingestion of raw potato or banana starch; RS<sub>2</sub>, and retrograded wheat or maize starch; RS<sub>3</sub>, although this effect was less noticeable in comparison with a corresponding amount of wheat bran (Cummings et al., 1992b). Thus the effect of resistant starch on transit time is unclear.



#### **1.6.4.2. Effects on bowel habit in animal studies**

The faecal bulking effects of resistant starch has been demonstrated in animal experiments by several investigators (Faulks et al., 1989; Gee et al., 1991; de Decker et al., 1995; Heijnen & Beynen, 1997*b* and Lajvardi et al., 1993). They noted that both raw potato starch; RS<sub>2</sub> and high amylose cornstarch; RS<sub>2</sub> significantly enhanced faecal weight compared with cooked potato starch in rats. Raw potato starch; RS<sub>2</sub>, however, due to a greater amount entering the colon, produced a 50% longer transit time. Calvert et al., (1989) and Mathers et al., (1995) found that raw potato starch; RS<sub>2</sub> increased faecal weight and prolonged transit time in rats.

### **1.7. Colonic fermentation**

Before considering the indirect effects of RS through its fermentation by colonic bacteria, I will first discuss colonic fermentation general.

Fermentation is the anaerobic degradation of oligosaccharides, polysaccharides and protein from the diet or mucin and glycoproteins from host secretions (Perman & Modler, 1982) by the colonic microflora (Edwards & Rowland, 1992; **Figure 1.5**). The main end products of fermentation are short chain fatty acids (SCFA), acetic, propionic and butyric acid. Gases, hydrogen, methane and carbon dioxide and hydrogen sulphide are also produced (Edwards & Rowland, 1992; **Figure 1.5**).

**Table 1.3. The effect of resistant starch on stool output in human**

No of subjects	Type of RS	Dose of RS	Period of RS (week)	Effect	References
8	cornstarch	—	1	increased	Shetty & Kurpad, 1986
12	glucosidase inhibitor <sup>a</sup>	—	4	increased	Scheppach et al., 1988a
8	cornflakes (RS <sub>3</sub> )	10.33 g	1	none	Tomlin & Read, 1990
11	RS <sub>2</sub> , RS <sub>3</sub> <sup>b</sup>	RS <sub>2</sub> :23.5 g , RS <sub>3</sub> :9.6 g	2	increased	Cummings & Englyst, 1991a
8	cornflakes (RS <sub>3</sub> )	10.33 g	1	none	Tomlin & Read, 1992
14	RS <sub>2</sub> <sup>c</sup>	45 g (62% RS)	2	increased	Van Munster et al., 1994a
11	RS <sub>1</sub> , RS <sub>2</sub> , RS <sub>3</sub> <sup>d</sup>	39.0 ± 3.0 g (x±SEM)	3	increased	Phillips et al., 1995
11	RS <sub>1</sub> , RS <sub>2</sub> , RS <sub>3</sub> <sup>e</sup>	39.0 ± 3.0 g (x±SEM)	3	increased	Muir et al., 1995a
12	RS <sub>2</sub> , RS <sub>3</sub> <sup>f</sup>	RS <sub>2</sub> :28.1g , RS <sub>3</sub> :18.2 g	2	increased	Cummings et al., 1996
24	RS <sub>2</sub> , RS <sub>3</sub> <sup>g</sup>	RS <sub>2</sub> :32 g , RS <sub>3</sub> :32 g	1	increased	Heijnen et al., 1997a

<sup>a</sup> acarbose (BAY g 5421)

<sup>b</sup> fresh banana (semiripe) , cooked and cooled potato

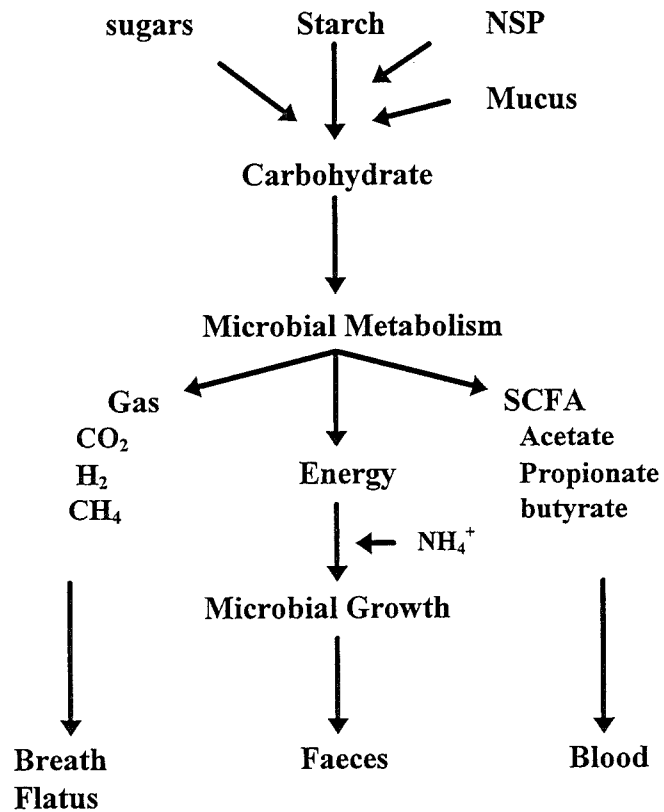
<sup>c</sup> uncooked high amylose maize starch (Hylon-VII)

<sup>d</sup> unprocessed coarse wheat seeds , raw green banana , cornbread from high amylose maize starch (Hi-maize)

<sup>e</sup> unprocessed coarse wheat seeds , uncooked green banana flour , cornbread from high amylose maize starch (Hi-maize)

<sup>f</sup> potato and banana granules , retrograded starch from wheat and maize

<sup>g</sup> uncooked high amylose maize starch (Hylon-VII) , extruded , retrograded Hylon-VII



**Figure 1.5** Fermentation in the human colon (Cummings, 1983; Englyst & Kingman, 1993)

### **1.7.1. Factors affecting colonic fermentation**

The fermentation of a particular polysaccharide is determined by several factors including the chemistry and structure of the carbohydrate, the presence of bacterial enzymes, environmental conditions such as mixing rate and the transit time through the gut, which will be explained in next section.

#### **1.7.1.1. Colonic bacteria**

Bacteria are the major component of the faeces and constitute 40-50% of faecal solids in people consuming a "Western" diet (low in fibre, high in protein and fat; Stephen & Cummings, 1980a). The large intestine is a complex community that contains more than 400 different species of bacteria (Finegold et al., 1983). These bacteria exist in high numbers, usually about  $10^{11}$  to  $10^{12}$  bacteria per gram of dry faeces (Moore et al., 1978). The predominant species are non-sporing, obligate anaerobes from the genera *Bacteroides*, *Fusobacterium* (Gram-negative rods), *Eubacterium* and *Bifidobacterium* (Gram-positive rods). Gram positive anaerobic cocci are also exist in large numbers (Drasar, 1988).

The endogenous source of substrate from the host secretions (mucin glycoproteins) has a major role for sustaining the colonic microflora and accounts for the difference between the daily amount of carbohydrate needed for the colonic microflora (60-70g) (Wolin, 1981), and the quantity of

substrate available from undigested dietary carbohydrates (NSP and RS) estimated from ileostomy studies as 25-35g (Schweizer et al., 1990).

The amount of material escaping from the ileum varies and an inverse relationship between caecal bacterial metabolism and upper intestinal nutrient absorption exists. Increasing the fibre content of the diet may increase the caecal bacteria mass, whereas, the types of bacteria do not alter (Eastwood, 1988).

#### **1.7.1.2. Substrates**

The amount and type of SCFA produced during colonic fermentation, are dependent on the substrate utilized. Acetate is the major SCFA produced during *in vitro* fermentation of all fermentable polysaccharides (Edwards & Rowland, 1992). Englyst et al., (1987*b*) showed that large proportion (29%) of SCFA produced during *in vitro* fermentation of starch was butyrate, whereas, butyrate accounted for only 2% of the SCFA generated from fermentation of pectin. Guar gum and pectin are associated with a large proportion of propionate (Adiotomre et al., 1990; Englyst et al., 1987*b*), whereas, oat bran and wheat bran are associated with large proportion of butyrate (Adiotomre et al., 1990; McBurney & Thompson, 1987).

#### 1.7.1.3. Transit time

Transit through the colon is largely determined by diet. A diet with low residue is associated with slow transit and small faecal output (Spiller et al., 1980). Diets supplemented with wheat bran or ispaghula, or a high mixed-fibre diet, had shorter transit times and increased stool output (Cummings et al., 1976; Eastwood et al., 1978; Spiller et al., 1980). NSP with a large water-holding capacity, retained after fermentation, increased the bulk of colonic contents the most (McBurney et al., 1985; Tomlin & Read, 1988) whilst fermentation of NSP is associated with acceleration of transit time (Tomlin & Read, 1988).

The mechanism by which fermentation accelerates transit is unclear.

Fermentation produces gases and SCFA. Gases may distend the bowel and cause propulsion (Chauve et al., 1976). The effect of SCFA is not well studied but Yajima (1985) showed that contraction of muscle strips, taken from the middle and distal colon of the rat, was stimulated by propionic and n-butyric acids. More recent studies in rat colon *in vitro* and *in vivo* suggest that SCFA inhibit colonic contractions (Cherbut et al., 1991; Squires et al., 1992). Thus the action of SCFA is not clear.

Another possible explanation is when the fibre is fermented, bile acids or fatty acids previously adsorbed into the fibre in the small intestine are released

(Vahouny et al., 1981). These are transformed into material with laxative properties such as deoxycholic acid and hydroxy fatty acid. Edwards et al., (1989) showed that infusion of low concentration of deoxycholic acid into rectum of healthy volunteers causes large rectal contractions with an intolerable urge to defecate and early studies indicated that bile acids stimulate colonic propulsion (Kirwan et al., 1975).

## **1.8. Colonic Fermentation products**

### **1.8.1. Short chain fatty acids**

SCFA are the principal products of fermentation in the colon of man and other animals. In man, acetate, propionate and butyrate contribute 85-95% of total SCFA in all regions of the colon (Cummings & MacFarlane, 1991b). The concentration of SCFA are greatest in the caecum and right colon of man and fall towards the distal colon (Macfarlane et al., 1992). Samples of colonic contents from sudden-death victims showed that the caecal SCFA concentration was 135 mmol/kg and declined to 120 mmol/kg in the transverse colon, and 101 mmol/kg in sigmoid/rectum region and finally the excretion of SCFA in faeces is very low about 10-20 mmol/day (Englyst & Kingman, 1993). Acetate and propionate are absorbed from the large intestine, enter into the portal circulation and transported to the liver (Cummings, 1981). Butyrate, however, is the preferred fuel for human colonocytes (Roediger, 1982).

Most SCFA are cleared by the liver, while only acetate passes into the peripheral circulation (Pomare et al., 1985).

McNeil, (1984) estimated that fermentation may provide up to 10% of daily energy requirement in the Western world. Energy liberated by fermentation of 1g hexose in the form of SCFA is 1.61 Kcal, 0.64 Kcal and 0.42 Kcal, from acetate, propionate and butyrate respectively. Therefore, a total of 2.67 Kcal is in the form of SCFA per gram of hexose (Wisker & Feldheim, 1994).

Cummings & Englyst, (1987a) noted that many factors influence the energy supplied by the fermentation, such as the amount and type of substrate entering the colon, the composition and the capacity of the colonic microflora, and the colonic transit time. The SCFA produced during fermentation are rapidly absorbed from the colonic lumen (Jorgensen et al., 1997), and represent about 70% of the energy available from the initial carbohydrate if it was hydrolysed and absorbed from the small intestine (Blaxter, 1962).

#### **1.8.1.1. Acetic acid**

Acetic acid is the major SCFA produced during fermentation (approximately 60% of total SCFA) and is reliably increased during fermentation. Although, acetate is also produced by the liver during fasting (Ballard, 1972; Knowles et al., 1974; Pethick et al., 1981), increases in plasma acetate in the fed state



result mainly from fermentation (Pomare et al., 1985; Scheppach et al., 1991b; Muir et al., 1995b).

Cummings et al., (1985) measured SCFA in portal, hepatic and peripheral venous blood in six people who were victims of sudden death. Blood was obtained within 4 hours of death in all cases. The amount of acetate was as follows ( $\mu\text{mol/l}$ ): portal blood ( $258 \pm 40$ ), hepatic blood ( $115 \pm 28$ ), peripheral venous blood ( $70 \pm 18$ ). Venous blood acetate was significantly higher than the amount observed in fasting healthy subjects ( $54 \pm 4 \mu\text{mol/l}$ ) but evidence of recently ingested food was seen in the gut of some of the sudden death victims. It is therefore clear that the concentration of acetate in portal blood is several times greater than peripheral venous blood, due to significant uptake of SCFA by the liver (Pomare et al., 1985).

Pomare et al., (1985) measured repeatedly (between 3 and 8 times) fasting blood acetate over 6 months in six healthy subjects. The subjects fasted for 12 hours before each study and plant polysaccharides and other potentially fermentable carbohydrates, such as starch, were excluded from their diet for the meal before the start of the fast. Fasting arterial blood acetates were significantly higher at  $125.6 \pm 13.5 \mu\text{mol/l}$  than venous blood  $61.1 \pm 6.9 \mu\text{mol/l}$  taken at the same time. Acetate is utilized by peripheral tissue in various mammals (Knowles et al., 1974), including man (Skutches et al.,

1979) and this study provided evidence for this in the significant fall in acetate concentration between arterial and venous blood in fasting subjects.

It was shown by Scheppach et al., (1991*b*) that fasting plasma acetate levels were significantly lower in ileostomy patients than in control subjects. The concentration of fasting blood acetate in 10 ileostomy patients was ( $21.3 \pm 0.8$   $\mu\text{mol/l}$ ) compared with that of 21 control subjects ( $48.0 \pm 4.2$   $\mu\text{mol/l}$ ). Thus the colon contributes significantly to venous plasma acetate concentration in man. However, this study also demonstrated that endogenous synthesis of acetate also occurs. Plasma acetate concentration was measured in eight healthy volunteers during 108 hours of continuous fasting. Plasma acetate concentration rose significantly from 12 hours ( $43.9 \pm 4.4$   $\mu\text{mol/l}$ ) to 108 hours of starvation ( $114.0 \pm 15.6$   $\mu\text{mol/l}$ ). Therefore, there are at least two major sources of acetate in man, an endogenous source and the colon which is more important when a fermentable substrate enters the colon.

The effect of two diets that differed markedly in the amount of RS on colonic fermentation were studied by Muir et al., (1995*b*). High and low RS diets were fed to eight healthy subjects in three meals for 1 day. Breath hydrogen and two blood samples were collected during 28 hours period. The first blood sample was a fasting sample and the second was taken to coincide with the peak of breath hydrogen. The high RS diet provided  $59.1 \pm 4.7\text{g}$  (  $\bar{x} \pm \text{SEM}$  )

RS and the low RS diet provided  $5.2 \pm 0.4$ g RS. The high resistant starch diet contained unprocessed coarsely wheat seeds; RS<sub>1</sub>, uncooked green banana flour; RS<sub>2</sub> and cornbread from high amylose maize kernels; RS<sub>3</sub>. In this study, changes in breath hydrogen and serum acetate were used as a qualitative markers of colonic fermentation. The results showed that breath hydrogen and average total serum acetate were significantly higher during the high resistant starch diet than during the low RS diet. This study provides evidence that RS is fermented in the colon by anaerobic microflora producing hydrogen and acetate.

#### **1.8.1.2. Propionic acid**

Propionic acid is absorbed from the colonic lumen into mucosa, enters the portal blood and is then transported to the liver. Hepatic propionate clearance from portal venous blood is nearly 90% and little appears in peripheral blood (Cummings et al., 1987*b*). Propionate is thought to have beneficial effects on glucose and lipid metabolism (Venter et al., 1990; Todesco et al., 1991).

Most of the studies conducted so far have been performed with dietary supplements of propionate. The effect of oral administration of propionate on carbohydrate metabolism, was studied by Todesco et al., (1991). Adding 3.3g sodium propionate per 50g available carbohydrate portion of bread

significantly reduced the postprandial glucose response in six healthy volunteers. Dietary supplementation for one week with 9.9g sodium propionate in bread/day reduced the blood glucose area compared with standard propionate-free bread. Propionate may act as an enzyme inhibitor of amylolytic activity (Elund, 1987), reducing the rate of starch digestion. Other possible mechanisms include a delay in the rate of gastric emptying in mammals, including man (Blum et al., 1976). In addition, propionic acid has been shown to increase glycolysis and decrease glucose production in isolated rat hepatocytes (Anderson & Bridges, 1982). Similar results, reduced fasting serum glucose after 7 weeks dietary supplementation with propionate in capsule form, were seen in healthy volunteers (Venter et al., 1990).

Berggren et al., (1996) studied the possible metabolic effects of propionic acid on cholesterol metabolism in obese hyperinsulinaemic rats during a 19 day test period. Sodium propionate was either fed orally through the diet (1g/day), or infused rectally (0.15g/day) to animals given diets high in cholesterol (20g/kg) and saturated fat (130g/kg). At the end of the test period, total liver cholesterol pools were significantly lower in rats given dietary or rectally infused propionate compared with the control group. Propionate has been shown to inhibit cholesterol synthesis in isolated rat hepatocytes (Chen et al., 1984), and this may result in lower blood cholesterol levels. However studies with rat hepatocytes have shown an increase in bile acid secretion by

propionate (Imaizumi et al., 1992).

#### **1.8.1.3. Butyric acid**

Butyrate like the other SCFA is absorbed from the colonic lumen. Butyric acid has been identified as the major source of energy for the colonocytes (Roediger, 1982). Residual butyrate enters the portal blood and is effectively cleared by the liver (Cummings et al., 1987*b*).

Butyrate has been emphasized as maintaining the health of the colonic mucosa. Scheppach et al., (1992) studied the effect of butyrate enemas on the colonic mucosa in 10 patients with distal ulcerative colitis. They were treated for 2 weeks with sodium butyrate (100 mmol/l) and 2 weeks with placebo in random order. After butyrate irrigation, stool frequency (n/day) significantly decreased and blood discharge stopped in 9 of 10 patients. The histological degree of inflammation also significantly decreased. On placebo, all of these parameters were unchanged. These findings suggest that butyrate deficiency may play a role in the pathogenesis of distal ulcerative colitis and butyrate supplement can improve this condition.

Butyrate is thought to play a key role in the prevention of colonic cancer. It prolongs doubling time and reduces the growth rate of human colorectal

cancer cell lines (Whitehead et al., 1986). Weaver et al., (1988) studied the distribution of SCFA in enemas samples between polyp-colon cancer subjects and normal subjects. A significantly lower ratio of butyrate to total SCFA was found for polyp-colon cancer subjects than for normal subjects. Similar results were found in an *in vitro* study reported by Clausen et al., (1991). The ratio of butyrate to total SCFA was reduced during faecal incubation of samples from colonic cancer patients with different fibres compared with healthy controls.

Therefore, butyrate has two contrasting effects on colonic epithelium. In normal colonic epithelium it is used as a preferable source of energy and stimulates the growth of colonic mucosa, whereas, in colonic tumor cell lines its role is as inhibitor for growing and inducing differentiate in colonic cancer cell lines (Hague et al., 1997). It has also been shown to stimulate apoptosis or programmed cell death (Hague et al., 1993; Hague & Paraskeva, 1995).

### **1.8.2. Gaseous products**

The production of hydrogen, carbon dioxide and methane in the large bowel has been extensively documented (Anderson et al., 1981; Cummings & MacFarlane, 1991b; Olesen et al., 1992; Rumessen et al., 1992). During fermentation, gas is eliminated both through the lungs and expelled as flatus. The excretion of gases per rectum occurs when the gas production is more

than the absorptive capacity of the colon, but at low level the gases are largely absorbed and excreted through the lungs (Calloway & Murphy, 1968; Levitt, 1971; Anderson et al., 1981).

#### **1.8.2.1. Hydrogen (H<sub>2</sub>)**

It was shown by Christl et al., (1992) that approximately 65% of total breath H<sub>2</sub> was expired in breath at production rates up to 200 ml/d, and at production rates of 400 ml/d or greater, the proportion falls to 20% in breath, with an overall average of 51% in breath. There are several ways of disposal of H<sub>2</sub>; methanogenesis, sulphate reduction, and acetogenesis (Gibson et al., 1990). In the ruminant, the major pathway for the H<sub>2</sub> disposal is reduction of CO<sub>2</sub> to CH<sub>4</sub> (Wolin, 1974). Similarly, in the human colon, methanogenic bacteria may use CO<sub>2</sub> and H<sub>2</sub> to form CH<sub>4</sub> (Bond et al., 1971).

The second potential mechanism for H<sub>2</sub> uptake in fermentation is sulphate reduction. Sulphate is known to be an important terminal electron acceptor in anaerobic environments (Jorgensen, 1982) and hydrogen can also act as an electron donor (Gibson et al., 1988a). The major end product of this process is sulphide, which is rapidly hydrolysed to H<sub>2</sub>S, thought to be toxic to methanogenic bacteria (Cappenberg, 1975). *In vitro* studies showed that the sulphate reducing bacteria can outcompete methanogenic bacteria for hydrogen if enough sulphate is available (Gibson et al., 1988b).

The third route which hydrogen can be used in the colon is acetogenesis, the reduction of CO<sub>2</sub> to acetate. This may occur in the human colon in the absence of methanogenesis (Lajoie et al., 1988). However, in an *in vitro* study by Christl et al., (1992) little acetogenic activity was seen in the faeces of healthy subjects, and no differences were found between the methanogenic and the sulphate-reducing groups. The optimum pH for acetogenesis is about 6 and at the neutral pH of faeces, acetogenic bacteria will be outcompeted by both methanogenesis and sulphate reduction (Gibson et al., 1990). Therefore, colonic acetogenesis is probably only significant in the caecum at a acidic pH. Gibson et al., (1990) reported an *in vitro* study which showed that acetogenic bacteria only become active when there is little uptake of hydrogen by sulphate reducing or methanogenic bacteria. Only low rates of acetogenesis were demonstrated but the rates were generally higher in the non-methanogenic group. Therefore, there are several possible competing pathways for hydrogen disposal in the colon and different factors such as colonic pH and sulphate availability can determine which route predominates.

#### **1.8.2.1.1. Fermentation of resistant starch as measured by breath hydrogen**

Tomlin & Read, (1990; 1992) studied the fermentation of resistant starch in eight healthy volunteers who took two dietary supplements for 1 week each in a random order with 1 week separation. The supplements provided 10.33g RS/d (cornflakes; RS<sub>3</sub>) and 0.86 g RS/d (rice krispies). Serial breath H<sub>2</sub>



measurement were made at 15 min intervals for 8 hours on day 1 of each supplement. Significantly more breath  $H_2$  was produced while eating cornflakes, than rice krispies, due to a larger amount of fermentable substrate reaching the colonic bacteria from cornflakes.

The effect of resistant starch on colonic fermentation was also reported by Van Munster et al., (1994a). They studied the effect of adding 45g native amylo maize (*Hylon-VII*; RS<sub>2</sub>) to a standardised diet (an average Dutch diet) in 14 healthy volunteers on colonic fermentation for 3 weeks. *Hylon-VII* ; RS<sub>2</sub> is a high amylo maize starch, containing 62% resistant starch. During amylo maize consumption, breath hydrogen excretion rose 85% ( $P = 0.002$ ), demonstrating fermentation of resistant starch in the colon.

Colonic fermentation of two diets with different amounts of RS was studied by Muir et al., (1995b). High and low RS diets were fed to eight healthy subjects in three meals for 1 day. The high RS diet provided  $59.1 \pm 4.7g$  ( $\bar{x} \pm SE$ ) RS and the low RS diet provided  $5.2 \pm 0.4g$ . The high RS meal contained unprocessed wheat seed (ground to  $< 3$  mm; RS<sub>1</sub>), uncooked green banana flour; RS<sub>2</sub> and cornbread from high amylose maize starch (Hi-maize; RS<sub>3</sub>). Breath hydrogen was significantly higher during the high RS diet than during the low RS diet.

The rise in breath hydrogen produced by RS may be delayed long after the meal is ingested. Achour et al., (1996) compared the fermentability of highly digestible maize starch and the same maize starch after retrogradation; RS<sub>3</sub> in healthy volunteers. 10 hours after the consumption of the test meals, breath was sampled for 3 hours. Breath H<sub>2</sub> excretion was significantly higher after the ingestion of retrograded starch; RS<sub>3</sub> than after ingestion of digestible starch in this post-absorptive period (10-13 hours) demonstrating that, in healthy humans, the digestion of retrograded maize starch; RS<sub>3</sub> in the small intestine is slow and its colonic fermentation continues 10-13 hours after its ingestion. Oleson study rise seen 11 hours after ingestion of raw potato starch (Oleson et al., 1994).

As pointed out by Cummings & Englyst, (1991a) food processing can alter the amount of starch which escapes digestion in the small intestine. Scheppach et al., (1991a), showed that the end products of RS fermentation *in vivo*, can be changed by food processing. 13 healthy volunteers were studied on four separate days about one week apart. The subjects consumed 375g potatoes containing 60g starch on three different occasions in random order: (A) potatoes boiled and consumed fresh at 60°C, (B) potatoes boiled frozen, thawed and consumed at 20°C, (C) potatoes boiled, frozen, thawed and reheated to 90°C, and consumed at 60°C. Breath H<sub>2</sub> was measured every 15 minutes for 10-14 hours. The extent of colonic fermentation ( $AUC = area$

*under the breath H<sub>2</sub> concentration vs time curve*) in experiment B and C were significantly higher than in experiment A, however, the breath H<sub>2</sub> AUC in experiment C was lower than in experiment B, due to large availability of starch in experiment B for fermentation by colonic microflora.

Malabsorption and influence of dietary fat on colonic fermentation was studied by Olesen & Gudmand-Hoyer, (1997). A dose-response study of wheat bread was performed in seven healthy volunteers. Malabsorption was evaluated by measuring breath hydrogen every 30 minutes over 10 hours. Test meals were: bread made from 25, 75, 100, 150, and 200g white wheat flour, and bread made from 100g white wheat flour served with 11 or 26g butter. The results showed that, all volunteers had a colonic bacterial flora which can ferment undigested residues of the bread. They found that mean hydrogen response was as follows: 100g bread > 75g bread > 25g bread. The effect of adding fat to the bread, was also measured. Adding 11g butter to the bread made from 100g white wheat flour induced a significantly higher breath hydrogen response than when the bread was served alone, whereas, adding 26g butter to 100g white wheat flour caused a response of same size as resulted from bread alone. It was clear from the results that the malabsorbed fraction of wheat bread was dependent on the amount ingested and the composition of the meal.

Chapman et al., (1985) demonstrated that slowing gut transit time increased

starch digestibility and speeding it up reduced digestibility. It seems that adding 26g butter to the bread, slowed the transit time, therefore, increasing the digestibility of carbohydrate reflected in a smaller breath hydrogen concentration. 11g butter in the meal was insufficient to slow the intestinal transit time.

#### 1.8.2.2. Methane (CH<sub>4</sub>)

In Western populations, between 30 and 50% of healthy people produce methane during fermentation in the colon (McKay et al., 1985). Non-methane producing individuals have populations of hydrogen-utilizing sulphate-reducing bacteria in their colons and these bacteria can outcompete methanogens for hydrogen Gibson et al., (1988 *a,b*). In methanogenic individuals, H<sub>2</sub> is used to form CH<sub>4</sub> by CO<sub>2</sub> reduction and the equation would be:

$$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$$

The partial pressure of H<sub>2</sub> and CH<sub>4</sub> in the gut is always higher than in the blood. The absorbed gases are cleared in the lung and excreted in the expired air (Rumessen, 1992).

The effect of food with a highly resistant starch content on breath hydrogen and methane production in methane and non-methane producers was assessed by Segal et al., (1993). Sixteen healthy volunteers (eight blacks and eight whites) took part in this study. All of the black subjects were methane

producers, in contrast to the white group who were non-producers of methane. Each subject consumed 600g peeled bananas (mainly green; 75% RS; RS<sub>2</sub>) after an overnight fast. Breath hydrogen and methane were measured over 6 hours. Both groups produced significant quantities of hydrogen after the banana meal, compared with a sucrose control meal. Breath hydrogen started to rise 30 minutes after the banana meal and peaked at 3½ hours in methane nonproducers, whereas, in methane producers, the rise began after 2 hours and peaked at 5 hours. Methane production was not significantly stimulated by test meal. Therefore, the main effect of banana fermentation, was on breath hydrogen, with minimal effect on methane production. A possible reason for less effect on methane could be the short duration of the study. In this study methane production was monitored for only 6 hours in all subjects. Methane production occurs mainly in the left colon (Flourie et al., 1991), and colonic transit is usually slow, therefore, the peak production of methane may have been missed. However, methane levels are usually steady throughout the day unlike hydrogen which rapid rises and falls after meals.

Van Munster et al., (1994b) studied the effect of native amylo maize (Hylon-VII; RS<sub>2</sub>) on breath hydrogen and methane excretion in healthy volunteers. 19 healthy volunteers were supplemented with 15g native amylo maize (Hylon-VII; RS<sub>2</sub>) three times a day, containing 28g RS, or with dextrins as a placebo for 7 days. Before the experiment, 11 subjects regularly produced breath

methane and 8 did not. The results showed that on the seventh day of supplementation with RS, the 24-h excretion of breath hydrogen increased, however, the mean rise relative to placebo was 35% ( $P = 0.03$ ) for all subjects and 60% ( $P = 0.02$ ) for eight subjects not producing methane. The 11 methane producers showed a 93% increase in breath methane excretion on RS. The lack of excretion of methane in non-producers of methane seems to be due to a different colonic bacterial composition rather than to a lack of fermentable substrate in the supplementation diet. From the results of this study it was noted that hydrogen excretion did not increase significantly in the methane producing subjects after supplementation with RS. This may be due to excess hydrogen being used for methane production. The difference in response of breath hydrogen to RS between methane and non-methane producers subjects was due to a difference in utilization of the hydrogen produced, rather than to a difference in colonic production of hydrogen from RS.

#### **1..8.2.3. Carbon dioxide (CO<sub>2</sub>)**

CO<sub>2</sub> is a major gaseous product of colonic fermentation of carbohydrate. During fermentation, gas is eliminated both through the lungs and expelled as flatus. Carbon dioxide is one of the main components of flatus, about 9% of flatus is composed of CO<sub>2</sub> (Levitt, 1971).

Florent et al., (1985) studied the effect of a chronic load of nonabsorbable

sugar on colonic bacterial metabolism in man (*In vivo*). Twice a day 20g of lactulose was given orally to eight normal volunteers during 8 days. In all of the subjects, breath hydrogen concentration was measured on days 1 and 8 after ingestion of the morning lactulose dose.  $^{14}\text{C}$ -lactulose was added to cold lactulose and  $^{14}\text{CO}_2$  breath was measured. The results showed that breath hydrogen excretion significantly fell from day 1 to day 8, whereas,  $^{14}\text{CO}_2$  breath excretion significantly increased. This was due to adaptation to the repeated amount of fermentable carbohydrates entering the colon, an increased bacterial population which can breakdown the lactulose in the colon and cause drop of caecal pH, an increase in total SCFA and also an increase in breath  $^{14}\text{CO}_2$  excretion. A decrease in pH of ceacal content caused the growth of bacteria which produce lactic and acetic acid (Hill, 1983). The molar ratio of acetic acid to total SCFA increased which may be responsible for the decreased breath  $\text{H}_2$ .

## **1.9. Fermentation of resistant starch**

### **1.9.1. Fermentation of different types of resistant starch**

Olesen et al., (1992; 1994) compared the fermentability of two different kinds of RS, RS<sub>2</sub> and RS<sub>3</sub> and lactulose. Four test meals were used: raw potato starch (RPS; 58% RS; RS<sub>2</sub>), corn flakes (CF; 5% RS; RS<sub>3</sub>), hylon VII high amylo maize starch, extrusion cooked and cooled (HAS; 30% RS; RS<sub>3</sub>),

highly retrograded hylon VII high amylo maize starch (HRA; 89% RS; RS<sub>3</sub>).

Seven healthy volunteers ingested in randomized order 50g RPS, 100g CF, 75 g HAS, 25g HRA. Breath hydrogen was measured every 30 minutes for 12 to 22 hours post-ingestion as a marker of fermentation. The results showed that RPS is fermentable, although the amount of breath hydrogen produced after 50g RPS was clearly less than after 10g lactulose. 100g of corn flakes showed a measurable increase in breath hydrogen, equivalent to 10-20g RPS, whereas, neither of the two samples of hylon VII amylo maize resulted any significant increase in hydrogen production, due to either inaccessibility by the colonic microflora, to a very low degree of fermentability, or to a fermentation pattern without producing H<sub>2</sub>. Therefore, the resistant starch in order of least *in vivo* fermentability are highly retrograded hylon VII high amylo maize starch (HRA; RS<sub>3</sub>), hylon VII high amylo maize starch, extrusion cooked and cooled (HAS; RS<sub>3</sub>), corn flakes (CF; RS<sub>3</sub>) and then raw potato starch (RPS; RS<sub>2</sub>).

*In vitro* fermentability of different types of resistant starch (RS<sub>2</sub> and RS<sub>3</sub>) in eight laboratories with a total of 40 individual faecal inocula was reported by Edwards et al., (1996b). It was shown that semipurified retrograded amylose (RS<sub>3</sub>; 69.8% RS) was less fermentable with the lowest SCFA production than raw potato starch (RS<sub>2</sub>; 54.1% RS). It seems that resistant starch may not only resist hydrolysis by amylase but also degradation by the enzymes of colonic bacteria in the large intestine.



### **1.9.2. Fermentation of resistant starch and dietary fibre**

At first it was thought that resistant starch would act very much like dietary fibre in the gastrointestinal tract. However, fermentation of RS is much slower than most dietary fibres. With fibre an increase in expiratory breath hydrogen is usually seen within 4 hours, the peak increase being reached within 6 hours. Whereas breath hydrogen did not rise until after approximately 9-11 hours after raw potato starch; RS2 and did not rise at all after ingestion of high amylose maize starch (HAS; RS<sub>2</sub>; 15% RS) and enriched retrograded amylomaize (ERA; RS<sub>3</sub>; 89% RS) during the 12 hours test period. These differences between dietary fibre and resistant starch may be due to either resistant starch passing much more slowly through the small intestine than dietary fibre or to a much slower rate of fermentation (Achour et al., 1996).

### **1.9.3. Fermentation of resistant starch and butyrate**

Both *in vivo* and *in vitro* studies have shown that fermentation of starch increases the proportion of butyrate formed compared with dietary fibres (Englyst et al., 1987b; Scheppach et al., 1988b; Weaver et al., 1992).

Butyrate in the colon is essential for the health maintenance of colonocytes (Roediger 1982; Weaver et al., 1988; Clausen et al., 1991) but the site for generation of butyrate may also be important in relation to colonic tumor development. Most naturally occurring tumours develop in the distal colon.

SCFA production usually decreases from the proximal to distal colon (Edwards & Parrett, 1996a). Distal production of butyrate therefore might be advantageous. McIntyre et al., (1993) found in a rat model that the concentration of butyrate in stools correlated significantly and negatively with tumour mass. RS is very slowly fermented and may provide a means of increasing distal butyrate.

The importance of butyrate site production in development of colonic tumorigenesis was shown by Young et al., (1996). They examined the effect of raw potato starch; RS<sub>2</sub> alone and in combination with wheat bran (insoluble NSP) on colonic tumorigenesis in the rat model. Three groups of rats received either a low RS/low fibre (basic) diet, raw potato starch as 20% carbohydrate content, or the potato starch diet plus 10% of wheat bran fibre. Epithelial proliferation and tumours were measured 6 and 20 weeks after a 10-week course of dimethylhydrazine. Rats on the potato starch diet had tumours more frequently and had larger tumours than rats consuming the wheat bran or low RS/fibre diets. These results are contrary to previous beliefs that RS would be beneficial, however, wheat bran produced higher distal colonic and faecal butyrate concentration in the rat (McIntyre et al., 1991) whereas raw potato starch; RS<sub>2</sub> has been shown to be fermented in the ileum to a greater extent than in the colon (Heijnen & Beynen, 1997b) in the rat. The relevance of these results to man however are not clear. There is higher colonisation of

the terminal ileum in rats. Moreover, less fermentable types of resistant starch may be more effective in increasing distal luminal butyrate concentrations.

#### **1.9.4. Effects of RS fermentation on nitrogen excretion**

It is estimated that with a Western diet, up to 12g/d protein reach the colon (Gibson et al. 1976; Chacko & Cummings, 1988) and is fermented there by the microflora to end products including phenol, indoles, amines and ammonia (MacFarlane & Cummings, 1991). Ammonia stimulates cell proliferation and can promote tumorigenesis (Lin & Vissek, 1991) and the growth of malignant cells (Vissek et al., 1978).

The effect of resistant starch on faecal concentration of ammonia in man, was reported by Birkett et al., (1996). Eleven subjects consumed a high RS diet ( $39 \pm 3\text{g/d}$ ) or low RS diet ( $5 \pm 0.4\text{g/d}$ ) for three weeks. The high RS diet was supplemented with cornbread from high amylose maize starch; RS<sub>3</sub>, unprocessed coarsely wheat seeds; RS<sub>1</sub> and raw green banana flour; RS<sub>2</sub>. During the high RS diet daily excretion of faecal nitrogen significantly increased. Faecal concentration of ammonia significantly decreased. RS significantly decreased the harmful byproducts of protein fermentation in the human colon. Ammonia may be used for bacterial protein synthesis and thus trap nitrogen for excretion in the faeces. The reduction in pH due to the colonic fermentation of RS should cause conversion of ammonia to

ammonium ( $\text{NH}_4^+$ ; Vissek, 1978). The ammonium ion is not well absorbed from the colon and is excreted in the faeces.

Similar results were found by Shetty & Kurpad, (1986; Muir et al., 1995*a*) and Cummings et al., (1996) in healthy volunteers and by Remesy & Demigne, (1989; Younes et al., 1995; Heijnen & Beynen, 1997*b*) in animals (rats, piglets).

Heijnen et al., (1997*a*) compared the effects of  $\text{RS}_2$  and  $\text{RS}_3$  on faecal ammonia concentrations in 24 healthy men. Each subject consumed a supplement (32g RS/d) with  $\text{RS}_2$ ; Hylon VII: i.e. uncooked high-amylose maize starch,  $\text{RS}_3$ ; extruded, retrograded Hylon VII and glucose for 7 days.  $\text{RS}_3$  lowered ammonium synthesis more than  $\text{RS}_2$  and glucose. Thus, the effect of RS on ammonium may be attributed to the  $\text{RS}_3$  component and to increased faecal output. Feeding resistant starch to reduce ammonia absorption may be beneficial for patients with impaired renal function (Rampton et al., 1984; Parillo et al., 1988).

#### **1.9.5. Effects of RS fermentation on colon cancer risk**

In the Western world, colon cancer is the second most common cancer in both males and females (Burkitt, 1971). In epidemiological studies of colorectal cancer, diet has a major role in the incidence of this disease (Cassidy et al.,

1994). Colon cancer risk increased in populations, consuming greater amounts of dietary fat and meat, and a reduction in risk is seen with consumption of high amounts of dietary fibre and vegetables (Lipkin, 1988; Bingham, 1990).

In an international survey (Cassidy et al., 1994) incidence of colorectal cancer in 12 populations world-wide was compared with dietary intakes of starch, non-starch polysaccharides (NSP), protein and fat. There were strong inverse associations between starch consumption and colon cancer incidence ( $r = -0.76$ ). The strong inverse relation found suggested a potentially important role of starch in protection against colorectal cancer. RS is hypothesised to reduce the risk of colon cancer in several ways:

(i) By increasing the bulk of colonic contents, diluting any carcinogens and tumour promoters, reducing their contact time with the colonic mucosa by speeding up intestinal transit (Burkitt, 1971; Stephen & Cummings, 1980b; Cummings et al., 1996).

(ii) By production of butyrate (see above).

(iii) By reducing colonic pH, preventing or reducing carcinogen formation such as deoxycholic acid and lithocholic acid (Thornton, 1981; van Dokkum et

al., 1983; Walker et al., 1986; Bruce, 1987). In the large intestine the primary bile acids, cholic acid and chenodeoxycholic acid, by 7 $\alpha$ -dehydroxylation, are converted in to the secondary bile acids, deoxycholic acid and lithocholic acid (MacDonald et al., 1983). Rat studies showed that the secondary bile acids may act as tumour promoters (Narisawa et al., 1974; Summerton et al., 1985). 7 $\alpha$ -dehydroxylase, produced by strictly anaerobic bacteria, has a pH optimum of 7-8. pH values below 6, depress its activity (MacDonald et al., 1978; Nagengast et al., 1988). Stool pH is higher in colorectal cancer patients than in healthy subjects (Vernia et al., 1989). SCFA produced during fermentation of resistant starch, decreased the pH of the colon, therefore, inhibiting the conversion of primary to secondary bile acids by depressing 7 $\alpha$ -dehydroxylation and decreasing the concentration of soluble deoxycholic acid (Van Munster et al., 1995).

Raw high amylose corn starch: 45g; RS<sub>2</sub>; 62% given to 14 healthy subjects for two weeks, decreased excretion of soluble deoxycholic acid by 50%, and the cytotoxic bile acids in the aqueous phase of faeces. Faecal water is the fraction of faeces which contains the water-soluble, not-bound components of faeces (Lapre & Van der Meer, 1992) which are in contact with the colonic mucosal cells (Bruce, 1987; Geltner Allinger et al., 1989).

However when Heijnen, (1997c) investigated the effects of RS on putative risk factors for colon cancer in 24 healthy men, who consumed a daily supplement in addition to their habitual diet for one week, he saw no effect of three supplements (32g RS/d containing RS<sub>2</sub> from uncooked high-amylose maize starch, RS<sub>3</sub> from retrograded high-amylose maize starch or glucose) on pH, bile acid concentrations and cytotoxicity of faecal water.

### **1.10. *In vivo* methods for fermentation assessment**

From the above discussions, it is obvious that to be able to predict the actions of resistant starch in the human intestine we must be able to determine the factors affecting its fermentability. However, it is very difficult to study fermentation *in vivo* due to the inaccessibility of the proximal colon where most fermentation takes place. Indirect approaches have therefore been used. The main methods will be discussed briefly here as they will be considered in more depth in later chapters.

#### **1.10.1. Breath hydrogen (H<sub>2</sub>)**

One of the indirect tests which is used for *in vivo* carbohydrate malabsorption (such as resistant starch) is breath hydrogen excretion. All H<sub>2</sub> produced in man results from the metabolism of the colonic microflora and a proportion of H<sub>2</sub> produced in the colon is absorbed into blood and then cleared as expired air (Levitt, 1969). Thus, a rise in breath H<sub>2</sub>, after ingestion of carbohydrate,

indicates carbohydrate entering into the colon and being fermented by colonic microflora (Strocchi & Levitt, 1991).

Although breath hydrogen increases in a dose dependent way for rapidly fermentable substrates such as lactulose, increases are not so clearly related to the amount and fermentation of a slowly digested carbohydrate (Olesen et al. 1994). Moreover, it has been shown, in intubation studies, that ingestion of a fermentable carbohydrate over a period of one week leads to more efficient fermentation with less hydrogen production (Florent et al., 1985). This is probably due to alternative routes for hydrogen disposal in the human colon including methanogenesis, sulphate reduction and acetogenesis (Gibson et al., 1990). This makes breath hydrogen alone a poor marker for the fermentability of resistant starch.

#### **1.10.2. Plasma acetate**

Another indirect method to assess *in vivo* carbohydrate fermentation is to measure the SCFA in plasma or faeces. For rapidly fermented carbohydrate most of the short chain fatty acids produced will be absorbed. For poorly fermented carbohydrate a larger proportion may escape absorption and appear in faeces.



#### **1.10.2.1. Portal blood**

Acetate is absorbed from the large bowel, enters into the portal circulation, and is transported to the liver. Portal acetate concentration can be measured in humans only at postmortem (Cummings et al., 1985) or in surgical patients (Dankert et al., 1981). Because of inaccessibility of portal blood, it is very difficult (ethically and practically) to obtain samples of portal venous blood from humans.

#### **1.10.2.2. Peripheral blood**

Acetate is the major SCFA produced during fermentation (approx 60%). Acetate is also produced by the liver during fasting (Ballard, 1972; Knowles et al., 1974; Pethick et al., 1981). However, increases in plasma acetate in the fed state result mainly from fermentation (Pomare et al., 1985; Scheppach et al., 1991*b*; Muir et al., 1995*b*).

Acetate is the only SCFA in man which remains in blood after passage through the liver (Pomare et al., 1985). Collection of serial blood samples over several hours is necessary for showing the pattern of carbohydrate fermentation. This needs medical supervision and is unpleasant for the subjects. Plasma acetate levels are much lower than faecal SCFA and therefore there are major problems in concentrating samples sufficiently for analysis. It is not clear how acetate from fermentation can be separated from endogenous production.

### **1.10.3. Use of isotopes**

A novel way of assessing fermentation *in vivo* is to use isotopes. There are two types of isotopes which are used in metabolic and nutrition research: radioactive isotopes and stable isotopes.

#### **1.10.3.1. $^{14}\text{C}$ radioactive isotope**

$^{14}\text{C}$  radio-isotope labelled substrates are largely used in fermentation research. The metabolism and fate of dietary plant cell walls labelled with  $^{14}\text{C}$  isolated from spinach cell cultures were followed in the rat (Gray et al., 1993*a, b*; Buchanan et al., 1994; 1995*a, b*). This method enabled the subsequent distribution of  $^{14}\text{C}$  in the host tissue, excretion products and expired gases (such as  $^{14}\text{CO}_2$ ) to be studied.

Use of radioactive isotopes for human research are not safe (they emit radiation in the process) (Wolf, 1992), especially for pregnant and lactating mothers as well as studies on newborns, infants and children.

#### **1.10.3.2. $^{13}\text{C}$ stable isotope**

One of the best potential ways for assessing fermentation in human studies is to use substrates which are labelled with  $^{13}\text{C}$  stable isotope.  $^{13}\text{C}$  stable isotope is safe because there is not any risk of radiation. This makes it safe for studies of newborns, infants, children, and pregnant and lactating women. Stable

isotopic tracers can be administered orally to human subjects and the metabolic products that enter into breath, milk, urine or faeces can be sampled (Shetty & Ismail, 1991). Several labelled substrates with stable isotopes can be given simultaneously to the same subject who can also be studied repeatedly. This option is restricted in human radiotracer studies because the limits of radioactivity are soon exceeded (Bier, 1982).

#### **1.10.3.2.1. $^{13}\text{C}$ stable isotope in nutritional research**

Stable isotopes have been used for labelling substrates as tracers for the investigation of metabolic pathways, to measure body composition, energy balance, protein turnover and for monitoring fuel metabolism (Rennie et al., 1991). Not all of the stable isotopes are useful as metabolic tracers. The ones that are useful are normally present in our environment in small amounts (less than 10% of the total) allowing us to distinguish easily between a compound labelled with the more abundant isotope (Rennie, 1986). The majority of elements which exist in a stable form have more than one stable isotope eg  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ ,  $^{18}\text{O}$ ,  $^{26}\text{Mg}$ ,  $^{70}\text{Zn}$  and these can be used as tracers (Rennie et al., 1991).

For measuring fermentation with stable isotopes  $^{13}\text{C}$  is the most useful tool. The percentage of natural abundance of  $^{13}\text{C}$  is 1.11%, whereas, that for  $^{12}\text{C}$  is 98.89%.

#### 1.10.3.2.2. Sources of $^{13}\text{C}$ substrates

Labelled nutrients with  $^{13}\text{C}$  can be produced in four ways:

a) by chemical synthesis of amino acids, fats, and carbohydrates labelled with  $^{13}\text{C}$ . In the last 20 years, these products have been used, but are useful only for studying the metabolism of individual nutrients. Most individuals consume diets containing many nutrients, not elemental mixtures.

b) photosynthetic fixation with  $^{13}\text{CO}_2$  into growing plants, during the grain seed or tuber filling stage of development.

c) photosynthetic incorporation of  $^{13}\text{CO}_2$  into unicellular organisms such as algae to produce labelled proteins and fats.

d) biotransformation of labelled organic and biosynthetic products e.g. metabolic processing of food of animals. Animals are fed with  $^{13}\text{C}$  enriched fodder resulting in the production of meat, milk and eggs which can be used as test substrates (Klein, 1991).

#### 1.10.3.2.3. $^{13}\text{CO}_2$ breath tests

Administration of  $^{13}\text{C}$  rich substrates results in the production of  $^{13}\text{CO}_2$  expired in breath as the substrate is oxidized. Because of the natural background of  $^{13}\text{CO}_2$ , the labelled  $\text{CO}_2$  is actually the amount of  $^{13}\text{CO}_2$  in excess of the  $^{13}\text{CO}_2$  abundance before the labelled substrate was administered.

Therefore, the test defines the difference between the initial abundance of  $^{13}\text{C}$  and that after consumption of a labelled substrate. If an adequate dose of labelled substrate is used in the test, the  $^{13}\text{CO}_2$  will always be detectable (Schoeller et al., 1980). In metabolic studies using  $^{13}\text{C}$  substrates as a test meal, subjects fast for 8 hours before starting the study and during the study, foods which have high amount of natural  $^{13}\text{C}$  should be forbidden, to reduce the influence of background fluctuations in the base-line. The isotopic abundance of  $^{13}\text{C}$  in common foodstuffs is shown in **Table 1.4**. (Schoeller et al., 1980).

Hiele et al., (1990) used the  $^{13}\text{CO}_2$  breath test for studying the digestibility of different starch preparations. They reported the effect of the amylose/amylopectin ratio and the degree of gelatinisation of starch on its hydrolysis rate, by measuring the rate of  $^{13}\text{CO}_2$  appearance in breath after the intake of different corn starch preparations by eleven healthy subjects. Starch is hydrolysed to glucose, which is subsequently oxidised to  $\text{CO}_2$ . Differences seen in  $^{13}\text{CO}_2$  excretion after ingestion of starchy foods must be due to differences in the rate of hydrolysis. The effect of the degree of chain branching between waxy starch, containing 98% amylopectin and high amylose starch, containing 30% amylopectin and normal crystalline starch, containing 74% amylopectin was studied. The effect of the extent of gelatinisation was also compared with extruded starch and crystalline starch.

**Table 1.4. Relative  $^{13}\text{C}$  abundances of common dietary constituents**

<b>Foodstuff</b>	<b><math>\delta_{\text{PDB}}^{13}\text{C}</math></b>	<b>% <math>^{13}\text{C}</math></b>
<b>Meat and meat substitutes</b>		
Pork	-13.5	1.0964
Chicken	-16.1	1.0935
Tuna	-16.9	1.0927
Peanut butter	-24.9	1.0838
<b>Dairy products</b>		
Egg	-16.2	1.0934
Cheddar cheese	-19.6	1.0897
American cheese	-20.3	1.0989
<b>Grains and cereals</b>		
Millet	-11.2	1.0989
Corn flour	-10.7	1.0995
Wheat flour	-22.7	1.0863
White bread	-21.6	1.0875
Rice	-24.2	1.0846
<b>Fruits</b>		
Pineapple	-12.8	1.0971
Orange	-27.5	1.0810
<b>Vegetables</b>		
Kidney bean	-22.9	1.0860
Lettuce (iceberg)	-24.4	1.0844
Cabbage (white)	-25.8	1.0829
Celery	-26.8	1.0818
Potato	-25.8	1.0829
<b>Sweeteners</b>		
Molasses	-12.6	1.0974
Honey	-23.5	1.0854

(Schoeller et al., 1980)

All starch preparations used in this study were corn derived. Corn carbohydrates are naturally enriched in  $^{13}\text{C}$ . After an overnight fast of about 13 hours, the subjects ate 50g carbohydrate in 250 ml water. The breath  $\text{CO}_2$  was monitored every 30 minutes for eight hours. The  $^{13}\text{CO}_2$  excretion rate after ingestion of high amylose starch was markedly lower than after ingestion of waxy starch, but there was no significant difference between waxy starch and normal crystalline starch.

Harding et al., (1994) obtained  $^{13}\text{C}$ -enriched wheat flour by the photosynthetic incorporation of  $^{13}\text{CO}_2$  during the grain filling period of wheat plants. 400 mg  $^{13}\text{C}$ -labelled flour was ingested by a healthy adult volunteer for measuring starch digestion and the effect of gelatinisation on  $^{13}\text{CO}_2$  recovery. Breath samples were collected, before the ingestion of the test substrate and at 30 minutes intervals continued for six hours. The timing of the peak recovery of  $^{13}\text{CO}_2$  varied and depended on to the load of starch ingested and the degree of gelatinisation.

#### **1.10.3.2.4. Measurement of $^{13}\text{CO}_2$ breath tests**

$^{13}\text{CO}_2$  enrichment is measured using gas isotope ratio mass spectrometry. The measurement is based on the behaviour of two ions of different mass in a strong magnetic field. The heavier mass is less deflected than the lighter mass.

The ratio of mass 45 to mass 44 is measured for determining the enrichment of  $^{13}\text{C}$  relative to  $^{12}\text{C}$ . Enrichment of the sample is compared with an international limestone standard, PeeDee Belemnite (PDB) (Craig, 1957) and delta ( $\delta$ ) per mil (‰) is used to express the difference in enrichment between the sample and PDB.

For expressing the recovery of  $^{13}\text{C}$  in breath samples after ingestion of a labelled substrate for a single time, the common and acceptable way is to use delta above baseline. This shows the rate of  $^{13}\text{C}$  appearance in the breath and also, the time to peak excretion. For measuring the actual proportion of the substrate eaten and then digested, results are best expressed as a percentage of  $^{13}\text{C}$  recovered (PDR) during the test time (Amarri & Weaver, 1995).

Although  $^{13}\text{C}$  labelled substrates have been used to measure digestion of starch in a number of small studies (Hiele et al., 1990; Harding et al., 1994) no-one has yet used this method for measurement of colonic fermentation.

### **1.11. Aims of this thesis**

In this thesis I have tried to develop methods for measuring colonic fermentation of resistant starch *in vivo*. I have also tested the hypothesis that repeated ingestion of resistant starch will increase the ability of the human



colonic flora to ferment it. This is important if resistant starch is to be used for increased colonic butyrate production to treat or prevent colonic disease.

The approaches I have used included breath hydrogen and plasma acetate measurements and the use of  $^{13}\text{C}$  labelled pea starch, grown as part of this thesis, for  $^{13}\text{CO}_2$  breath tests.

# CHAPTER TWO

## Methods

In this chapter I will describe the general methods used for experiments.

Methods used for single experiments will be described in the relevant chapter.

## **2.1. Hydrogen (H<sub>2</sub>) breath test**

The breath hydrogen (H<sub>2</sub>) test is one of the indirect tests for measuring carbohydrate malabsorption (Rumessen, 1992). The basis of this test is that all the H<sub>2</sub> produced in man results from the fermentation processes of the anaerobic bacterial flora in the colon. A proportion of the H<sub>2</sub> produced is absorbed into the blood and then excreted in breath (Levitt, 1969). Therefore, a rise in breath H<sub>2</sub>, after consumption of carbohydrate, indicates that carbohydrate has reached the microflora in the colon, whereas, no rise in breath H<sub>2</sub> indicates that not enough carbohydrate entered the colon for fermentation (i.e. near complete absorption) (Strocchi & Levitt, 1991).

H<sub>2</sub> breath tests are non-invasive and non-radioactive, and they do not disturb normal physiological functions. Breath tests are generally carried out after an overnight (8-12 hours) fast to bring breath H<sub>2</sub> concentration to below 20 ppm in healthy subjects (Rumessen, 1992) ideally below 5 ppm.

During the first part of the test fasting levels may decline further. The lowest H<sub>2</sub> concentrations obtained before a sustained increase can be used as a

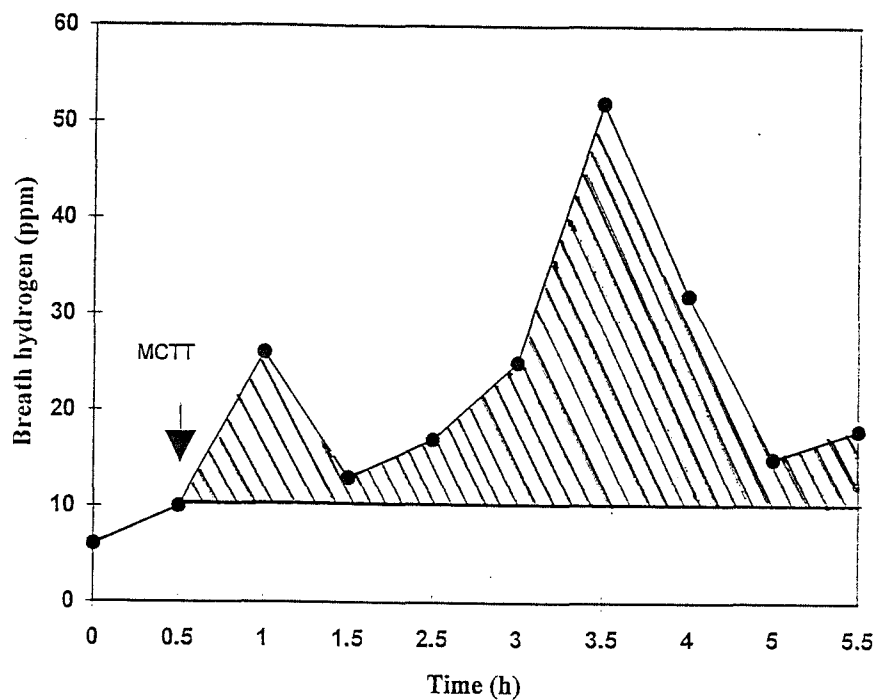
baseline (Barr et al., 1981). It has been suggested that a usable indication of carbohydrate malabsorption is demonstrated when breath hydrogen concentration increases 20 ppm or 10 ppm above the baseline (Barr et al., 1981). Mouth-to-caecum transit time (MCTT or OCTT; oro-caecal transit time) is defined as the time interval between ingestion and the first sustained rise in breath H<sub>2</sub> concentration (Bond & Levitt, 1975). The rise in breath H<sub>2</sub> concentrations after MCTT can be quantified by the maximal rise from baseline or more accurately by the area under the curve (AUC) in a sufficient period of time (Rumessen, 1992) for the hydrogen to return to baseline or at least to approach baseline values.

But as discussed in chapter 1, breath hydrogen measurement values are not a good quantitative measure of carbohydrate malabsorption because there are several other routes for hydrogen disposal. It is also important to choose an appropriate control carbohydrate. Lactulose, which is most often used, is not a good model for slowly fermented carbohydrates such as resistant starch. In our studies we have used both lactulose and guar gum.

#### **2.1.1. Breath hydrogen (H<sub>2</sub>) test used in this thesis**

Breath samples were collected every 30 minutes in 20 ml syringes with a small plastic T-piece. The breath H<sub>2</sub> monitor used in our studies was a selective electrochemical cell (GMI, Renfrew, Scotland) which was calibrated with a 96

ppm standard gas. End-expiratory breath  $H_2$  in parts per million (ppm) was measured. The subjects who participated in our studies fasted for at least 10 hours before each experiment. Breath  $H_2$  production in a healthy adult after ingestion of 20g lactulose during 5.5 hours is shown in **Figure 2.1**. Area under the curve; AUC is shown in shade and mouth to caecum transit time; MCTT is indicated.



**Figure 2.1.** Breath hydrogen concentration (ppm) after ingestion of 20g lactulose in a healthy subject. MCTT: mouth-to-caecum transit time. AUC: area under the curve is shown in shade.

## **2.2. Measurement of plasma acetate**

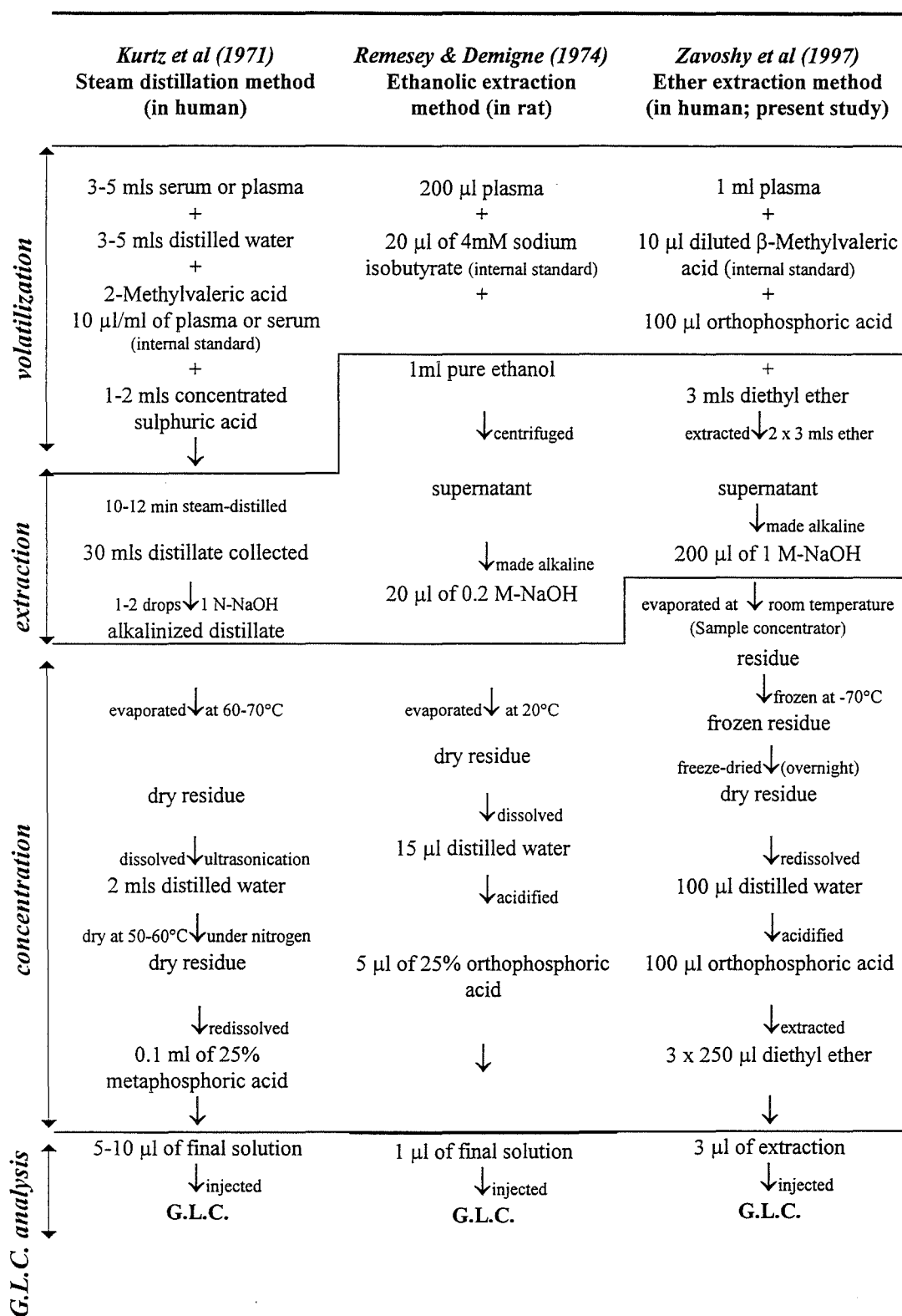
Acetate is the major short chain fatty acid produced from fermentation of carbohydrate (Cummings & Englyst, 1987*a*). Acetate is the only short chain fatty acid to enter the peripheral blood, although a large amount is removed by the liver (Pomare et al., 1985). Plasma acetate can therefore be used as an indirect method for assessment the colonic fermentation. Acetate can be made in the liver and tissues but this is very low under fed conditions. Acetate in peripheral blood during fed conditions is mostly from colonic fermentation.

### **2.2.1. Assay of plasma acetate**

There are two approaches to extract acetate from plasma. The amount of acetate in plasma is very low and sample must be concentrated. This can be achieved either by steam distillation or a solvent extraction method.

Most techniques used to measure the concentration of plasma acetate utilise steam distillation (Perry et al., 1970; Kurtz et al., 1971) of the plasma samples. This requires at least 3 ml of plasma and the vacuum distillation (Tyler & Dibdin, 1975; Tangerman et al., 1983) is much more complicated procedure than the solvent extraction method (Remesy & Demigne, 1974) (**Figure 2.2**) .

**Figure 2.2.** Comparison between different methods for measurement of blood acetate.



We modified the extraction method of Remesy & Demigne, (1974). Remesey & Demigne, (1974) used an ethanolic extraction method for measuring plasma volatile fatty acids in rats. We modified this method for measurement of plasma acetate in human studies. In our method ether is used instead of ethanol because it is dried faster than ethanol and only 1 ml of plasma is needed for procedure.

### **2.2.2. Plasma acetate analysis**

2 ml of heparinized blood samples were centrifuged (2500 rpm for 10 minutes) immediately after collection and 1 ml of plasma was taken for the assay.

#### **2.2.2.1. Internal standard**

1.075 ml of  $\beta$ -methyl valeric acid (Sigma, Chemical Company Ltd., Poole, UK) was made up to 100 ml with distilled water. This was then brought to pH 7.0 with 0.34g of NaOH (Merck, Loughborough, UK). For this assay, the internal standard was diluted with distilled water (1 ml of distilled water was added to 1 ml of internal standard (V/V). This was done immediately before the assay to avoid deterioration.



#### **2.2.2.2. Procedure of plasma acetate assay**

10 µl of diluted internal standard (Sigma, Chemical Company Ltd., Poole, UK) and 100 µl of orthophosphoric acid (Merck, Loughborough, UK) and 3 ml of diethyl ether (Analar, Merck, Loughborough, UK) were added to the 1 ml of fresh plasma. The mixture was centrifuged at 2500 rpm for 5 minutes. The ether layer, at the top of the tube was removed by pastette and transferred to a clean tube. This extraction procedure with 3 ml of diethyl ether was repeated twice more. The extracted ether layers were pooled in a clean tube and then made alkaline with 200 µl of 1M-NaOH (Merck, Loughborough, UK) to produce the ionised form of the short chain fatty acid which would remain in the aqueous phase. The ether was evaporated at room temperature under N<sub>2</sub> (oxygen free) using a sample concentrator (Techne, Cambridge, UK). The small amount of aqueous fluid left was frozen at -70°C and then freeze-dried (Micro Modulyo; Edwards, Crawley, UK) overnight. The dry residue was redissolved in 100 µl of distilled water, acidified with 100 µl of orthophosphoric acid (Merck, Loughborough, UK), and extracted by 3 × 250 µl diethyl ether (Analar, Merck, Loughborough, UK). 3 µl of extraction was run on a gas liquid chromatograph (GLC).

#### **2.2.2.3. External standard**

0.954 ml of glacial acetic acid (BDH, Poole, UK) was made up to 100 ml with distilled water. The pH was adjusted to pH 7 with 1M-NaOH (Merck, Loughborough, UK). This standard was made freshly for each experiment to avoid any deterioration. Four sets of external standards were prepared as 10 µl, 20 µl, 50 µl and 100 µl and made up to 10 ml with distilled water then 1ml of each was taken and treated same as the samples mentioned in section 2.2.2.2 this chapter.

#### **2.2.2.4. GLC conditions**

Acetate was measured using a Pye Unicam 304 series GLC (Gas Liquid Chromatograph, Unicam Ltd, Cambridge), this was connected to a Phillips pm257 (Unicam Ltd, Cambridge) chart recorder. A glass packed column, 1.2m in length, external diameter was 60mm and internal diameter 4mm was used. The packing material was 10% SP1000 + H<sub>3</sub>PO<sub>4</sub> on Chromosorb WAW 80-100 mesh (Phase Separation Ltd, Deeside). Acetate was visualised with a flame ionisation detector.

Conditions were as follows:

Gas flow rates

carrier gas	nitrogen: 60 ml/min
flame gases	air: 30 ml/min

hydrogen: 20 PSI

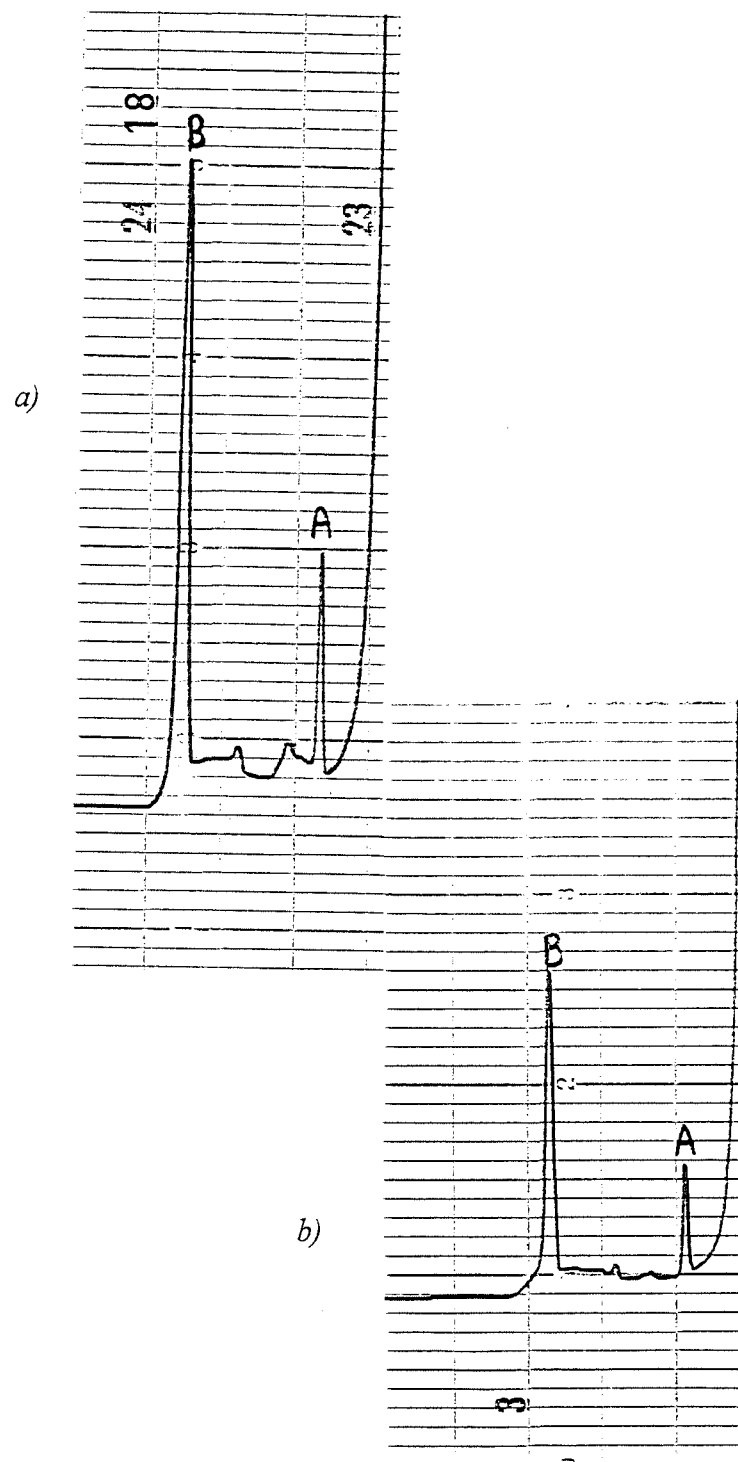
#### Temperature

column	200°C
injector	180°C
detector	200°C

#### Running conditions

lower temperature	125°C
upper temperature	150°C
start hold time	2 minutes
end hold time	1 minute
rate	10°C per minute

Samples produced from acidified ether extraction were run on the GLC. Peaks recorded on a chart recorder for standards, two peaks were recorded representing with the acetate in the external standard and internal standard. The first peak in the samples was acetate, the second the internal standard (**Figure 2.3**). The peaks which were produced by chromatograms were narrow. Peak height was measured and a ratio was obtained for acetate peak height /internal standard peak height. This enabled us to quantify the volume of acetate in plasma samples. A complete run under these running conditions was 10 minutes.



**Figure. 2.3.** Acetic acid peak analysed by G.L.C.

*a)* Plasma sample

*b)* External standard

A: Acetate peak

B: Internal standard peak

#### **2.2.2.5. Precision of plasma acetate assay**

The precision of our method was determined. The same sample was extracted on three occasions on the same day and the plasma acetate measured. The concentration of plasma acetate was (Mean  $\pm$  SEM)  $150.3 \pm 2.7 \mu\text{mol/l}$ .

#### **2.2.2.6. Recovery of plasma acetate**

To test the accuracy of the method, the recovery of acetate from samples spiked with a standard solution was measured.  $10 \mu\text{l}$  of freshly made external standard was added to 1 ml fresh plasma and the procedure was continued as described in plasma acetate assay, section 2.2.2.2. in this chapter.

Samples taken from one subject under both fasting condition and during experiments with lactulose ingestion (20g) were used for this purpose. Fasting sample showed 89% recovery of the spiked standard. Samples after lactulose ingestion showed 95.5% recovery of spiked standard (n=5).

#### **2.2.2.7. Stability of plasma samples**

The effect of freezing on plasma acetate concentration was determined. Tollinger et al., (1979) reported that storage of plasma at  $-20^{\circ}\text{C}$  for 24 hours significantly increased acetate concentration from  $26 \pm 6 (\mu\text{mol/l})$  to  $63 \pm 4 (\mu\text{mol/l})$ . We needed to assess the impact of freezing and storage on

our samples to optimise our assay method. Fasting plasma acetate concentration was measured in samples from 3 volunteers. Two sets of experiments were designed. In the first experiment, blood was collected from 3 subjects, immediately centrifuged (2500 rpm for 10 minutes) and the plasma removed. Plasma acetate was analysed (as described in section 2.2.2.2. in this chapter) in aliquots of this fresh plasma. Aliquots of plasma were stored at -20°C and -70°C for different periods of time. For measuring the concentration of acetate in frozen plasma, the plasma was thawed at room temperature and was processed as described in plasma acetate assay in this chapter (section 2.2.2.2). The results of this experiment are shown in **Table 2.1**.

A similar study was carried out but this time samples were stored at -70°C. The results of this experiment are shown in **Table 2.2**. Quite different values of plasma acetate concentration were obtained for one sample, when it was fresh and when it was frozen. This could be due to the length of storage or the freezing of plasma in freezer. Therefore, I used fresh plasma in all studies to eliminate this problem.

My results compare reasonably well with normal ranges quoted in the literature. My samples for fasting were  $80 \pm 12.6$  (n=4) for fasting compared

with published data 28-172  $\mu\text{mol/l}$  (Tangerman et al., 1983; Pomare et al., 1985). For fed samples my values were  $65 \pm 11.4$  compared with published data 44-189  $\mu\text{mol/l}$  (Tangerman et al., 1983; Scheppach et al., 1991*b*). For my values this was a blood sample taken 2 hours after the subjects had their normal lunch for the comparable published data this was after a standard breakfast.

**Table 2.1** The effect of storage at -20°C and -70°C on plasma acetate concentration (μmol/l)

Plasma acetate concentration (μmol/l)				
Samples	Fresh	-70°C (after 5 days)	-20 °C (after 10 days)	-70°C (after 10 days)
No.1	187	153	119	119
No.2	119	153	102	136
No.3	187	153	170	-

**Table 2.2** The effect of storage at -70°C on plasma acetate concentration (μmol/l)

Plasma acetate concentration (μmol/l)					
Samples	Fresh	after 1 day	after 2 days	after 3 days	after 5 days
No.1	187	136	90	119	-
No.2	170	119	136	-	110
No.3	204	111	119	-	170



### **2.3. Starch digestibility**

The physiological effect of dietary starch is mainly characterised by the rate and extent of its digestion and fermentation in the intestine (Cummings & Englyst, 1987*a*). Starch that resists digestion and becomes available for fermentation in the colon has different physiological effects than that of starch which is hydrolysed and absorbed in the small intestine. Characterisation of starchy foods is therefore related to their digestibility.

Dietary starch is divided into three main groups which have different physiological characteristics (Englyst et al., 1992). Rapidly digestible starch; RDS: this type of starch is rapidly and completely digested and absorbed in the small intestine of man. Slowly digestible starch; SDS: completely digested in the small intestine but at a slower rate. Resistant starch; RS: this type of starch escapes digestion in the small intestine and becomes available for fermentation in the colon. Many factors influence the digestion of starch which were explained in detail in chapter one section 1.4.

#### **2.3.1. Principle**

We had to measure the digestibility of the starches used in our test meals. For this the Englyst method (Englyst et al., 1992) was used. The various types of starch are quantified by controlled enzymic hydrolysis and measurement of the

released glucose using glucose oxidase. RDS and SDS are measured after incubation with pancreatic amylase and amyloglucosidase at 37°C. RDS is measured as the glucose released after 20 minutes and SDS as glucose released after a further 100 minutes incubation. RS is calculated as the starch not hydrolysed after 120 minutes incubation.

### **2.3.2. Measurement of rapidly digestible, slowly digestible and resistant starch fractions**

**Glucose standard 25 mg/ml** (BDH, Poole, UK): 5000 mg glucose was weighed then made up to 200 ml with sodium acetate buffer.

**Sodium acetate buffer 0.1M**, pH 5.2: 13.6g of sodium acetate trihydrate (BDH, Poole, UK) were dissolved in 250 ml saturated benzoic acid solution, (BDH, Poole, UK) and made to 1 litre with distilled water. This was adjusted to pH 5.2 with 0.1M acetic acid (BDH, Poole, UK) then 4 ml of 1M calcium chloride (BDH, Poole, UK) was added to 1 litre of buffer.

For preparation of **enzyme mixture 1**: 3.0g of pancreatin was weighed into 4 centrifuge tubes and a magnetic stirring bar added. Then into each tube 20 ml of distilled water was added. The solution was mixed well, stirred magnetically for 10 minutes, then centrifuged for 10 minutes at 3000 rpm. 13.5 ml of the cloudy layer were taken from each tube. Then into this solution

6 ml of amyloglucosidase solution 1 and 4 ml of invertase were added and mixed well.

**Amyloglucosidase solution 1:** 1.4 ml of amyloglucosidase was taken and was diluted to 4 ml with distilled water.

#### **2.3.2.1. The procedure of the assay**

50 mg of guar gum were weighed and 5 glass balls were added into three 50 ml centrifuge tubes. Into two of these tubes, 20 ml of glucose standard were added and into the third one (blank) 20 ml of acetate buffer were added. Then the tubes were shaken well.

2g of our test meal (raw potato starch biscuits), 800 mg raw potato starch; RPS and 800 mg of our pea flour were used in this assay. For each sample, 50 mg pepsin powder and 10 mls of 0.05 M hydrochloric acid were mixed well.

Then 10 ml of this solution was added to each sample, after vortex mixing, the tubes were put in a water bath at 37°C for 30 minutes. After the samples were removed from the water bath, 5 glass balls were added to each one. Then 10 ml of 0.25 M sodium acetate (34g of sodium acetate trihydrate,  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$  dissolved in 1 litre of distilled water) were added into each one. The tubes were shaken gently and placed in a water bath at 37°C to equilibrate. Sample tubes were removed from the water bath, one at time, immediately

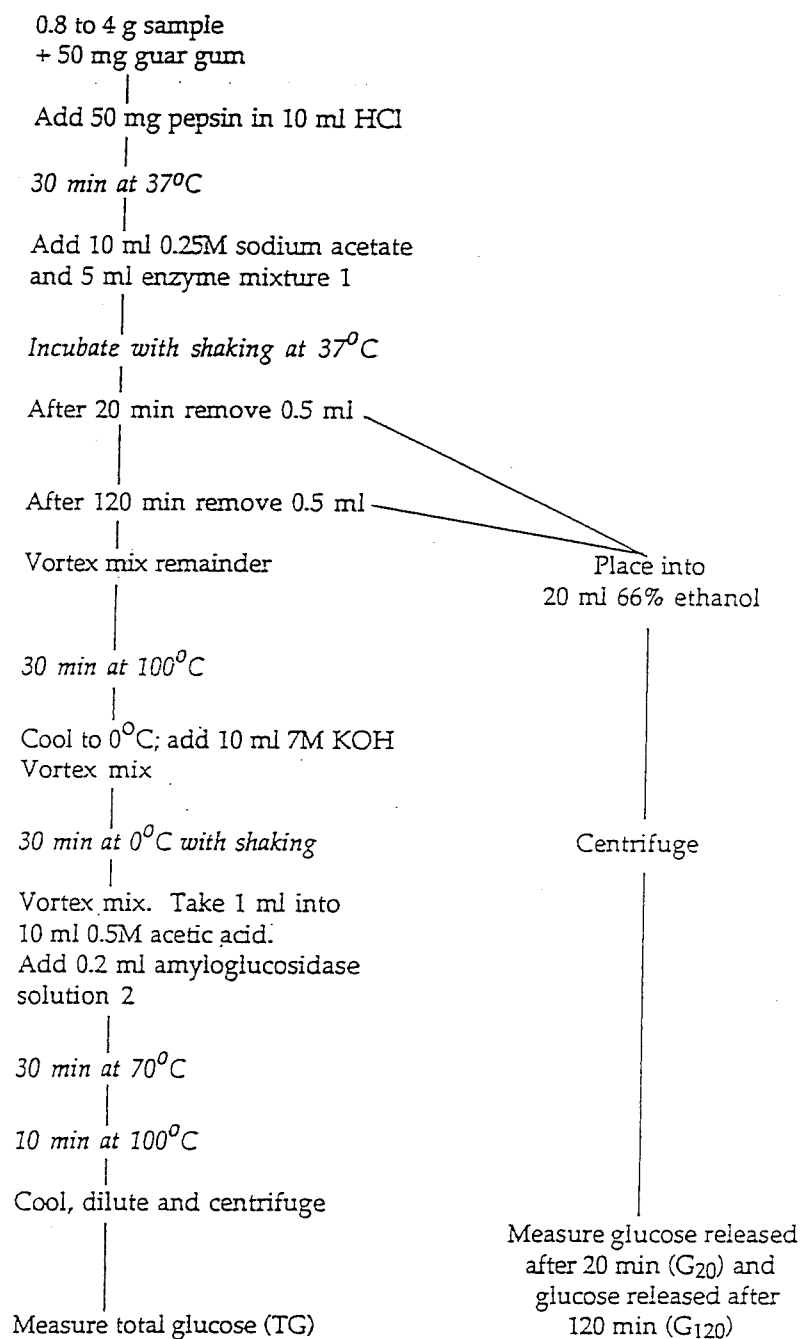
after 5 ml of enzyme mixture 1 was added to each one. Tubes were then placed horizontally in a shaking water bath at 37°C (160 strokes/ minute; stroke length 35 mm). After 20 minutes, 0.5 ml sample was taken from each tube into a labelled tube containing 20 ml 66% ethanol, (for measurement of the amount of glucose released after 20 minutes:  $G_{20}$ ). The incubation tubes were immediately replaced in the shaking water bath and after a further 100 minutes, a second 0.5 ml sample was taken in the same way. Then the  $G_{20}$  and  $G_{120}$  portions were centrifuged at low speed for 5 minutes.

The tubes containing the remainder of the sample mixture were removed from the shaking water bath and were put in a boiling water bath for 30 minutes. After vortex mixing, the tubes were cooled in ice. 10 ml of 7M potassium hydroxide (BDH, Poole, UK) were added and the tubes mixed well. The tubes were then placed horizontally for 30 minutes in the shaking ice water bath. A third set of centrifuge tubes were prepared in advance containing 10 ml of 0.5 M acetic acid (BDH, Poole, UK). After the the samples were removed from the shaking water bath, 1 ml of the contents was added into the 10 ml of acetic acid and mixed well. Then 0.2 ml of amyloglucosidase solution 2 (0.5 ml amyloglucosidase was taken and was diluted to 4 ml with distilled water), were added to each sample and mixed well. Tubes were put in a water bath at 70°C for 30 min, and then removed to a boiling water bath for 10 minutes. After the tubes were removed from boiling water bath, they were cooled at

room temperature, 40 ml of water was added into each one, and mixed well.

The tubes were centrifuged for 5 minutes at 3000 rpm; these were ready for measuring the total glucose: TG.

All of the stages for analysing of total, rapidly digestible, slowly digestible and resistant starch fractions are shown in **Figure 2.4**.



**Figure 2.4.** Procedure of analysis of total, rapidly digestible, slowly digestible and resistant starch fractions (Englyst et al., 1992).

#### 2.3.2.2. Determination of glucose

Glucose was measured by GOD-PAP kit(diagnostic kit, BDH, Poole, UK) based on the glucose oxidase reaction. 100 µl of distilled water, samples and standards in duplicate were added into labelled test tubes. 2 ml of GOD-PAP reagent were added to each tube and mixed well. Then the tubes were put into a water bath at 37°C for 20 minutes. The absorbance of the standards and samples was measured by spectrophotometer (ATI unicam 86/20 spectrometer) at 510 nm. Glucose in g/100 g sample was given by the following equation:

$$\% \text{ glucose} = \frac{A_t \times V_t \times C \times D \times 100}{A_s \times W_t}$$

where             $A_t$  is absorbance of the test solution  
                     $V_t$  is the total volume of the test solution  
                     $C$  is the concentration (in mg glucose/ml) of the standard used  
                     $A_s$  is the absorbance of the standard used  
                     $W_t$  is the weight (in mg) of sample taken for analysis  
                     $D$  is a dilution factor (usually 1)

#### 2.3.2.3. Calculation

The values obtained for  $G_{20}$  and  $G_{120}$  and TG were used to achieved values for RDS, SDS and RS.

$$RDS = G_{20} \times 0.9$$

$$SDS = (G_{120} - G_{20}) \times 0.9$$

$$RS = (TG - G_{120}) \times 0.9$$

where

TG: total glucose

$G_{20}$ : glucose measured in sample after 20 minutes

$G_{120}$ : glucose measured in sample after 120 minutes



## **2.4. Development and validation of test meals**

### **2.4.1. Test meals in plasma acetate study**

#### **2.4.1.1. Raw potato starch biscuits**

To investigate the effects of resistant starch on plasma acetate concentration and breath hydrogen response in man, raw potato starch; RS<sub>2</sub> was tested incorporated into biscuits as test meal. The recipe is shown below.

This recipe provided 911 Kcal.

#### **Recipe:**

Raw potato starch flour	100 g (Roquette, UK Ltd, Tunbridge Wells)
Sugar	28 g
Margarine	60 g
egg	1
Water	1 dessert spoonful (tap water)
Vanilla flavour	1/4 teaspoon

The biscuits were cooked by microwave oven; 1350 watt using a combination of oven and grill at level 7 for 3 minutes one side and for 1 minute the other side. The amount of rapidly digestible starch; RDS, slowly digestible starch; SDS and resistant starch RS in each test meal was measured by the Englyst method (Englyst et al., 1992) (explained in this chapter, section 2.3.2). The biscuits contained 38g/100g RDS, 2.8g/100g SDS and 34.4g/100g RS. The amount of RS in the original raw potato starch; RPS was 58g/100g.

Therefore, a large amount of resistant starch was preserved in our test biscuits.

#### **2.4.1.2. Guar gum biscuits**

In our research plan, guar gum, a readily fermentable fibre, was used as control. Thus, in the biscuits recipe, raw potato starch; RPS was replaced with low molecular weight guar gum (M30, Meyhall, Kreuzlingen, Netherlands) and wheat flour. The recipe is shown below.

This recipe provided 726 Kcal.

#### **Recipe:**

Guar gum	12 g
Wheat flour	65 g
Sugar	28 g
Margarine	40 g
Egg	1
Water	200 ml (tap water)
Vanilla flavour	1/4 teaspoon

The biscuits were cooked by microwave oven; 1350 watt was used at level 7 for 17 minutes one side and 8 minutes the other side.

## 2.4.2. Test meals in $^{13}\text{C}$ study

### 24.2.1. $^{13}\text{C}$ pea flour biscuits

To study the small intestine digestion and colonic fermentation of carbohydrate, enriched pea flour with  $^{13}\text{C}$  was incorporated into the biscuits.

The recipe is shown below.

This recipe provided 466 Kcal.

#### **Recipe:**

$^{13}\text{C}$ pea flour	300 mg
Wheat flour	55 g
Sugar *	14 g
Margarine	20 g
Egg	1
Water	1 dessert spoonful (tap water)
Vanilla flavour	1/4 teaspoon

\* : beet sugar used in this recipe, because cane sugar is naturally enriched with  $^{13}\text{C}$  (Schoeller et al., 1980).

The biscuits were cooked by microwave oven; 1350 watt using a combination of oven and grill at level 7 for 3 minutes one side and for 1 minute the other side.

The amount of RDS, SDS and RS in pea flour was measured by the Englyst

method (Englyst et al., 1992; explained in this chapter, section 2.3.2). The pea flour contained 14.4% RDS, 63.7% SDS and 21.9% RS.

#### **2.4.2.2. $^{13}\text{C}$ lactose-ureide biscuits**

In further studies of colonic fermentation,  $^{13}\text{C}$  lactose-ureide was used as a test ingredient in biscuits. The  $^{13}\text{C}$  pea flour was therefore replaced with non-labelled pea flour and  $^{13}\text{C}$  lactose-ureide.  $^{13}\text{C}$  lactose-ureide was a gift from Douglas Morrison (Bell College of Technology, Hamilton). The recipe is shown below. The biscuits were cooked by microwave oven; 1350 watt using a combination of oven and grill at level 7 for 3 minutes one side and for 1 minute the other side.

This recipe provided 466 Kcal.

#### **Recipe:**

$^{13}\text{C}$ lactose-ureide	500 mg
Unlabelled pea flour	300 mg
Wheat flour	55 g
Sugar *	14 g
Margarine	20 g
Egg	1
Water	1 dessert spoonful (tap water)
Vanilla flavour	1/4 teaspoon

\* : beet sugar used in this recipe, because cane sugar is naturally enriched with  $^{13}\text{C}$  (Schoeller et al., 1980).

## 2.5. Measurement of $^{13}\text{C}$

### 2.5.1. $^{13}\text{CO}_2$ breath test

To investigate the small intestinal digestion and colonic fermentation of carbohydrate,  $^{13}\text{CO}_2$  breath test was used. Breath samples, before and after consumption of the test meal (explained in this chapter, section 2.4.2) were collected at 30 minutes intervals for up to 30 hours. The subjects blew into a plastic mask (Laerdal face masks, Ambu bags) (**Figure 2.5**). The breath sample was transferred to a vacutainer (Labco, High Wycombe, UK) with a 20 ml plastic syringe. Breath  $^{13}\text{CO}_2$  in vacutainers is stable for two months.

$^{13}\text{CO}_2$  excretion in breath was determined by continuous-flow isotope ratio mass spectrometry (CF-IRMS) (Europa Scientific Crewe) in collaboration with Scottish Universities Research and Reactor Centre, East Kilbride (Preston & McMillan, 1988).

There is a natural background of  $^{13}\text{CO}_2$ , therefore, the labelled  $\text{CO}_2$  is actually the amount of  $^{13}\text{CO}_2$  in excess of the  $^{13}\text{CO}_2$  abundance before administration of the labelled substrate. The test specified the difference between the initial abundance of  $^{13}\text{C}$  and that after ingestion of labelled substrate. Ratio of mass 45 to mass 44 is measured to determine the enrichment of  $^{13}\text{C}$  relative to  $^{12}\text{C}$ .



**Figure 2.5.** Demonstration of collecting breath  $^{13}\text{CO}_2$  in a plastic mask.

In this thesis the results of  $^{13}\text{C}$  studies are expressed as abundance in units of atom%. This is a measurement of the number of atoms of the isotope in 100 atoms of the element. The unit of  $^{13}\text{C}$  enrichment, atom % excess, is determined by subtracting the natural abundance from measured  $^{13}\text{C}$  enrichment. At low enrichment, parts per million (ppm) and ppm excess can be used.

$$\text{Atom\% } ^{13}\text{C} = \frac{^{13}\text{C}}{^{13}\text{C} + ^{12}\text{C}} \times 100$$

### 2.5.2. $^{13}\text{C}$ enrichment in potato and pea flour

Samples (1.00-1.50 mg) of labelled pea and potato flour (which will be explained in detail in chapter 4) were weighed into tin capsules (8×5 mm, Elemental Microanalysis, Okehampton, Devon, UK) by a 5 figure balance (Mettler A7201, Greifensee, Switzerland). The value of  $^{13}\text{C}$  enrichment in pea and potato flour was determined by continuous-flow isotope- ratio mass spectrometer (CF-IRMS) (Europa Scientific Crewe) in collaboration with Scottish Universities Research and Reactor Centre, East Kilbride (Preston & McMillan, 1988).

In this thesis the amount of  $^{13}\text{C}$  enrichment in labelled pea and potato flour is expressed in units of atom %  $^{13}\text{C}$  excess.

$$\text{Atom \% excess} = \text{sample atom \%} - \text{background atom \%}$$

$$\text{ppm excess} = \text{atom \% excess} \times 10000$$

## CHAPTER THREE

*In vivo* fermentation of resistant starch by  
measurement of plasma acetate and breath hydrogen test



### 3.1. Introduction

Resistant starch is not digested and absorbed in the small intestine and is available for fermentation in the colon by anaerobic bacteria to short chain fatty acids (SCFA) including acetate, propionate and butyrate, and gases such as hydrogen methane and CO<sub>2</sub> (Cummings & Englyst, 1987*a*). Hydrogen produced during fermentation is absorbed into the circulation and excreted in breath. Of the SCFA, acetate is the only one that passes in to peripheral circulation and is the major SCFA produced during fermentation. Although acetate is also produced by the liver during fasting (Scheppach et al., 1991*b*), increases in plasma acetate in fed state result mainly from fermentation (Pomare et al., 1985).

Fermentation in the colon can be assessed indirectly by several methods (refer to chapter 1 and 2). Previous studies of *in vivo* fermentation of resistant starch (Scheppach et al., 1991*a*; Segal et al., 1993; Olesen et al., 1994) relied mainly on measuring breath hydrogen. Breath hydrogen was proposed as a quantitative measure of fermentation by Bond & Levitt, (1972). However, although breath hydrogen increases in a dose dependent way for rapidly fermentable substrates such as lactulose, increases are not clearly related to slowly fermented substrates, such as wheat bran (Levitt et al., 1987) and resistant starch (Olesen et al., 1994). This may be due to several potential routes for hydrogen disposal in the human

colon (methane, H<sub>2</sub>S, or acetate; Gibson et al., 1990) but may also be related to the speed of transit and rate of delivery of the substrate to the colon, the time taken for fermentation and the site of fermentation in the colon. More methane is produced in the distal colon (Gibson et al., 1990). In addition the amount of hydrogen produced from the same substrate may change with time. Florent et al (1985) showed that ingestion of lactulose for a week reduced the amount of hydrogen produced although fermentation efficiency actually increased. This makes breath hydrogen alone a poor marker for measurement of the fermentation of resistant starch.

Oleson et al., (1994) has shown that breath hydrogen does not rise after raw potato starch until at least 9-11 hours. This is much longer than the time taken for a rise in breath hydrogen after dietary fibre. However, it is not clear whether this delay is due to a slower transit or some characteristic of breath hydrogen production during resistant starch fermentation.

Plasma acetate is an alternative method for assessing fermentation. Previous researchers (Pomare et al., 1985) have used acetate to monitor the fermentation of dietary fibre and a few studies have noted a general increase in blood acetate after resistant starch fermentation (Muir et al., 1995*b*). However, no study has yet

assayed serial plasma samples for acetate after resistant starch ingestion and related this to breath hydrogen.

In this thesis , *in vivo* fermentation of resistant starch was therefore determined by combination of measurements of breath hydrogen and plasma acetate. This combination should provide more robust measurements of resistant starch fermentation than either alone.

The following studies describe the validation of a new method for measuring RS fermentation assaying plasma acetate using guar gum as a standard, the plasma acetate increases seen after ingestion of raw potato starch and an experiment to test the hypothesis that ingestion of raw potato starch for one week increases the fermentation capacity of individuals and shortens the time taken for breath hydrogen and plasma acetate to rise.

### **3.2. Breath H<sub>2</sub> and plasma acetate after lactulose**

In one subject the relationship between breath hydrogen and plasma acetate was assessed after ingestion of lactulose to identify the appropriate standard for studies with resistant starch. Lactulose is a non-absorbable disaccharide.

Subject 1 (female aged 37 years) was a nonsmoker and had not taken antibiotics for at least 2 months. She was asked to consume a diet low in fibre and resistant starch and fasted from 9pm the night before the study to 9am on the morning of study day. Then 20g lactulose were ingested as a drink. Measurement of breath hydrogen (explained in chapter 2, section 2.1) was performed every 30 min during the experimental day and blood samples were taken for plasma acetate (explained in chapter 2, section 2.2) at intervals of 0.5-2 hours (h) related to the expected rise of acetate, more frequent samples being taken when the rise was expected (**Figure 3.1**). At 1pm, lunch was eaten which consisted of rice (165g) and chicken (one = 617 Kcal. portion: 180g). This meal was chosen as it was low in fibre, RS and fat and in previous studies had been shown to have no effect on breath hydrogen (Dr Christine Edwards personal communication). The study lasted for 6 hours after the ingestion of lactulose. A rise in breath hydrogen was seen at 0.5-1 h and the rise in acetate was evident at 1 hour showing good agreement between the two indices of colonic fermentation. However, this short transit time and rapid fermentation was not at all comparable with the characteristics of fibre or resistant starch fermentation. The use of lactulose as the standard carbohydrate for further studies was therefore rejected.

### **3.3. Guar gum study**

In this study, the use of the fermentable dietary fibre guar gum as a standard for the plasma acetate and breath hydrogen response was explored.

#### **3.3.1. Study design**

Five healthy subjects participated in this study (two females and three males; aged 25-58 years) from the Department of Human Nutrition and Biochemistry at University of Glasgow after informed written consent. All subjects were non-smokers and had not taken antibiotics for at least 2 months. Ethical approval for the work was obtained from the Glasgow University Ethics Committee.

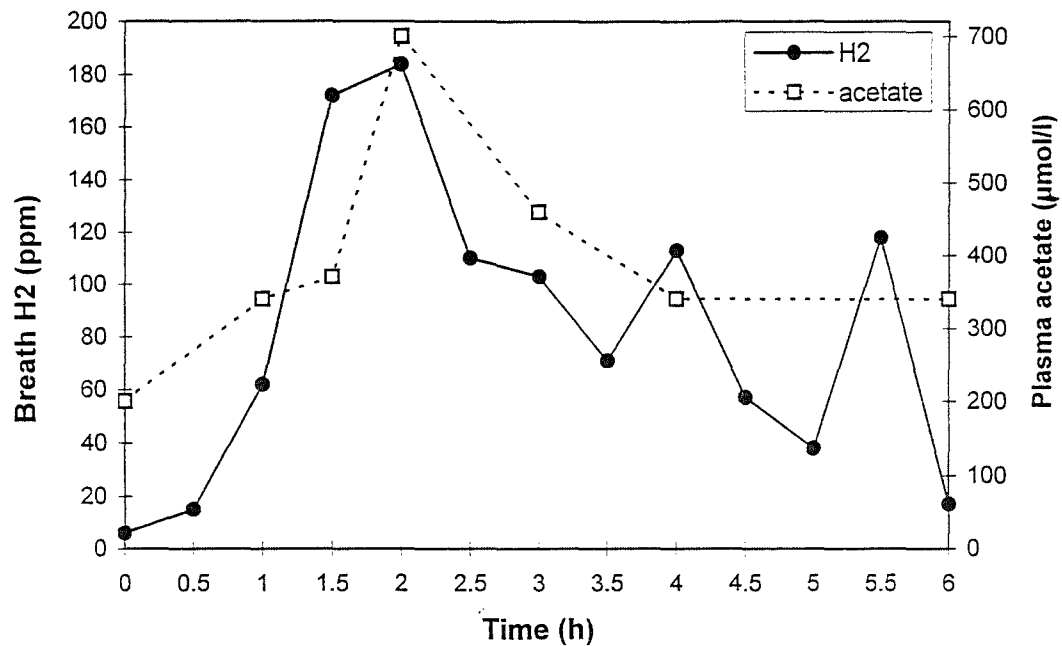
Subjects were asked to consume a diet low in fibre and resistant starch the night before the study and fasted from 9pm the night before to 9am on the morning of study day. Then 12 g guar gum incorporated into biscuits (explained in chapter 2, section 2.4.1) were ingested. Measurement of breath hydrogen (explained in chapter 2, section 2.1) was performed every 30 min during the experimental day and blood samples were taken for plasma acetate (explained in chapter 2, section 2.2) at intervals of 1-3 hours depending on the subject and the expected rise of acetate. More frequent samples being taken when the rise was expected (**Figures 3.2-3.6**). At 1pm the low fat, low fibre lunch was eaten (see above). The study

lasted for 7 hours after the test meal. The assays for breath hydrogen and plasma acetate are described in detail in chapter 2. All data are presented as mean  $\pm$  standard error of mean. Differences in all parameters were assessed by Student's paired *t* test.

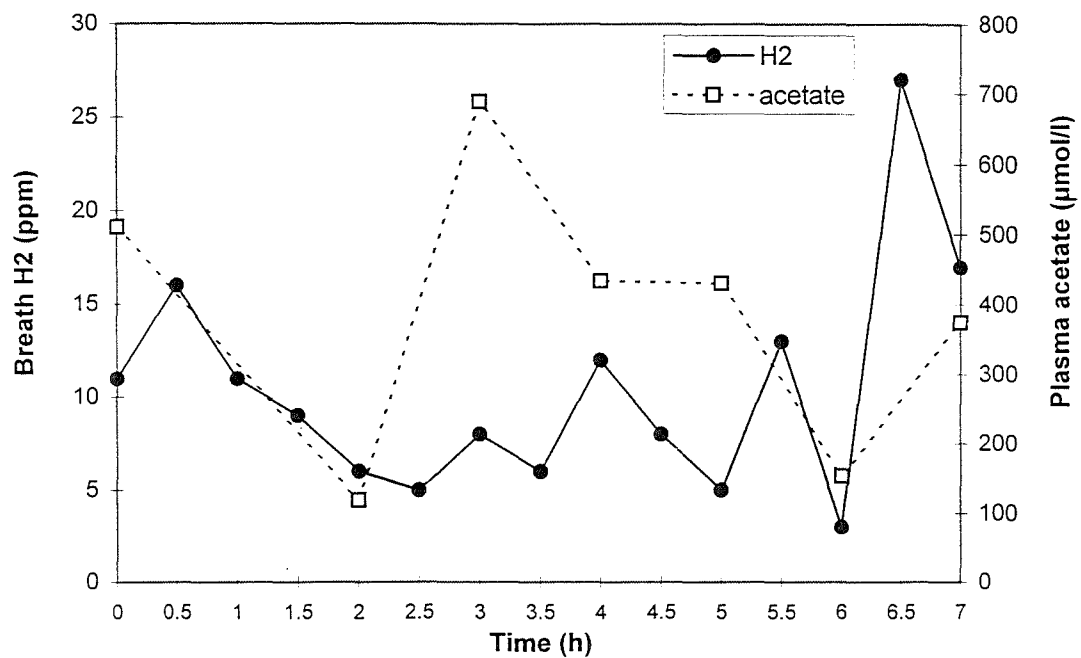
### 3.3.2. Results

Individual curves for breath hydrogen and plasma acetate are shown (**Figures 3.2-3.6**). There was much variation in the breath hydrogen and plasma acetate responses. The rise in plasma acetate did not always correspond with the rise in breath H<sub>2</sub>. Although in 4 of the 5 subjects the acetate rose earlier than the hydrogen, in subject 2 the rise in hydrogen occurred 1.5 hours before the acetate rise (**Table 3.1**). Moreover, the rise in these parameters was not related to the relative times for the acetate and hydrogen peaks. In 3 subjects the acetate peak occurred before the hydrogen peak by upto 3.5 hours and in 1 subject the acetate peak occurred after the hydrogen peak by upto 2 hours and in 1 subject both peak occurred at the same time (**Table 3.1**). The apparent mean transit time (TT) using breath hydrogen for all subjects was  $4.7 \pm 0.5$  h (mean  $\pm$  SEM), whereas using plasma acetate TT was  $3.4 \pm 0.7$  h (mean  $\pm$  SEM). The mean peak for breath hydrogen in all subjects was at  $6.5 \pm 0.4$  h (mean  $\pm$  SEM), whereas the peak of plasma acetate occurred at  $5.2 \pm 0.8$  h (mean  $\pm$  SEM).

This suggests that acetate may be a better marker of fermentation than hydrogen as in most cases it rises first but given such variation between the two measurements, it would be wise to consider both in future studies.



**Figure 3.1.** Breath H<sub>2</sub> and plasma acetate after ingestion of 20g lactulose in subject 1



**Figure 3.2.** Breath H<sub>2</sub> and plasma acetate after ingestion of 12g guar gum in subject 1

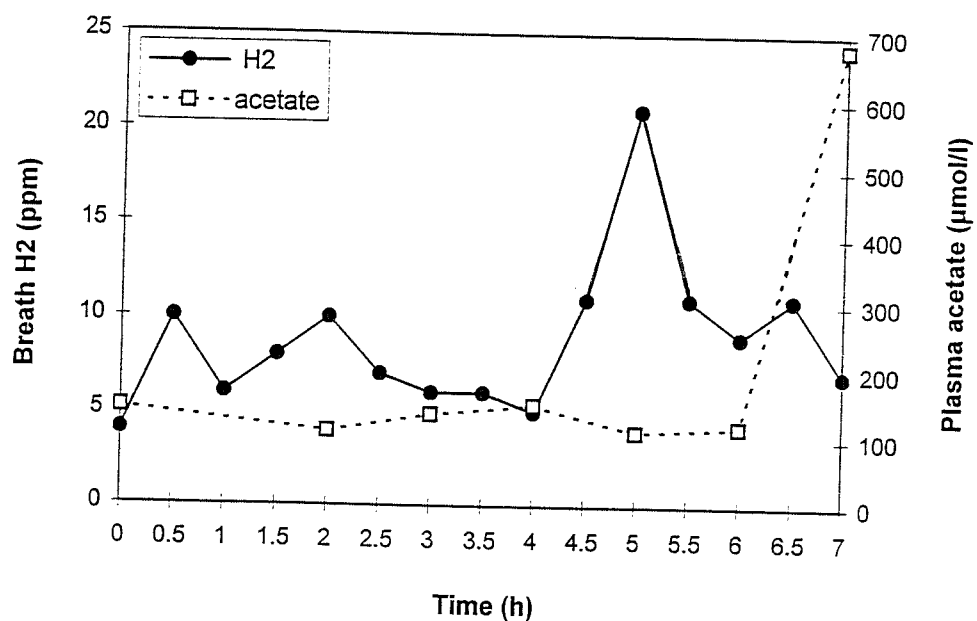


Figure 3.3. Breath H<sub>2</sub> and plasma acetate after ingestion of 12g guar gum in subject 2

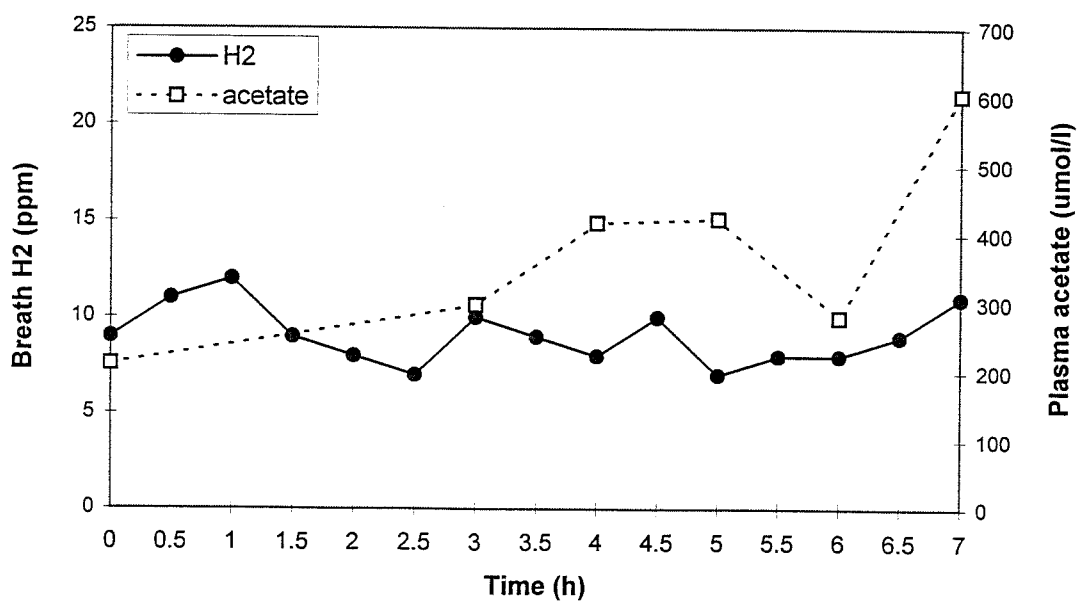


Figure 3.4. Breath H<sub>2</sub> and plasma acetate after ingestion of 12g guar gum in subject 3



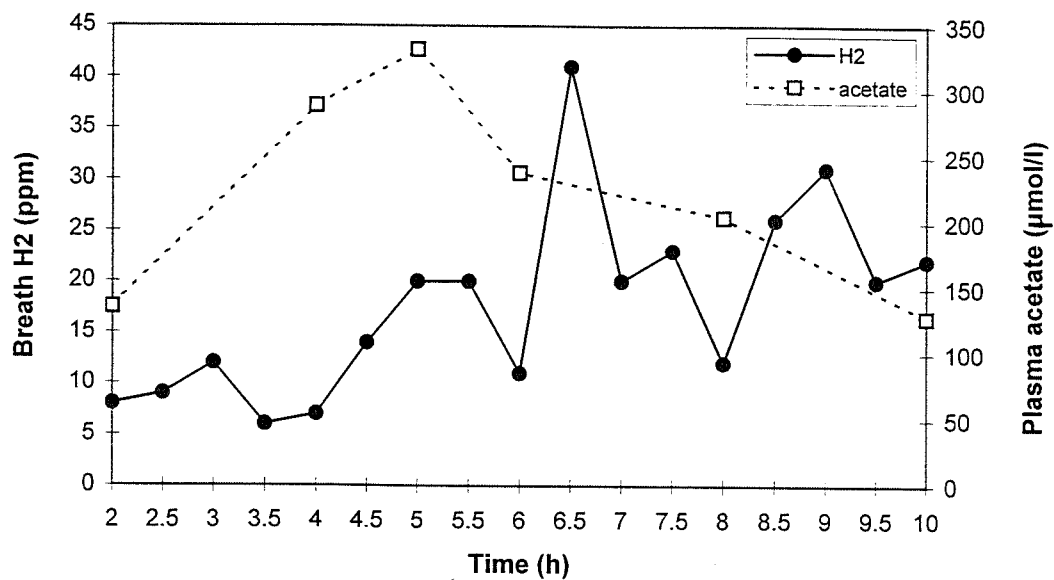


Figure 3.5. Breath H<sub>2</sub> and plasma acetate after ingestion of 12g guar gum in subject 4

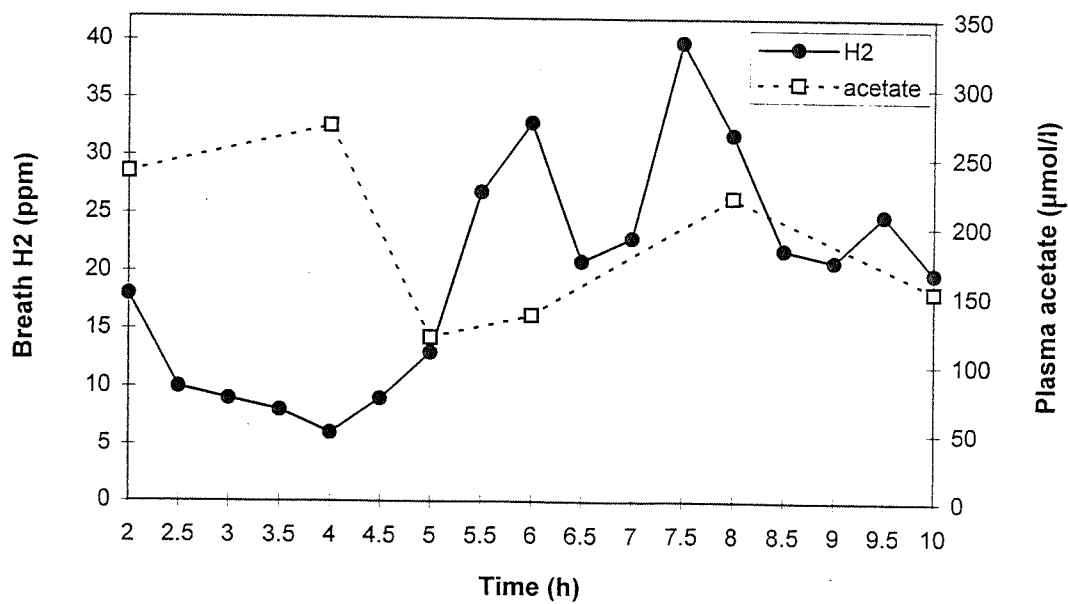


Figure 3.6. Breath H<sub>2</sub> and plasma acetate after ingestion of 12g guar gum in subject 5

**Table 3.1.** Plasma acetate ( $\mu\text{mol/l}$ ) and breath hydrogen (ppm) after ingestion of 12g guar gum.

Subjects	Transit time H <sub>2</sub> (h)	Transit time acetate (h)	AUC* H <sub>2</sub> (ppm . h)	AUC* acetate ( $\mu\text{mol/l}$ . h)	H <sub>2</sub> peak (ppm)	Acetate peak ( $\mu\text{mol/l}$ )	H <sub>2</sub> peak (h)	Acetate peak (h)
1	3.5	2.0	24.0	1287.0	27.0	689.0	6.5	3.0
2	4.5	6.0	20.0	838.0	21.0	680.0	5.0	7.0
3	6.5	3.0	um <sup>φ</sup>	766.0	11.0	603.0	7.0	7.0
4	4.5	4.0	94.0	825.0	41.0	332.0	6.5	5.0
5	4.5	2.0	107.0	618.0	40.0	272.0	7.5	4.0
Mean*	4.7 ± 0.5	3.4 ± 0.7	61.3 ± 20.4	866.8 ± 112.0	28.0 ± 5.7	515.2 ± 88.8	6.5 ± 0.4	5.2 ± 0.8

\* area under the curve

<sup>φ</sup> unmeasurable

\* mean ± SEM

### **3.4. Fermentation of resistant starch**

Previous studies have shown that resistant starch fermentation is slow with breath hydrogen rising 9-11 hours after ingestion of the starch (Oleson et al., 1994). This late rise in hydrogen makes the practical aspects of studying fermentation difficult because the study must be very long and the compliance of subjects is likely to be a problem. In these studies, we first investigated the best timing for the experiment; whether the meal should be ingested in the morning, with a long study day, or whether we could feed the meal the night before and collect samples from the morning of the following day and so include only the later part of the transit of the meal through the intestine.

In these studies, subjects were asked to consume 100 g raw potato starch; RS<sub>2</sub> incorporated into biscuits; 34.4% RS. Then blood and breath were sampled for 0-10 hours (study in the morning) and 10-18 hours (study with meal eaten the night before) post-ingestion.

#### **3.4.1. Study in the morning**

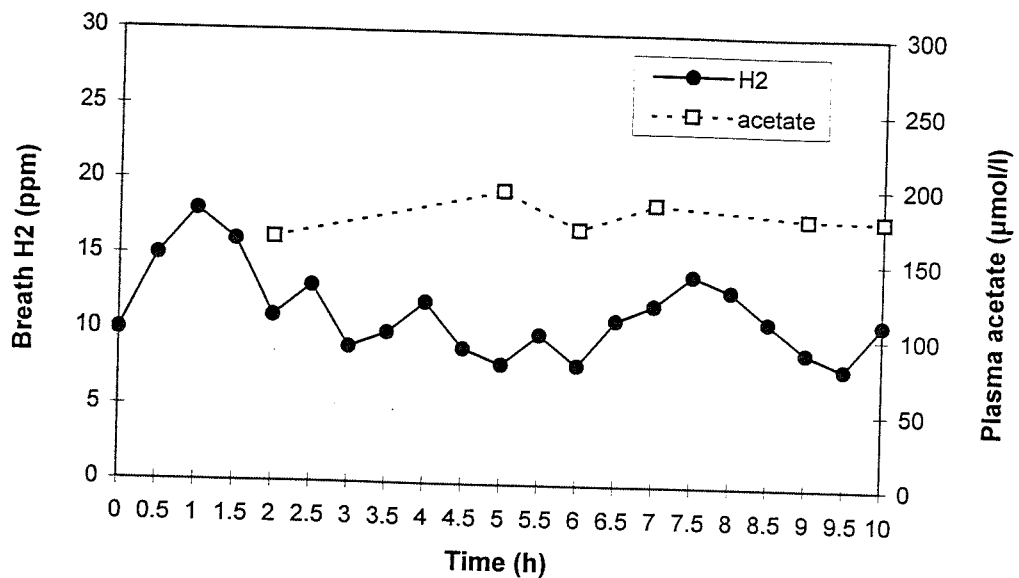
##### **3.4.1.1. Study design**

This study was carried out in the same subject as the lactulose study. Again, the subject was asked to consume a diet low in fibre and resistant starch the night

before the study and fasted from 9pm to 7am on the morning of study day. 100g raw potato starch; RS<sub>2</sub>, 34.4% RS, incorporated into biscuits (explained in chapter 2, section 2.4.1) were then ingested. The first breath H<sub>2</sub> samples were taken by subject at home. The subject was asked to attend at the Department of Human Nutrition at 9am. Measurement of breath hydrogen (explained in chapter 2, section 2.1) was performed every 30 minutes during the experiment day and blood samples were taken for plasma acetate (explained in chapter 2, section 2.2) at intervals of 1-3 hours as before (**Figure 3.7**). At 1pm the standard lunch was eaten (see above). The study lasted for 10 hours after the test meal. All data are presented as mean  $\pm$  standard error of mean. Differences in all parameters were assessed by Student's paired *t* test.

#### **3.4.1.2. Results**

No sustained rise in breath H<sub>2</sub> (ppm) and plasma acetate concentration ( $\mu\text{mol/l}$ ) was seen during the 10 hours after ingestion of raw potato starch biscuits (**Figure 3.7**). Thus, the study design needed to be changed to provide enough time for the increase in breath hydrogen and plasma acetate to be demonstrated after ingestion of raw potato starch biscuits. To achieve this we decided to feed the test meal the night before the study day and thus measure fermentation the following morning.



**Figure 3.7.** Breath H<sub>2</sub> and plasma acetate after ingestion of 100g raw potato starch in subject 1

### 3.4.2. Study with meal eaten the night before

#### 3.4.2.1. Study design

The same five healthy subjects participated in this study. Again, subjects were asked to consume a diet low in fibre and resistant starch the day before the study and an evening meal at 4pm was prepared at Department of Human Nutrition the night before the experiment day. This meal was low in fibre and resistant starch and consisted of: fish 156g with mornay sauce (milk powder, butter, cheddar cheese, salt and pepper), rice 125g, margarine 10g, white bread 2 slices,

= 1001 Kcal.

ice cream 33g and one small carton of orange juice 130g. Then subjects fasted until 11pm that night. At 11pm, 100 g raw potato starch incorporated into biscuits (explained in chapter 2, section 2.4.1) was consumed. The next meal was eaten at 1pm the next day as lunch (chicken 180g and rice 165g as in previous studies see above). Subjects were asked to attend at the Department of Human Nutrition at 9am. Measurement of breath hydrogen (explained in chapter 2, section 2.1) were performed every 30 minutes during the experimental day and blood samples were taken for plasma acetate (explained in chapter 2, section 2.2) after 10 hours ingestion of test meal at intervals 1-2 hours depending on the subjects and the expected of rise of acetate. More frequent samples being taken when the rise was expected. The study lasted for 18 hours after the test meal. All data are presented as mean  $\pm$  standard error of mean. Differences in all parameters were assessed by Student's paired *t* test.

#### **3.4.2.2. Results**

In two cases the time for the rise in acetate or hydrogen obviously occurred before the first sample. This was therefore given a nominal value of 9.9 hours. This reduced the validity of the results but did allow changes in TT to be identified. The apparent mean transit time (TT) using breath hydrogen for all subjects was  $11.9 \pm 1.0$  h (mean  $\pm$  SEM), whereas using plasma acetate TT was  $11.3 \pm 0.7$  h

(mean  $\pm$  SEM). The mean peak for breath hydrogen in all subjects was at  $14.0 \pm 1.2$  h (mean  $\pm$  SEM), whereas the peak of plasma acetate occurred at  $13.4 \pm 0.9$  h (mean  $\pm$  SEM) (**Table 3.2**).

Again, the rise in plasma acetate did not always correspond with the rise in breath  $H_2$ . Although in 1 subject the acetate and breath hydrogen rose at the same time, in 3 of the five subjects the acetate rose earlier than the hydrogen, and in subject 1 the rise in hydrogen occurred before the acetate rise (**Table 3.2**). However, this relationship was not related to the relative times for the acetate and hydrogen peaks. In 3 subjects the acetate peak occurred before the hydrogen peak by upto 4 hours and in 1 subject the acetate peak occurred after the hydrogen peak by upto 3.5 hours and in 1 subject both peaks occurred at the same time (**Table 3.2**).

The results showed that apparent mean transit time (TT) using breath hydrogen for all subjects after ingestion of 100g raw potato starch, was significantly longer than after ingestion of 12g guar gum:  $11.9 \pm 1.0$  h,  $4.7 \pm 0.5$  h (mean  $\pm$  SEM) ( $P=0.0012$ ), respectively. Apparent mean transit time (TT) using plasma acetate after ingestion of 100g raw potato starch was also significantly longer than after ingestion of 12g guar gum:  $11.3 \pm 0.7$  h,  $3.4 \pm 0.7$  h (mean  $\pm$  SEM) ( $P = 0.003$ ),

respectively. The mean time for peak in breath hydrogen in all subjects after ingestion of 12g guar gum was significantly earlier than after ingestion of 100g raw potato starch  $6.5 \pm 0.4$  h,  $14.0 \pm 1.2$  h (mean  $\pm$  SEM) ( $P = 0.0018$ ), respectively. The mean peak for plasma acetate in all subjects after ingestion of 12g guar gum was significantly earlier than after ingestion of 100g raw potato starch  $5.2 \pm 0.8$  h,  $13.4 \pm 0.9$  h (mean  $\pm$  SEM) ( $P = 0.0005$ ), respectively.



**Table 3.2.** Plasma acetate ( $\mu\text{mol/l}$ ) and breath hydrogen (ppm) after ingestion of 100g raw potato starch in study with meal eaten the night before

Subjects	Transit time H <sub>2</sub> (h)	Transit time acetate (h)	AUC* H <sub>2</sub> (ppm . h)	AUC* acetate ( $\mu\text{mol/l}$ . h)	H <sub>2</sub> peak (ppm)	Acetate peak ( $\mu\text{mol/l}$ )	H <sub>2</sub> peak (h)	Acetate peak (h)
1	9.9	13.0	29.0	1083.0	17.0	578.0	14.0	14.0
2	10.0	9.9	32.0	525.0	14.0	289.0	10.5	14.0
3	13.0	9.9	36.0	1255.0	26.0	600.0	17.0	16.0
4	14.5	11.0	55.0	397.0	42.0	247.0	16.0	12.0
5	10.0	10.0	57.0	507.0	24.0	272.0	12.5	11.0
Mean*	11.9 $\pm$ 1.0	11.3 $\pm$ 0.7	41.8 $\pm$ 5.9	753.4 $\pm$ 173.2	24.6 $\pm$ 4.9	397.2 $\pm$ 78.7	14.0 $\pm$ 1.2	13.4 $\pm$ 0.9

\* mean  $\pm$  SEM

\* area under the curve

### **3.5. Adaptation study**

#### **3.5.1. Study design**

This study was planned to determine whether consumption of 100g raw potato starch biscuits; RS<sub>2</sub>, 34.4%: RS over one week had any effect on the digestibility and fermentability of raw potato starch in the gut. Five healthy subjects who participated in previous study took part in this study; two females and three males aged 25-58. Subjects were asked to consume 100g raw potato starch incorporated into biscuits (explained in chapter 2, section 2.4.1) every day for one week. The last test biscuits were eaten 10 hours prior to the study day. One day before the test, subjects were asked to consume a diet low in fibre and resistant starch and an evening meal (as above) at 4pm was prepared at Department of Human Nutrition the night before the experiment day. Then subjects fasted to 11pm that night. As in the previous study at 11pm, 100g raw potato starch incorporated into biscuits (explained in chapter 2, section 2.4.1) were consumed. Subjects then fasted until lunch next day. Subjects were asked to attend at the Department of Human Nutrition at 9am. Measurement of breath hydrogen and plasma acetate (explained in chapter 2, section 2.1) were performed every 30 minutes during the experimental day and blood samples were taken for plasma acetate (explained in chapter 2, section 2.2) after 10 hours ingestion of test meal at intervals 1-2 hours depending on the subjects and the expected of rise of acetate.

At 1pm, subjects ate the standard lunch. The study lasted for 18 hours after the test meal. As this was the same subjects as in the previous study the subjects acted as their own controls and results were compared by Student's paired *t* test.

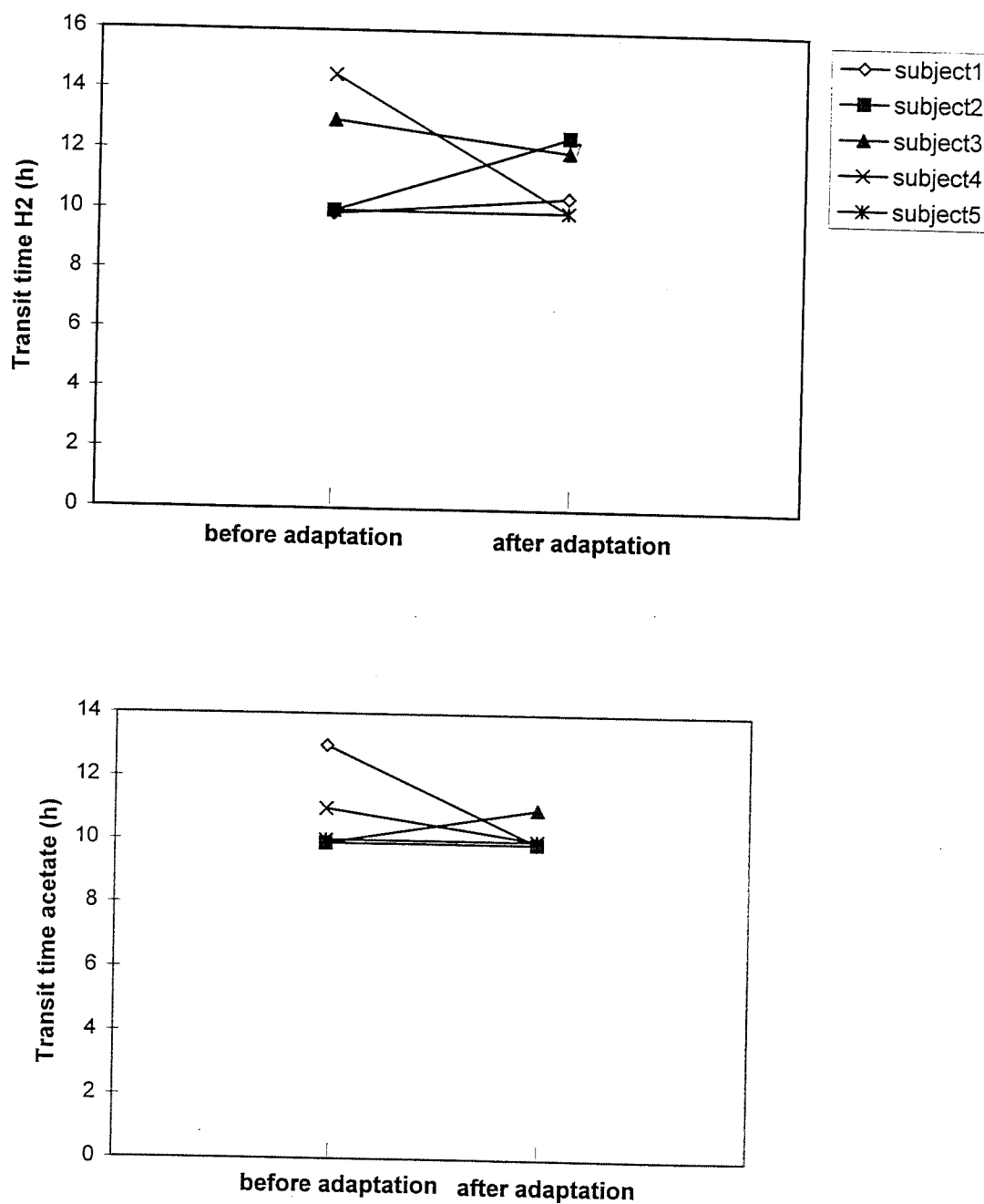
### 3.5.2. Results

The transit time (TT) of breath hydrogen (first sustained rise) and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in five subjects are shown in **Figure 3.8**. In 2 subjects the TT of breath hydrogen was decreased after adaptation period whereas, in another 2 subjects it was increased and no change was seen in one subject. The TT of plasma acetate in 2 subjects was decreased after adaptation period whereas, in one subject was increased. In one subject the TT of plasma acetate was less than 10h both before and after adaptation. The area under the curve (AUC) of breath hydrogen and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in five subjects are shown in **Figure 3.9**. In 4 subjects the AUC of breath hydrogen after adaptation period was smaller than before the adaptation period. The AUC of plasma acetate after adaptation period was greater for 2 subjects but not for the other 3 subjects.

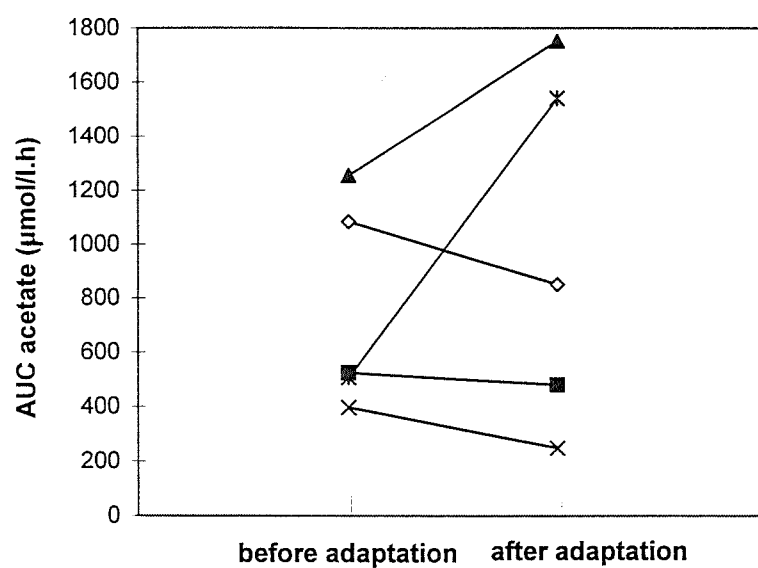
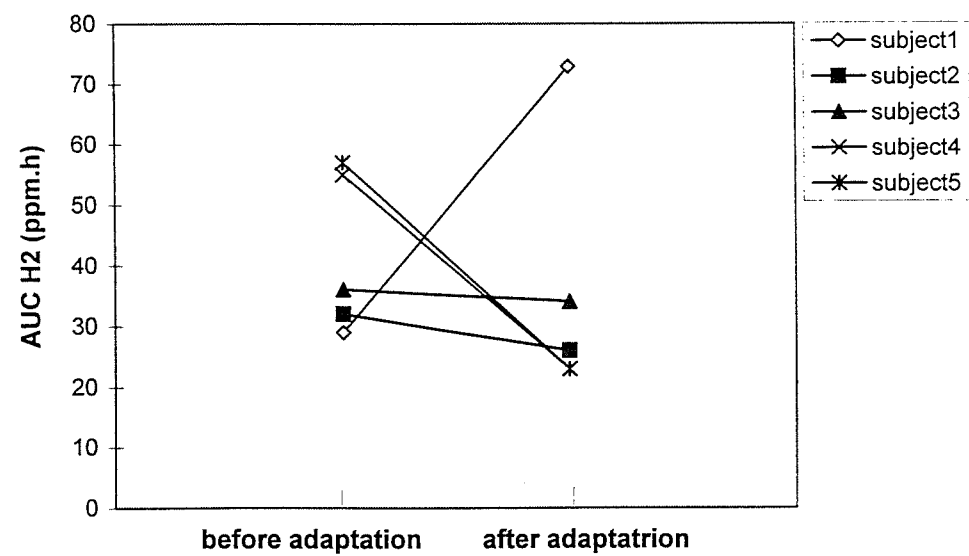
The height of the peaks of breath hydrogen and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in five subjects are

shown in **Figure 3.10**. The height of the peak of breath hydrogen in 3 subjects was decreased after adaptation period and it was increased in 2 subjects. The height of the peak of plasma acetate in 3 subjects was increased after adaptation period and in the other 2 subjects was decreased. The time of the peaks in breath hydrogen and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in five subjects are presented in **Figure 3.11**. Except in one subject the peak in breath hydrogen after the adaptation period occurred earlier than before adaptation period. In 3 subjects of five subjects the peak in plasma acetate after adaptation period happened earlier than before adaptation period, although in 2 subjects the acetate rose at the same time in both situations. Breath hydrogen after ingestion of 12g guar gum and 100g raw potato starch before and after adaptation period in five subjects are presented in **Figure 3.12**. Plasma acetate after ingestion of 12g guar gum and 100g raw potato starch before and after adaptation period in five subjects are demonstrated in **Figure 3.13**. The peak in plasma acetate in all the three situations occurred earlier than the breath hydrogen (**Figures 3.12-3.13**).

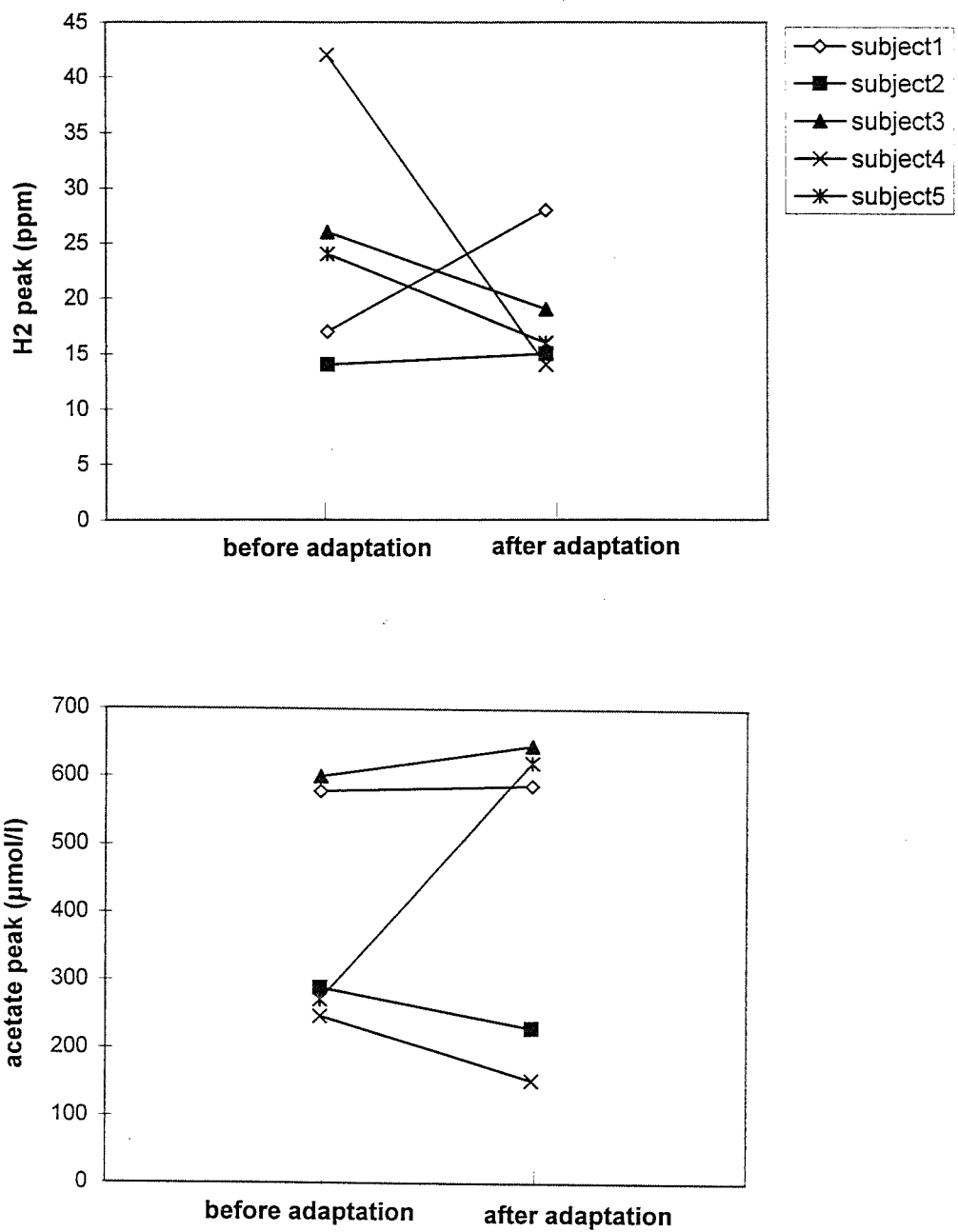
Although the apparent TT after adaptation had shortened the values gained either from plasma acetate or breath hydrogen were still significantly longer than for guar gum (**Tables 3.1, 3.3**).



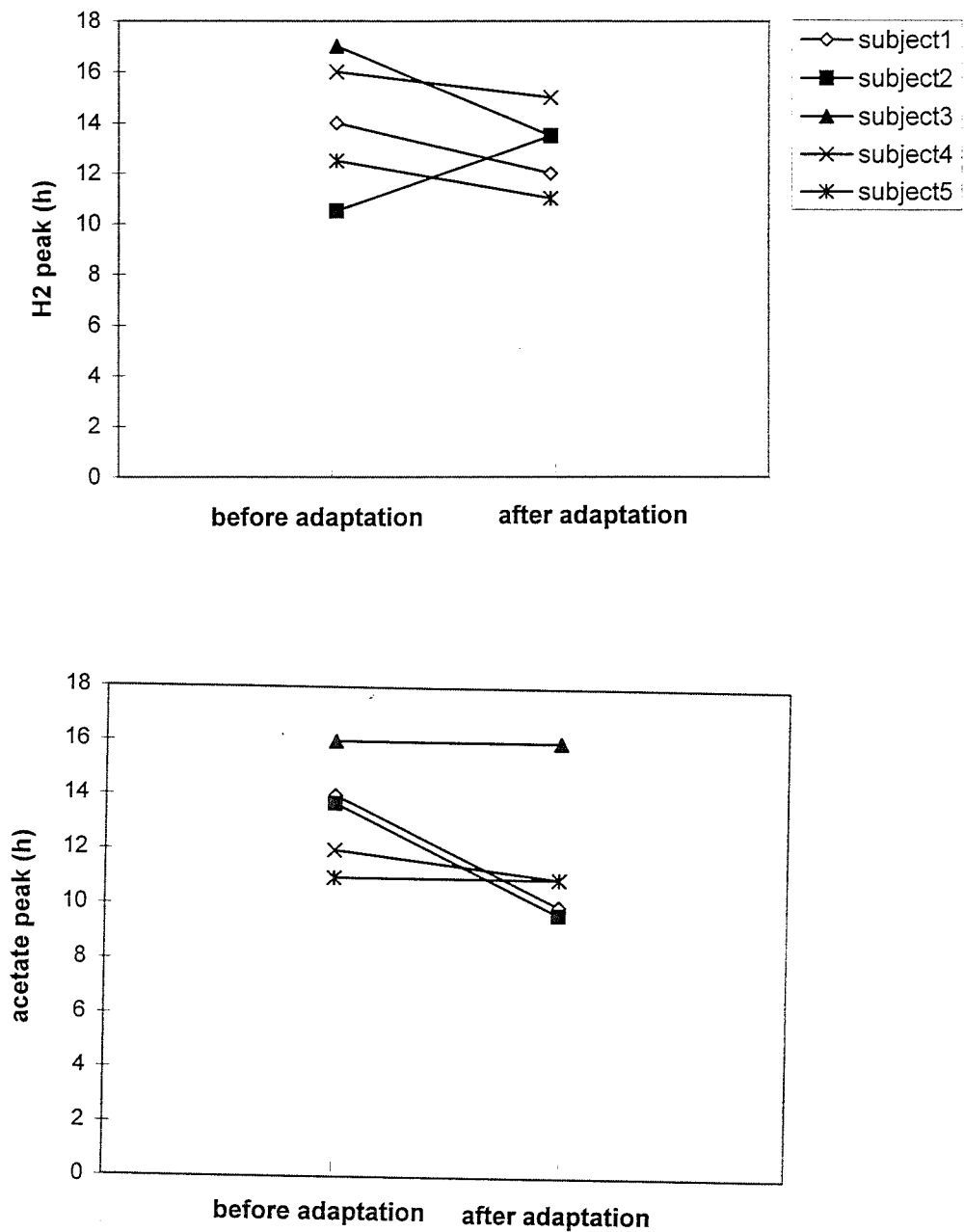
**Figure 3.8.** The transit time (first sustained rise) of breath H<sub>2</sub> and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in 5 subjects.



**Figure 3.9.** The area under the curve (AUC) of breath H<sub>2</sub> and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in 5 subjects.

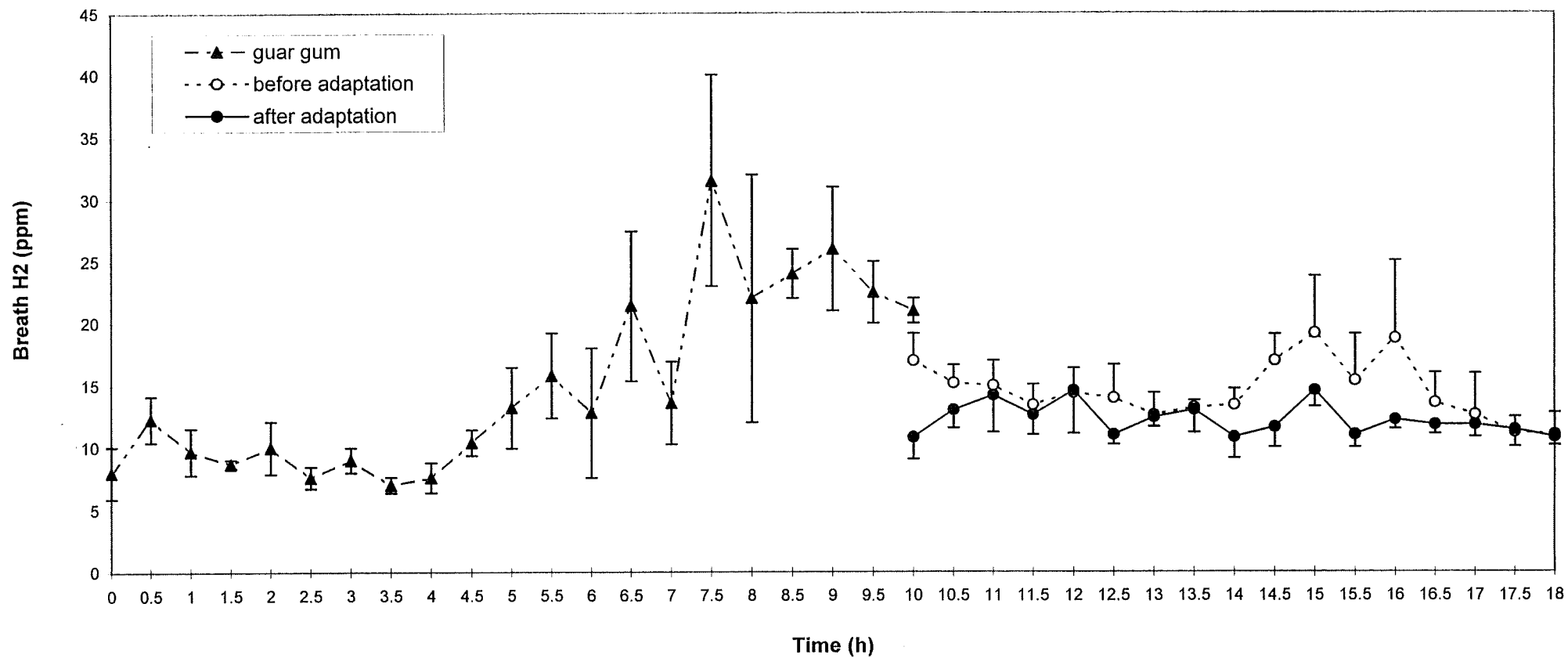


**Figure 3.10.** The height of the peaks of breath H<sub>2</sub> and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in 5 subjects.

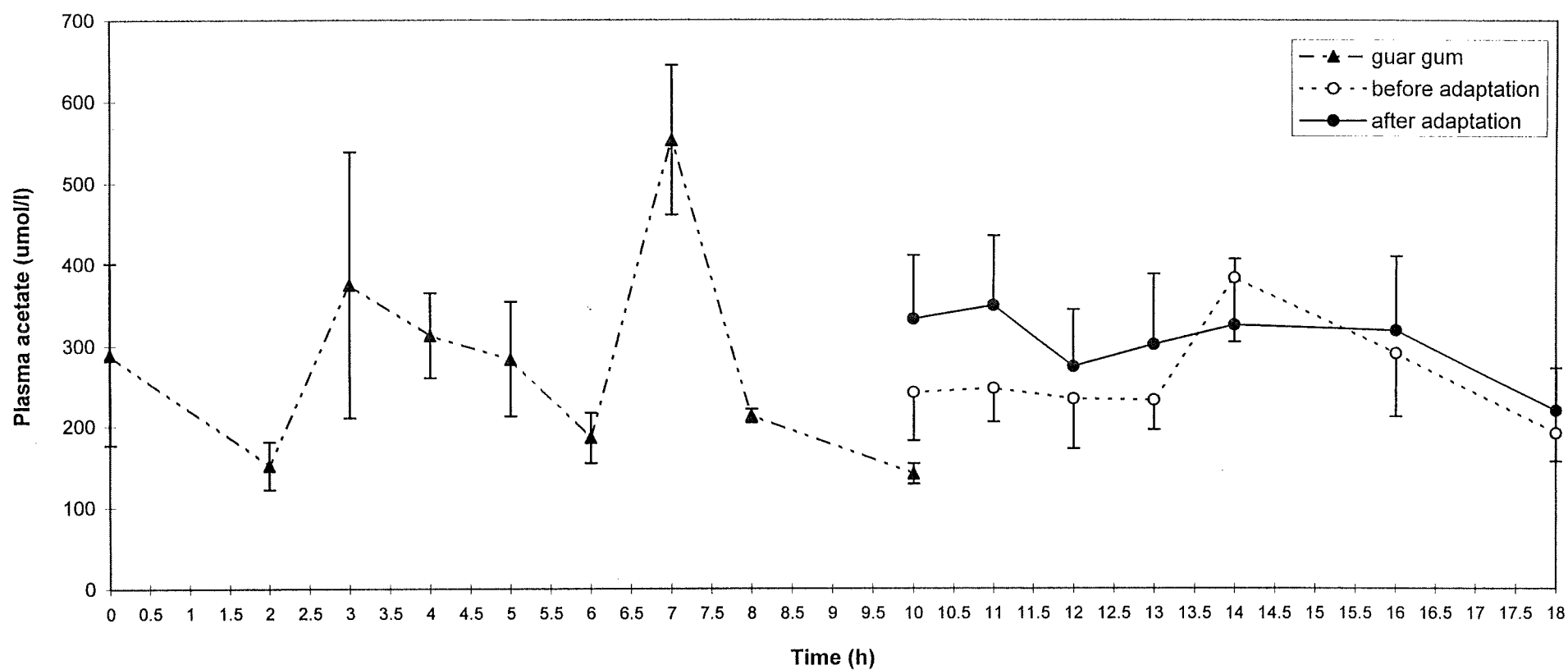


**Figure 3.11.** The time of the peaks in breath H<sub>2</sub> and plasma acetate after ingestion of 100g raw potato starch before and after adaptation period in 5 subjects.





**Figure 3.12.** Breath H<sub>2</sub> production after ingestion of 12 g guar gum, 100 g raw potato starch before and after adaptation period. Values: means  $\pm$  SEM; five subjects, except at 0, 0.5, 1, 1.5h (n=3) and at 7.5, 8, 8.5, 9, 9.5 and 10h (n=2).



**Figure 3.13.** Plasma acetate concentration after ingestion of 12g guar gum, 100g raw potato starch before and after adaptation period. Values: means  $\pm$  SEM; five subjects, except at 2h (n=4), at 0,3 & 7h (n=3), at 8 and 10h (n=2).

**Table 3.3.** Plasma acetate ( $\mu\text{mol/l}$ ) and breath hydrogen (ppm) after ingestion of 100g raw potato starch after adaptation period

Subjects	Transit time H <sub>2</sub> (h)	Transit time acetate (h)	AUC* H <sub>2</sub> (ppm . h)	AUC* acetate ( $\mu\text{mol/l}$ . h)	H <sub>2</sub> peak (ppm)	Acetate peak ( $\mu\text{mol/l}$ )	H <sub>2</sub> peak (h)	Acetate peak (h)
1	10.5	9.9	73.0	851.0	28.0	587.0	12.0	10.0
2	12.5	9.9	26.0	480.0	15.0	230.0	13.5	10.0
3	12.0	11.0	34.0	1752.0	19.0	646.0	13.5	16.0
4	10.0	10.0	23.0	250.0	14.0	153.0	15.0	11.0
5	10.0	10.0	23.0	1541.0	16.0	621.0	11.0	11.0
Mean*	11.0 $\pm$ 0.5	10.3 $\pm$ 0.3	35.8 $\pm$ 9.5	974.8 $\pm$ 292.4	18.4 $\pm$ 2.5	447.4 $\pm$ 105.5	13.0 $\pm$ 0.7	11.6 $\pm$ 1.1

\* area under the curve

\* mean  $\pm$  SEM

### 3.6. Discussion

In this chapter, I have described studies using plasma acetate and breath hydrogen to measure colonic fermentation of resistant starch. To develop these methods it was first necessary to identify a suitable fermentable standard. An initial study investigated the use of lactulose as a standard. However, breath hydrogen and plasma acetate measurements after ingestion of 20g lactulose showed that the short transit time (TT) and rapid fermentation of lactulose was completely different from that expected for the fermentation of RS. *In vivo* (Olesen et al., 1994) and *in vitro* (Edwards et al., 1996b) studies of fermentation of RS, showed a very slow fermentation which could occur several hours after lactulose fermentation. Lactulose was therefore rejected as a standard. Guar gum was tested next and produced slower rises in breath hydrogen and plasma acetate over a longer time period. Guar gum was therefore chosen as the standard for all other studies in this chapter.

Breath hydrogen and plasma acetate have been used in previous studies to monitor colonic fermentation but in this study we have tried to relate serial plasma acetate levels with breath hydrogen production after resistant starch which has not been reported before. Pomare et al., (1985) studied H<sub>2</sub> and plasma acetate over 24 hours after pectin and found a good match between the two measurements. As RS fermentation is much slower than pectin (11 hours after the test meal) the use of

plasma acetate as a marker may be less clear than after a fermentable dietary fibre.

In fact in our study, there was much variation in the relative profiles of breath hydrogen and plasma acetate responses. The rise in plasma acetate did not always correspond with the rise in breath hydrogen. Moreover, the rise in these parameters were not related to the relative times for the acetate and hydrogen peaks. The mean time for a rise in acetate occurred earlier than that for breath hydrogen, for all fermentable substrates and before and after adaptation. The mean time for the peak of plasma acetate occurred earlier than the mean time for the peak of breath hydrogen, although these differences were not statistically significant due to the large individual variation. This makes it difficult to use these measurements for quantitative estimations of fermentation. In the study of Pomare et al., (1985) they fed meals every 4 hours and drinks, which may have contained sugar, every 2 hours which will have suppressed endogenous acetate production. On a low fibre diet they saw very little variation in plasma acetate. In future studies it would be better to provide small meals often to improve on the use of plasma acetate as a measure of RS fermentation. However in Pomare's study they gave only mean values for rises in breath hydrogen and acetate which matched well. The mean TT in our study also matched well (**Tables 3.2, 3.3**);  $11.9 \pm 1.0$  (H<sub>2</sub> rises) vs  $11.3 \pm 0.7$  (acetate rises); before adaptation, and  $11.0 \pm 0.5$  (H<sub>2</sub> rises) vs  $10.3 \pm 0.3$  (acetate rises); after adaptation. It was the individual values which varied.

In agreement with Olesen et al., (1992; 1994) we demonstrated that fermentation occurred approximately 9-11 hours after ingestion of 100g raw potato starch, although in most of our subjects this occurred after 11 hours from ingestion of RS. Plasma acetate also rose 10 hours after ingestion of RS. This delay in breath hydrogen and plasma acetate after ingestion of raw potato starch could be related either to slow passage through the small intestine or to a slow rate of colonic fermentation of raw potato starch.

This delayed fermentation made it difficult to study the rise and duration of the breath hydrogen and plasma acetate. To try and overcome the problems of a very long study we carried out experiments with the meal eaten at the latest time in the night before the study day. This was around 11pm, breath hydrogen and plasma acetate were measured the following morning which was 10 hours after the test meal and should have been the right time for capturing the beginning of fermentation. However we still missed the initial rise in breath hydrogen and plasma acetate in one or two subjects.

This is one of the problem with *in vivo* fermentation study of RS. Moreover, the duration of the study in one day was very long, and the subjects should fasted about 14 hours after ingestion of RS test meal. Furthermore, for collecting serial blood samples, the intravenous catheter was positioned in each subjects' forearm during the whole study day. The potential for catheter blockage as well as subject

discomfort was very high.

Our *in vivo* RS fermentation studies showed that after ingestion of raw potato starch breath hydrogen and plasma acetate increased approximately within 11 hours, and peaked within 14 hours, whereas after administration of guar gum breath hydrogen and plasma acetate increased within approximately 4 hours, a peak being reached within 6 hours. The guar gum results were typical of a fermentable dietary fibre.

The delayed fermentation of the resistant starch was in agreement with a previous *in vivo* study (Olesen et al., 1992). In addition, *in vitro* fermentation models using human faecal microflora showed that raw potato starch; RS<sub>2</sub> had a slow rate of fermentation (Edwards et al., 1996b) compared with digestible starch. Six times as much unfermented starch in cultures of raw potato starch after 8 hours than in cultures with precooked digestible starch.

This suggests that the delayed fermentation of RS *in vivo* is due to slower fermentation rather than a delayed small bowel transit. This slower fermentation is probably due to the complex physical structure (intact starch granules and super helix of type B starch) so that RS is more resistant to bacterial enzymes degradation in the colon than fibre.

Slower fermentation could also be due to the lack of certain bacterial species or metabolic activities of those species present which enable the fermentation of resistant starch. To investigate this hypothesis we carried out an adaptation experiment measuring starch fermentation before and after 7 days ingestion of daily raw potato starch.

The results of this study showed that the area under the curve (AUC) of breath hydrogen after one week ingestion of raw potato starch was smaller in 4 out of 5 subjects than before the adaptation period. However in the 5th subject it was much higher (although these differences were not statistically significant due to large individual variation). This finding is similar to that seen in previous adaptation studies, that demonstrated after several days of ingestion of lactulose (Florent et al., 1985) and high amylose maize starch; RS<sub>2</sub> (Van Munster et al., 1994a) breath hydrogen production was decreased from the first day of ingestion although in the case of lactulose samples from the proximal colon showed more efficient fermentation. In contrast to our results, Olesen et al., (1994) reported that daily intake of 50g raw potato starch to the volunteers usual diet for 3 week periods increased the basic level of breath hydrogen (ie. fasting levels). They did not measure post-ingestion hydrogen after adaptation. In our study we fed 100g of RPS per day for 1 week and saw no significant differences in any parameter but as discussed above in most subjects there appeared to be a reduction in H<sub>2</sub> production after 1 week. There is therefore very little data indicating adaptation to



prolonged feeding with RS, but this needs to be investigated further with larger numbers of subjects.

Our study suffered from the small number of subjects. However, it was difficult to persuade people to take part in such a long study involving blood samples. Moreover the variation seen in 5 subjects makes it unlikely that a clearer picture would have been seen if the number of subjects was increased by 2 or 3. A much larger number would be needed and this was not practicable in this project. It was really hard to recruit subjects, we had many problems with the catheters and we often had difficulty arranging medical cover for the extended length of the study.

In conclusion, the main result of these studies was that there was much variation between breath hydrogen and plasma acetate responses to resistant starch. Neither seemed a good candidate for quantitative studies of resistant starch fermentation. These results prompted the development of an alternative method for measuring fermentation *in vivo* using stable isotopes. The development of this method is described in the next two chapters.

## CHAPTER FOUR

Production of  $^{13}\text{C}$  labelled starchy foods for *in vivo* studies

#### **4.1. Introduction**

A useful method to follow the fate of certain chemical compounds in the human body utilises tracer molecules that are isotopes of the molecule of interest. A tracer is chemically similar to the compound of interest, but has one different characteristic which enables its detection (Wolf, 1992). Isotopes are atoms (iso: same, topes: place) which hold the same place in the periodic table (Koenlynsberg, 1989). All isotopic forms of an element have the same atomic number (same number of protons) but differ in atomic weight (different number of neutrons) (Amarri & Weaver, 1995). Isotopes are either stable or radioactive (Shetty & Ismail, 1991). In stable isotopes the neutrons are stable with no evidence of spontaneous degradation, whereas in radioactive isotopes, spontaneous rearrangement of protons and or neutrons in an unstable atomic nucleus occurs (Wolf, 1992). Radioactive isotopes are potentially harmful to living organisms (Shetty & Ismail, 1991). The health of subjects who ingest substantial amounts of radioactive tracer may be at risk. Stable isotopes, however, are safe because of the absence of risks of radiation. This makes stable isotopes safe for studies on pregnant and lacting mother as well as for newborns, infant and children (Shetty & Ismail, 1991).

Stable isotope-labelled substrates have been used as tracers for the investigation of metabolic pathways (Rennie et al., 1991) such as protein

turnover and energy metabolism. Similar techniques could be used to study the digestibility and fermentability of starch. In our studies we have utilised the stable isotope of  $^{12}\text{C}$  which has an atomic weight of 12. The percentage of natural abundance of  $^{13}\text{C}$  is 1.11%, whereas, that for  $^{12}\text{C}$  is 98.89%. Thus,  $^{13}\text{C}$  can be used as a tracer in metabolic research of carbon containing compound (Rennie et al., 1991). In starch digestion experiments naturally enriched foods ( $\text{C}_4$  plants) can be used although large amounts are needed.  $\text{C}_4$  plants which have a  $\text{CO}_2$  concentrating mechanism for the formation of starch which enables them to grow very fast, these are plants of tropical origin, such as corn, millet, pineapple and cane sugar. To study other non  $\text{C}_4$  plants, it is necessary to enrich the  $^{13}\text{C}$  content artificially. This can be achieved by growing the plants in a  $^{13}\text{CO}_2$  environment.

Measurement of breath  $^{13}\text{CO}_2$  after ingestion of  $^{13}\text{C}$  starch (if some of this is resistant starch; RS) can differentiate between digestion and fermentation, which occur at different times. During digestion of  $^{13}\text{C}$  starch, the starch is hydrolysed to  $^{13}\text{C}$  glucose, this is absorbed and then oxidised to  $^{13}\text{CO}_2$ , which is excreted in breath.  $^{13}\text{CO}_2$  is also produced during colonic fermentation of RS by colonic bacteria.  $^{13}\text{CO}_2$  can also be produced indirectly from  $^{13}\text{C}$  SCFA which are produced by the bacteria and metabolised in the body.

Starchy foods labelled with  $^{13}\text{C}$  can be given by mouth and the  $^{13}\text{C}$  tracer

detected in breath  $\text{CO}_2$ , the metabolic end product. Breath  $^{13}\text{CO}_2$  is a better bio-marker than breath  $\text{H}_2$  for measurement of colonic fermentation because there are several disposal routes for  $\text{H}_2$  including  $\text{CH}_4$ ,  $\text{H}_2\text{S}$  and acetate (Gibson et al., 1990) as discussed in chapter one, and total breath  $\text{H}_2$  production is not always related to the amount of fermentation of a carbohydrate. When I started this work,  $^{13}\text{C}$ -labelled foods were not commercially available. Sufficient enrichment of the food is needed to produce a level of enrichment in the  $^{13}\text{CO}_2$  to be detectable above noise levels (baseline variation). In the past an enrichment of 2.6% of wheat starch have been shown to be sufficient (Harding et al., 1994). In this chapter I will explain how peas and potatoes were labelled with  $^{13}\text{C}$ .

## **4.2. Labelling peas and potatoes with $^{13}\text{C}$**

### **4.2.1. Photosynthetic fixation with $^{13}\text{CO}_2$ into pea plants**

Forty pea plants were grown in individual pots (12.5 cm diameter) with compost (Livingston multi purpose compost) in a greenhouse at Department of Botany, University of Glasgow. Baccara pea seed was used, because it contains a high level of starch (more than 50%).

Ten days later, the seeds started to germinate and fifteen days afterwards, were transferred to bigger pots (25 cm diameter), with a cane supporting them

(Figures 4.1-4.2). Forty four days later, metal rings were fixed to support the stems and some of the pea plants started to flower. On 65th day, three pea plants were chosen, which had small pods of premature pea seeds formed. For labelling these pea plants with  $^{13}\text{CO}_2$ , three bags were made from coated polypropylene film (Propylene CD 35; ICI, Welwyn Garden City, UK) approximately 154×30×45 cm to fit individual plants and sealed air-tight with heat sealer. This material was used because it has a low permeability to  $\text{CO}_2$  (Harding et al., 1994). This film was quite difficult to form into bags and to seal completely. At first a domestic vacuum bag sealer (Salton, No.1101) was used but this was unwieldy and difficult to manoeuvre, especially when sealing the bag with a plant inside. It was difficult to get a complete seal with no gaps and each bag had to be checked several times. A hand held sealer (Avon Cosmetics Ltd !!) made the sealing process much easier, however, it still took 2 people 1-1.5 hours to make and seal each bag.

The three plants were stripped of a few tendrils, to reduce non-photosynthetic plant material, watered and enclosed in the bags. Then each one was put in a bucket of water, to prevent any  $^{13}\text{CO}_2$  escaping out of any leaks in the underneath of each pot, which was a potential site of stress on the bottom seam.

Plastic valves were inserted through the wall of the bag to allow injection of

$^{13}\text{CO}_2$ . Air was delivered through the plastic valve into the bags by an air pump to provide enough air for the plant and to check if there were any holes in the seals (**Figure 4.3**). 250 ml  $^{13}\text{CO}_2$  (99 atom%, Cambridge Isotope Laboratories, supplied by Promochem Ltd., Welwyn Garden City, UK) was introduced to each bag through the valve (**Figure 4.4**) and the bags were left in a controlled environment room (growth cabinet) (**Figure 4.5**). The growth cabinet was used to provide controlled light (14 hours) and temperature ( $20 \pm 2$ ), not possible in a Scottish greenhouse. After six days, the bags were removed and the three pea plants rested for one week in the greenhouse. Any ripe pea pods were harvested. A second incubation was carried out to test the optimal conditions for enrichment. The peas were replaced in the bags and a further 250 ml  $^{13}\text{CO}_2$  were added to each bag. Six days later, the bags were removed. The pea pods were allowed to ripen under normal conditions and were then harvested and frozen. The frozen peas then were freeze-dried (by a food grade freeze-dryer, College of Food Technology, Glasgow). The pea seeds were separated from the skins, then ground to achieve pea flour for analysis. Three other pea plants were grown under normal condition and used as a control.

#### **4.2.2. Photosynthetic fixation with $^{13}\text{CO}_2$ into potato plants**

Potato contains more starch than peas with less protein and fibre. It is also much more resistant to digestion in the raw state (see chapter 1). Thus we

attempted to label potato plants with  $^{13}\text{CO}_2$ . Six potato plants were grown in individual pots (25 cm diameter) with compost (Livington Multi purpose Compost) in a greenhouse at Department of Botany, University of Glasgow. Record potato was used, because it contains high levels of starch (80%).

Sixty five days after planting, two potato plants were enclosed in bags, as for the peas, but the bags were much bigger needing two sheets per side instead of one. To allow for larger plants in bags, this time 500 ml  $^{13}\text{CO}_2$  (99 atom%, Cambridge Isotope Laboratories, supplied by Promochem Ltd., Welwyn Garden City, UK) was injected into each bag. Bags were left in greenhouse for six days (**Figure 4.6**). One of the potato plant was enclosed in bag only for 3 days because a hole was found at the sealed edge. At this time the bags were removed and potato were allowed to ripen under normal condition and then harvested and frozen.

The potato plants were labelled only once, because they could not tolerate the conditions in the polypropylene bags. After six days of incubation with 500 ml  $^{13}\text{CO}_2$ , most of the leaves turned yellow because of the excess condensation (**Figure 4.6**) and the leaves touching the plastic bag. A more compact variety would have been better.



Two other non labelled potato plants were grown under normal condition and used as control. After the potatoes were harvested some of them were frozen without any skin. The frozen potatoes then were freeze-dried (by a food grade freeze-dryer, College of Food Technology, Glasgow). The potatoes were ground to achieve potato flour for analysis.



**Figures 4.1. and 4.2.** Pea plants started to germinate after ten days and transferred to bigger pots fifteen days later.





**Figure 4.3.** Air was delivered through the plastic valve into each bag by an air pump (pea plant).



**Figure 4.4.**  $^{13}\text{CO}_2$  was injected through the plastic valve into each bag (pea plant).





**Figure 4.5.** After  $^{13}\text{CO}_2$  was injected into each bag, the pea plants left in growth cabinet.



**Figure 4.6.** Six days after  $^{13}\text{CO}_2$  was injected into each bag (potato plants).

### 4.3. Results of enrichment with $^{13}\text{C}$

Samples (1.00-1.50 mg) of labelled pea and potato flour were analysed for  $^{13}\text{C}$  enrichment by continuous-flow isotope- ratio mass spectrometer (CF-IRMS) (Europa Scientific Crewe) in collaboration with Scottish Universities Research and Reactor Centre, East Kilbride (Preston & McMillan, 1988). In this thesis the amount of  $^{13}\text{C}$  enrichment in labelled pea and potato flour is expressed in units of atom %  $^{13}\text{C}$  excess.

**Table 4.1.**  $^{13}\text{C}$  enrichment of pea plants

Plant	atom % $^{13}\text{C}$ excess
Unlabelled peas	0.00
Labelled peas 1 (once <sup>*</sup> )	2.09
Labelled peas 2 (once)	2.71
Labelled peas 3 (once)	2.28
Labelled peas 1 (twice <sup>†</sup> )	7.37
Labelled peas 2 (twice)	8.77
Labelled peas 3 (twice)	9.78

<sup>\*</sup>Once labelled with  $^{13}\text{C}$     <sup>†</sup>Twice labelled with  $^{13}\text{C}$

The mean atom % excess of  $^{13}\text{C}$  enrichment in our pea flour was 8.64%. By twice labelling the peas the  $^{13}\text{C}$  enrichment was increased more than double.

Very poor enrichment was achieved with the potato plants.

**Table 4.2.**  $^{13}\text{C}$  enrichment of potato plants

<b>Plant</b>	<b>atom % <math>^{13}\text{C}</math> excess</b>
Unlabelled potato (no skin)	0.00
Labelled potato (no skin)	0.73
Labelled potato (with skin)	0.69
Labelled wheat (from Cambridge*)	3.63

*\*MRC Dunn Nutrition unit, Cambridge*

#### **4.4. Attempts to separate protein and carbohydrates**

To find out the amount of  $^{13}\text{C}$  enrichment of starch in the labelled pea flour, we attempted to separate the protein from carbohydrate and to remove starch from fibre in the pea flour. To retain the majority of the labelled pea flour for human studies, some of this work was carried out with unlabelled pea flour.

##### **4.4.1. Separation of protein from starch and fibre**

At first we attempted to remove the protein from the peas using digestive enzymes. Two dialysis bags (size 5-24/32") of 5cm were made, one filled with 500 mg of labelled pea flour and the other with 500 mg of non labelled pea flour. 0.01g pepsin (Sigma, Poole, 3100 units/mg solid) and 0.1g pancreatin enzymes (Creon 25000, Solvay, Dublin, 1000 Ph.Eur.units protease) dissolved in 0.5 ml of distilled water, were added. The amounts of the enzymes were chosen from previous experience of studies measuring protein digestibility

(R. Varley personal communication). The bags were suspended in a beaker containing distilled water and incubated in a shaking water bath at 37°C (60 strokes/min) for 120 minutes (to mimic small intestinal motility). Then the content of the bags was washed with distilled water, transferred into two universal bottles and centrifuged at 2500 rpm at room temperature for 1 hour. The precipitate was collected, frozen at -70°C and freeze-dried. The percentage of protein was measured by continuous flow isotope-ratio mass spectrometer (CF-IRMS) (Europa Scientific Crewe) in collaboration with Scottish Universities Research and Reactor Centre, East Kilbride. The results are shown in **Table 4.3**.

**Table 4.3.** The results of first attempt to separate protein from carbohydrates

Pea flour	%Protein	atom % <sup>13</sup> C excess
Whole pea flour*	20.39	0
Protein removed pea flour*	13.04	0
Protein and starch removed pea flour*	13.79	0
Whole pea flour*	19.67	9.75
Protein removed pea flour*	10.22	9.04
Protein and starch removed pea flour*	11.83	9.21

\* *unlabelled pea flour*                      \* *labelled pea flour*

Despite the prolonged digestion with pancreatic enzymes protein removal was not complete with 10% still contaminating the pea flour. As this method was not completely successful, another attempt was made. This time in a test tube to 500 mg of unlabelled pea flour, 1 ml of distilled water was added and the mixture incubated in a boiling water bath for half an hour (the boiling should cause disruption of plant cell wall, helping to increase the hydrolysis of protein). The mixture was cooled down to 37°C (enzymes can be active in this temperature). 0.01g pepsin and 0.1g pancreatin enzymes dissolved in 0.5 ml of distilled water were added and incubated in a shaking water bath at 37°C (60 strokes/min) for at least 4 hours. The content of the test tube was then washed with distilled water, (to remove the released amino acids), transferred into a universal bottle and centrifuged at 2500 rpm at room temperature for 1 hour. The precipitate was then collected, frozen at -70°C and freeze-dried. The results are shown in **Table 4.4**.

**Table 4.4.** The results of second attempt to separate protein from carbohydrates

Pea flour	%Protein
Unlabelled whole pea flour	18.25
Unlabelled protein removed pea flour	21.87
Unlabelled protein and starch removed pea flour	17.96



In fact the removal of the protein by this method was not as successful as the first attempt.

#### **4.4.2. Separation of starch from fibre**

To remove starch from the pea flour so that the enrichment of the residual fibre content could be estimated. 5 ml of DMSO (Dimethyl Sulphoxide: can hydrolysis all type of starch, even resistant starch) was added to 50 mg of sample from the last stage of the dialysis above and incubated in a shaking boiling water bath for 30 minutes (boiling condition increases the hydrolysis of starch). The tubes were then centrifuged at 2500 rpm at room temperature for 30 minutes. The precipitate was washed with distilled water and recentrifuged. This was carried out several times. The precipitate was collected, frozen at -70°C and freeze-dried. The results are shown in **Table 4.3-4.4**. Attempts at both protein and starch removal had little effect on the degree of enrichment of the residue.

#### **4.4.3. Separation of starch and protein with chemical method**

As we did not achieve complete separation of protein and starch in our pea flour, others (Christine Slater, at Scottish Universities Research and Reactor Centre, East Kilbride) attempted to separate the starch and protein fractions of pea flour with chemical method.

The sample (60 mg of once labelled pea flour) was dissolved in 5 ml 0.3 M NaOH, and left overnight at 37°C in a weighed glass centrifuge tube. The tube was centrifuged at 3000 rpm for 5 minutes, and the supernatant transferred to a pre-weighed centrifuge tube. The residue was washed 3 times with 3 ml 0.1 M NaOH. The residue was freeze-dried.

This should have achieved the following separation:

Alkali insoluble portion = non-starch polysaccharides (dietary fibre)

Alkali soluble portion = starch and protein

The alkali insoluble portion was contaminated by salts, therefore 2 ml 0.1 M HCl was added (removes carbonates and bicarbonates), and the sample freeze-dried again.

The protein was precipitated from the alkali soluble portion by adding 3 ml 10% TCA (trichloroacetic acid). The tube was centrifuged and the supernatant poured into a weighed centrifuge tube. The protein residue was washed 3 times with 6M HCl.

The residues should have then contained the following:

Alkali soluble, acid insoluble portion = protein

Alkali soluble, acid soluble portion = starch

Deionised water (1 ml) was added to the protein fraction and the sample was freeze-dried.

The alkali and acid soluble portion was neutralised with 3 M NaOH and ultrafiltered using an Amicon Centriplus Concentrator (25000 MW cut off).

The starch was washed with deionised water and freeze dried.

Freeze dried fractions (1-2 mg) were weighed in tin capsules for analysis by Continuous Flow Isotope Ratio Mass Spectrometry with Dumas combustion. The results are shown in **Table 4.5**.

**Table 4.5.** The results of once labelled pea flour analysis with chemical method

sample	%protein	<sup>13</sup> C atom % excess
Pea flour	28	2.1
Residue (fibre)	2.7	2.45
Protein	13	2.31
Starch 1	5.9	2.43
Starch 2	9.3	2.35

Separation of starch and protein was incomplete as indicated by the protein content of the starch fractions. Enrichment of all fractions was the same, indicating that all parts of the peas were equally enriched.

#### **4.5. Measurement of resistant starch in pea flour**

To estimate the proportion of digestible and resistant starch in the raw pea flour, the Englyst method was used. The amount of rapidly digestible starch; RDS, slowly digestible starch; SDS and resistant starch; RS in pea flour was measured (Englyst et al., 1992) (explained in chapter 2, section 2.3.2). The pea flour content 14.4% RDS, 63.7% SDS and 21.9% RS.

The Englyst method (Englyst et al., 1992) is designed to mimic small intestinal digestion of starch, but uses precise timing and shaking speeds. The method is very sensitive to shaking speed and in this study it took several days to calibrate the shaking speed of water bath to give correct values for the standards provided. This method needs a lot of apparatus (ie. several water bath) and it took at least one long day for each assay. The assay was calibrated with standards of known starch digestibility.

#### **4.6. Pilot study—<sup>13</sup>C labelled pea flour fed to one subject**

##### **4.6.1. Introduction**

To test the feasibility of using the pea flour in *in vivo* studies, <sup>13</sup>C labelled pea flour was incorporated into biscuits (explained in chapter 2, section 2.4) and fed to one normal human volunteer. Breath CO<sub>2</sub> and H<sub>2</sub> were monitored for up to 16½ hours.

#### 4.6.2. Study design

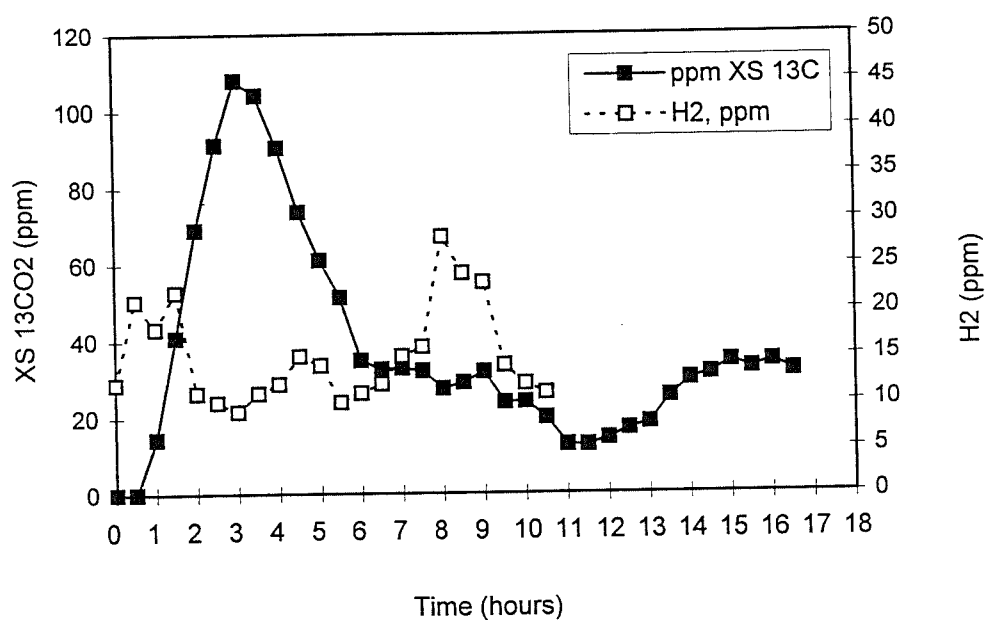
Subject 1 (female aged 37 years) was a non-smoker and had not taken antibiotics for at least 2 months. She was asked to consume a diet low in fibre and resistant starch the night before the study, avoid foods which are naturally enriched with  $^{13}\text{C}$ . She fasted from 9pm the night before the study to 7am on the study day. Then 300 mg of enriched pea flour incorporated into biscuits were ingested at 7am. Breath samples were collected at 30 minutes intervals up to 16½ hours after the biscuits, and analysed for hydrogen (explained in chapter 2, section 2.1) and  $^{13}\text{CO}_2$  enrichment (explained in chapter 2, section 2.5). The subject's mean volume  $\text{CO}_2$  production was determined at 1pm by a Deltatrac Metabolic Monitor <sup>TM</sup> (Datex Instrumentarium Corporation, Helsinki). For calculation of atom % excess  $^{13}\text{C}$  in the breath the volume of breath  $\text{CO}_2$  is needed. For simplicity it was assumed that  $\text{CO}_2$  production was constant throughout the study and an activity level of 1.4 RMR was applied.

Lunch and an evening meal, consisting of rice (165g) and chicken (one portion: 180g) were eaten at 2pm and 7pm. This meal was chosen as it was low in fibre, RS and fat, to prevent any stimulation of colonic motility.

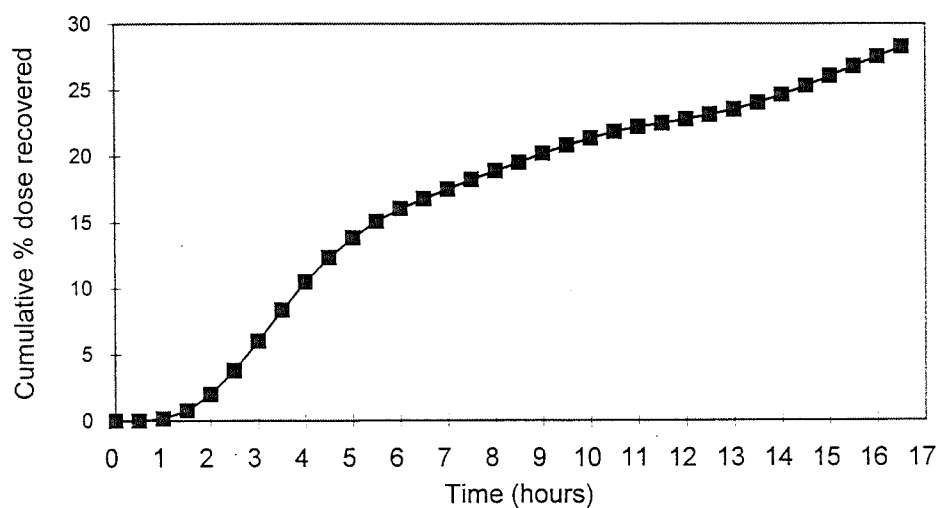
Ethical approval for the work was obtained from the Glasgow University Ethics Committee.

#### 4.6.3. Results

Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in one human volunteer are shown in **Figure 4.7**. After intake of the biscuits containing 300 mg labelled pea flour, the breath  $^{13}\text{CO}_2$  started to rise over the first 3 hours with the highest value (peak) reached at 3 hours. The breath  $^{13}\text{CO}_2$ , started to rise again at 8 hours with a second peak at 9 hours, coincident with the rise in breath hydrogen. For a third time, the breath  $^{13}\text{CO}_2$  started to rise at 12 hours after ingestion of labelled test meal and it was greater than basal after 16½ hour when the subject went to sleep for the night. The cumulative  $^{13}\text{C}$  percentage dose recovery (PDR) is shown in **Figure 4.8**. The total 16½ hours recovery of  $^{13}\text{C}$  in breath was 28.27%.



**Figure 4.7.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 1



**Figure 4.8.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 1

#### 4.6.4. Discussion

In this study we successfully produced a high level of  $^{13}\text{C}$ -labelled pea flour by photosynthetic incorporation of  $^{13}\text{CO}_2$ . The mean atom % excess of  $^{13}\text{C}$  in once labelled pea flour was 2.4%. Whereas, that in twice labelled one was 8.64%. In previous studies (Harding et al., 1994) showed that 400 mg of  $^{13}\text{C}$ -labelled wheat flour with mean atom % excess enrichment of 2.6% was enough to produce measurable amount of  $^{13}\text{CO}_2$  for breath test analyses. If the expense of 2 litres of  $^{13}\text{CO}_2$  is considered (£1000), it may be sufficient to label the peas only once. With twice labelled pea flour we need a very small quantity of labelled substrate, a larger amount of once labelled pea could be used and still have enough enrichment to produce measurable amount of  $^{13}\text{CO}_2$  for breath test analyses in future studies.

To really interpret the human studies, it is important to know what is labelled in the pea flour. To test this we tried both enzymic and chemical separation of the protein, starch and fibre. These attempts were not completely successful. However, we did achieve some removal of protein and starch and yet the % enrichment of the residue remained the same. This indicated that the label was distributed throughout the pea flour and so we decided to go ahead with the *in vivo* studies knowing that the  $^{13}\text{CO}_2$  would also be derived from protein digestion and fibre fermentation.



Further separation studies were not possible due to the small amounts of labelled pea flour available. In future studies it would be necessary to characterise the labelled constituents more definitely.

The pea flour was tested initially in one subject. During 16½ hour after ingestion of  $^{13}\text{C}$ -labelled pea flour, the profiles of  $^{13}\text{CO}_2$  enrichment in breath showed a complex of three apparent peaks. Thus, this small quantity (300 mg) of our labelled-pea flour was quite enough to produce measurable  $^{13}\text{CO}_2$  excretion for breath test analysis. It was hypothesised that the *in vitro* measurements of digestible starch was related to the first peak and the 2nd two peaks which occurred after the rise in breath hydrogen were caused by fermentation. This is explored further in chapter 5.

We therefore concluded that for measurement of the digestibility and fermentability of starchy foods this small quantity (300 mg) of our  $^{13}\text{C}$  labelled pea flour could be used in an *in vivo* study of more human subjects over a longer period.

Our  $^{13}\text{C}$ -labelled potato, however, was not highly enriched because the potato plant could not tolerate a long time in the bags. After six days incubation with  $^{13}\text{CO}_2$ , most of the potato leaves turned yellow. The bags were removed, and

the potato plants rested for one week. Then we planned to incubate those plants for the second time with  $^{13}\text{CO}_2$  for another six days, but during the resting period, all the leaves of potato plants turned yellow and it was clear that the once labelled potato plants could not survive in the bags. Thus the potato plants were not incubated long enough with  $^{13}\text{CO}_2$  and were poorly enriched (the mean atom % excess of  $^{13}\text{C}$  enrichment in our potato flour was 0.71%). Further studies should use a more compact potato plant and a bigger container for incubation with  $^{13}\text{CO}_2$ .

# CHAPTER FIVE

Pea flour digestibility and fermentability

measured with  $^{13}\text{C}$  isotope

## 5.1. Introduction

It is now generally accepted that some of starches in the ordinary daily diet escape digestion and absorption in the human small intestine (Englyst et al., 1992). Starches that remain undigested in the upper gastrointestinal tract enter the colon where they may be fermented by the colonic microflora to short chain fatty acids and gases ( $\text{CO}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ) (Englyst & Macfarlane, 1986b). As discussed in previous chapters *in vivo* measurement of digestibility and fermentability of starchy food in healthy human subjects is difficult and most of the current knowledge is based on the results of *in vitro* or animal studies.

Fermentation *in vivo* can be monitored by measurement of bacterial products in the breath. Previous *in vivo* studies have relied mainly on breath hydrogen measurements. However, it has been shown that breath hydrogen alone is a poor marker for the malabsorption of slowly fermentable substrates such as resistant starch (Olesen et al., 1994). Furthermore, there are several routes for hydrogen disposal in the human colon (Gibson et al., 1990) such as methane,  $\text{H}_2\text{S}$  and acetate. The measurement of the  $^{13}\text{C}$  enrichment of breath  $\text{CO}_2$  ( $^{13}\text{C}$ -breath tests), after a  $^{13}\text{C}$  labelled starchy food is an alternative way to quantify the digestibility and fermentability of starch foods.

Although some previous *in vivo* studies have measured starch digestion with

$^{13}\text{CO}_2$  no-one has previously used  $^{13}\text{CO}_2$  to measure fermentation in adults. Naturally  $^{13}\text{C}$ -enriched corn has been used, to measure the digestibility of various types of corn starch (waxy starch, gelatinized starch) in healthy volunteers (Hiele et al., 1990) and similar studies have evaluated starch digestion in children with cystic fibrosis (Dewit et al., 1992). Harding and colleagues (1994) used artificially enriched  $^{13}\text{C}$  labelled wheat flour to measure starch digestion but the results presented were for only one subject, followed for just 6 hours after ingestion of a  $^{13}\text{C}$  labelled test meal. The authors were not interested in fermentation.

$^{13}\text{C}$  labelled starch offers the possibility to measure fermentation of resistant starch. In this chapter I will describe how we used the enriched pea flour, produced as described in chapter 4, to estimate the digestibility and fermentability of raw pea flour.

## **5.2. *In vivo* study of digestibility and fermentability of $^{13}\text{C}$ pea flour**

### **5.2.1. Study design**

In this study  $^{13}\text{C}$  labelled pea flour incorporated into biscuits was fed to six normal human volunteers (four males and two females aged 25-57) and breath  $\text{CO}_2$  and  $\text{H}_2$  were monitored for up to 34 hours. All the subjects were non-smokers and had not taken antibiotics for at least 2 months. Subjects were asked to consume a diet low in fibre and resistant starch the night before the study and avoid foods which are naturally enriched with  $^{13}\text{C}$ . Subjects fasted from 9pm the night before the study to 7am on the morning of study day. Then 300 mg of enriched pea flour incorporated into biscuits (explained in chapter 2, section 2.4) were ingested. Breath samples were collected at 30 minutes intervals for up to 34 hours, and analysed for hydrogen (explained in chapter 2, section 2.1) and  $^{13}\text{CO}_2$  enrichment (explained in chapter 2, section 2.5). The subjects' mean volume  $\text{CO}_2$  production was determined at 1pm by Deltatrac Metabolic Monitor<sup>TM</sup> (Datex Instrumentarium Corporation, Helsinki). Lunch and the evening meal which consisted of rice (165g) and chicken (one portion: 180g) were provided and eaten at 2pm and 7pm. This meal was chosen as it was low in fibre, RS and fat to avoid it interfering with the colonic processing of the test meal during the study. Subjects were asked to continue taking breath samples for as long as possible but could then have a

sleep. Most subjects slept for 6-8 hours after which they continued taking samples. The study lasted for up to 34 hours after the ingestion of test meal.

In one subject (female aged 37 years) lactose- $^{13}\text{C}$ ureide was used as an additional marker for colonic fermentation. In a similar separate study to the pea flour, 500 mg of lactose- $^{13}\text{C}$ ureide incorporated into biscuits (explained in chapter 2, section 2.4) were ingested at 7am in the morning. Breath samples were collected at 30 minutes intervals for up to 25 hours, and analysed for hydrogen (explained in chapter 2, section 2.1) and  $^{13}\text{CO}_2$  enrichment (explained in chapter 2, section 2.5). As before, the subject's mean volume  $\text{CO}_2$  production was determined by Deltatrac Metabolic Monitor <sup>TM</sup> (Datex Instrumentarium Corporation, Helsinki). Lunch and the evening meal which consisted of rice (165g) and chicken (one portion: 180g) were eaten. The study lasted for 25 hours after the ingestion of test meal. Ethical approval for the work was obtained from the Glasgow University Ethics Committee.

## **5.2.2. Results**

### **5.2.2.1. Breath $^{13}\text{CO}_2$ and breath hydrogen profiles**

Profiles of the breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in six subjects are shown in **Figures 5.1, 5.3, 5.5, 5.7, 5.9, 5.11**. The excretion of  $^{13}\text{CO}_2$  in breath showed a complex of three apparent peaks. The first peak happened over the first 6 hours, the second

peak occurred between 7 and 11 hours appearing as a shoulder on peak 1 and the third one happened much later between 12 and 20 hours in individual subjects. The second peak coincided with the rise in breath hydrogen. The third peak tended to occur when subjects were asleep so information about the exact duration and height of this peak was lost. The  $^{13}\text{CO}_2$  in breath returned to baseline after 28 h in subject 3 and after 34 h in subject 4. Data collection finished before the  $^{13}\text{CO}_2$  returned to baseline in all other subjects.

#### **5.2.2.2. Percentage dose recovered from three peaks**

The cumulative percentages of administered dose of  $^{13}\text{C}$  recovered (PDR) in breath for the six subjects are shown in **Figures 5.2, 5.4, 5.6, 5.8, 5.10, 5.12**. The final cumulative PDR were very similar for four out of six subjects of the study (44.05-47.18%) during the period of time up to 34 h. Total recoveries of  $^{13}\text{C}$  in breath in subject one and three were 28.27% and 37.11%, respectively.

To examine the contribution of small intestinal digestion and colonic fermentation to total PDR, the three peaks were analysed in two ways. First the area under the curve (AUC) of breath  $^{13}\text{CO}_2$  (ppm XS  $^{13}\text{C.h}$ ) was calculated for each peak. The third peak was extrapolated from the data points available. Secondly, values of estimated percentage of total dose of  $^{13}\text{C}$  recovered were calculated using a mathematical model (Morrison et al., 1998) (**Table 5.1**). Morrison et al., (1998) reported that cumulative PDR can



be calculated mathematically using the equation:

$$y = a.(t-d)^b .e^{[-c (t-d)]}$$

where a is a scaling factor and variables b and c describe the shape of the curve and the parameter  $t_{\max} (b/c+d)$  was used to define the time of maximal  $^{13}\text{CO}_2$  excretion for a given peak (Ghoos et al., 1993) (**Figure 5.16**).

According to the AUC of breath  $^{13}\text{CO}_2$  (ppm XS  $^{13}\text{C.h}$ ), if total  $^{13}\text{CO}_2$  collected in the 3 peaks is taken as 100%, 40% was due to the second and third peaks. As this occurred after the rise in breath hydrogen, this could be all due to colonic fermentation. Using the mathematical calculation (Morrison et al., 1998) the value for sum of peak 2 and 3 was 42.11%, again suggesting than over 40% of the  $^{13}\text{CO}_2$  was produced from fermentation or fermentation products.

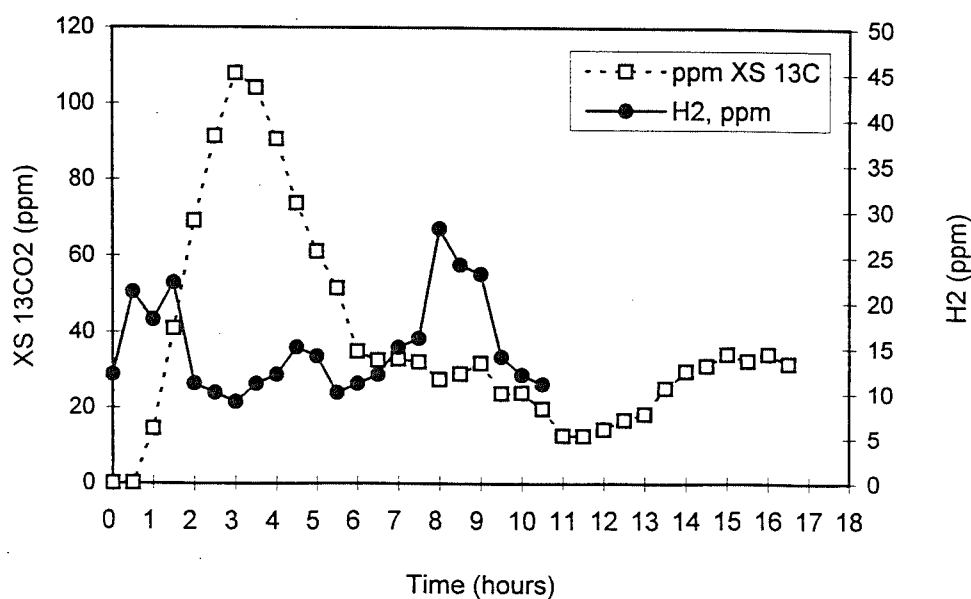
#### **5.2.2.3. Relationship of $^{13}\text{C}$ peaks with *in vitro* results**

The first peak of  $^{13}\text{CO}_2$  in the breath should be equivalent to the amount of rapidly digestible starch; RDS and slowly digestible starch; SDS. The second and third peaks should be equivalent to the amount of RS. However, our measurements of starch digestibility using the *in vitro* assay (Englyst et al., 1992; chapter 4) showed values of RDS; 14.4% and SDS; 63.7% equal to 78.1%. The  $^{13}\text{CO}_2$  breath test shows the first peak accounted for only 57.9% of the accumulated PDR. Conversely, the second and third peaks corresponded

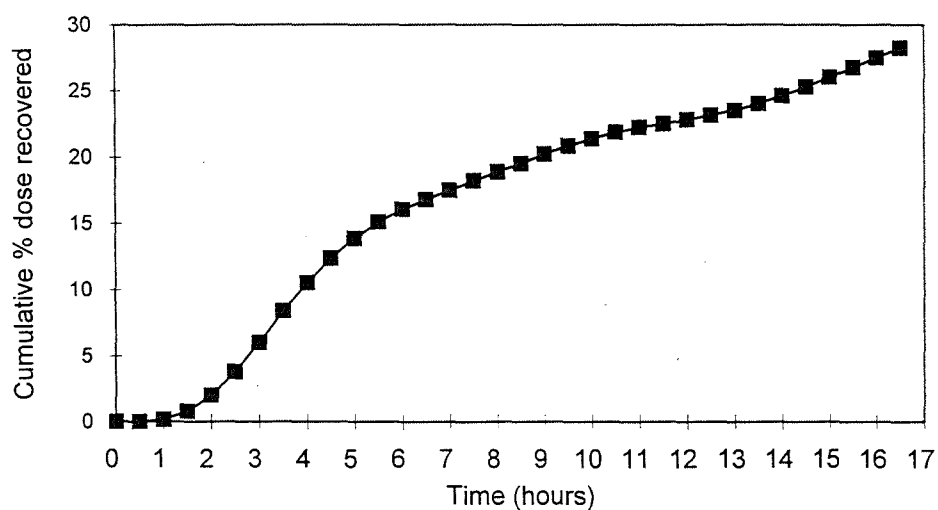
to 42.1% and RS *in vitro* was only 21.9%.

#### 5.2.2.4. Measurement of arrival of meal into colon by lactose- $^{13}\text{C}$ ureide

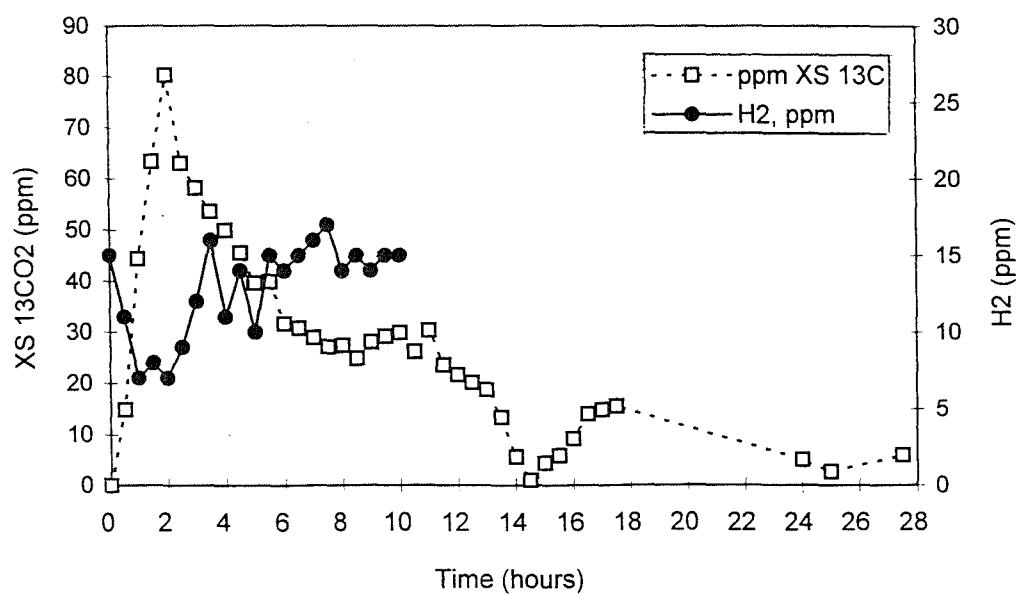
The result of breath  $^{13}\text{CO}_2$  and hydrogen after ingestion of biscuits containing 500 mg lactose- $^{13}\text{C}$ ureide in subject 1 is shown in **Figure 5.13**. After intake of labelled lactose-ureide, the breath  $^{13}\text{CO}_2$  and hydrogen began to rise at 6 hours, and the peak of breath hydrogen happened at 7 hours. The peak breath  $^{13}\text{CO}_2$  however was at 10½ hours and began to decline towards the baseline at 25 hours. The total recovery of  $^{13}\text{C}$  in breath during 25 hours was 50.19% (**Figure 5.14**). A comparison of the breath  $^{13}\text{CO}_2$  after ingestion of biscuits containing 500 mg lactose- $^{13}\text{C}$ ureide and after biscuits containing 300 mg  $^{13}\text{C}$  labelled pea flour in subject 1 is shown in **Figure 5.15**. The second peak coincided with the highest value of  $^{13}\text{CO}_2$  in breath after ingestion of 500 mg lactose- $^{13}\text{C}$ ureide.



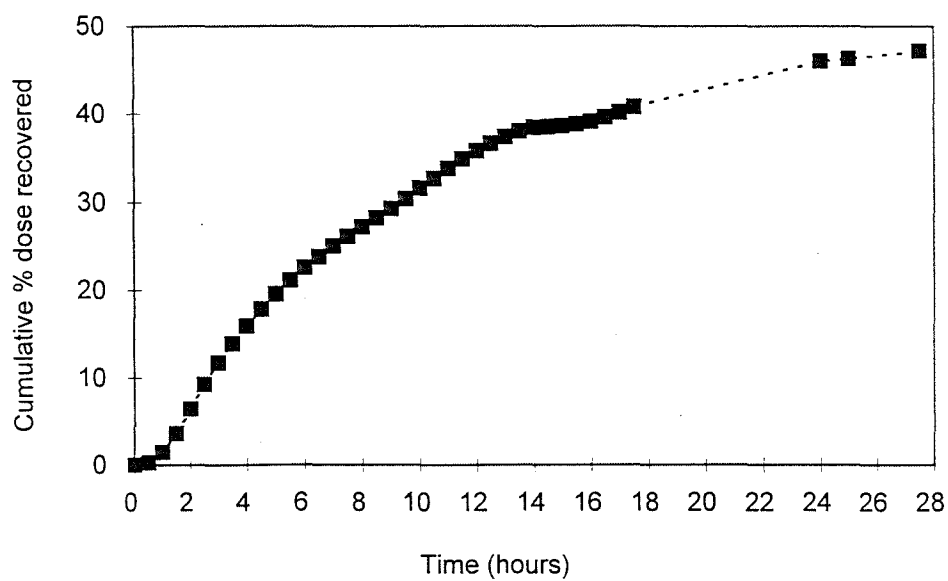
**Figure 5.1.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 1



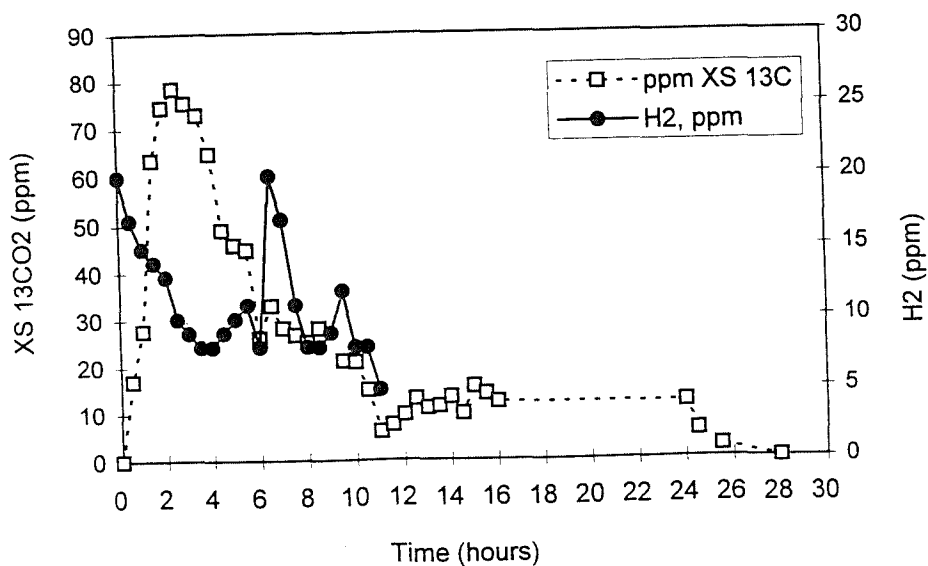
**Figure 5.2.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 1



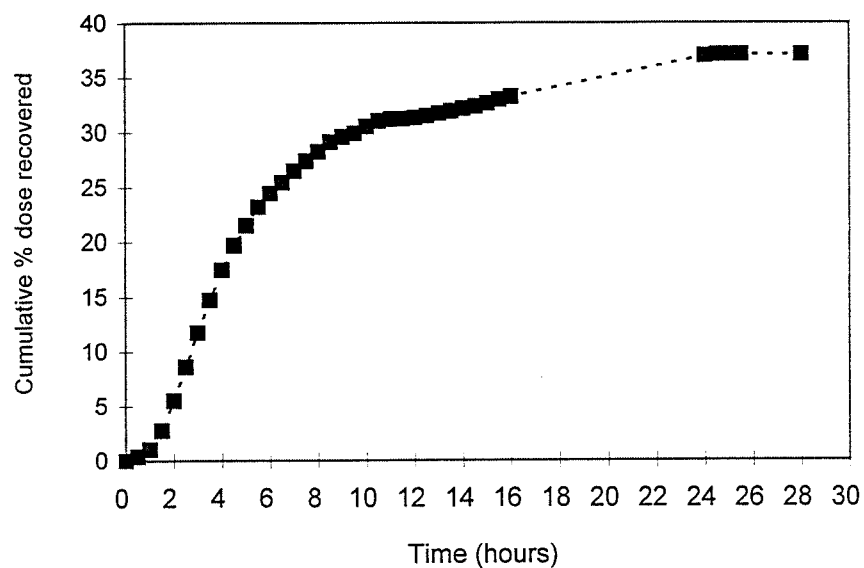
**Figure 5.3.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 2



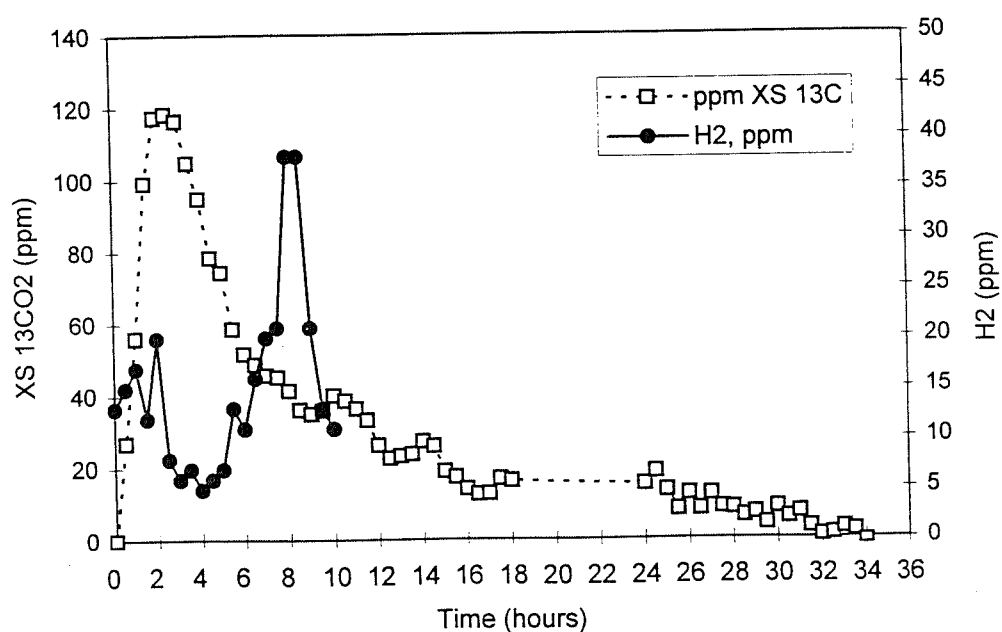
**Figure 5.4.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 2



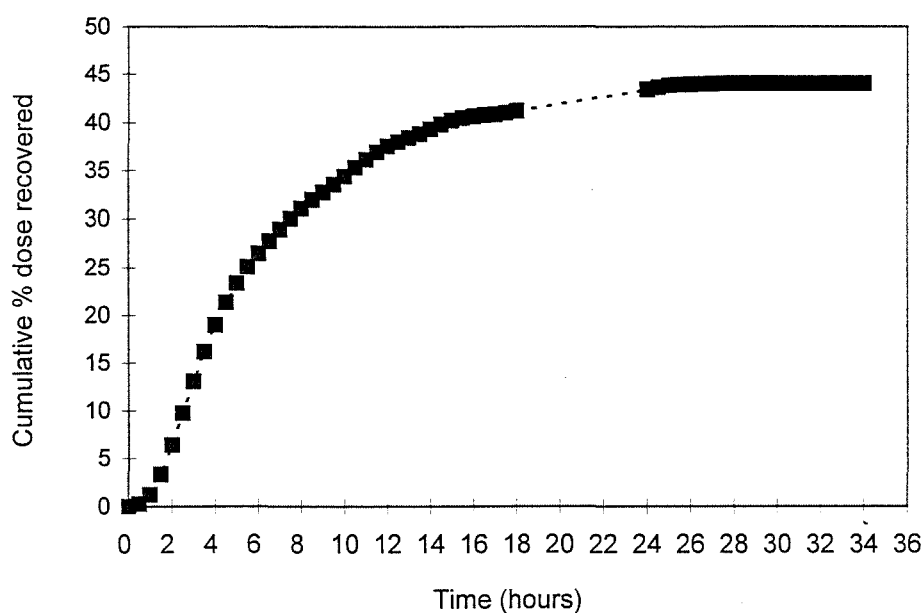
**Figure 5.5.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 3



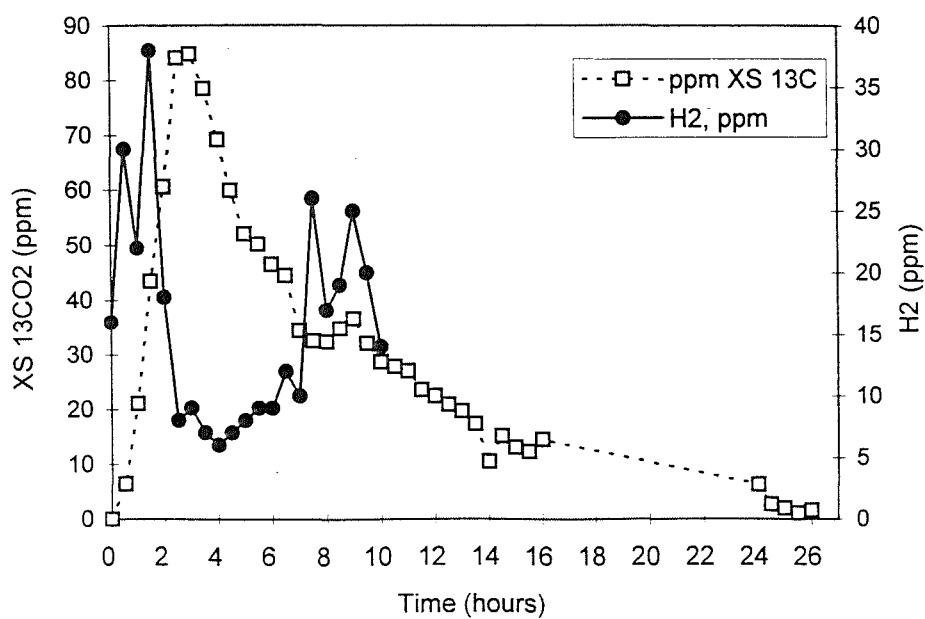
**Figure 5.6.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 3



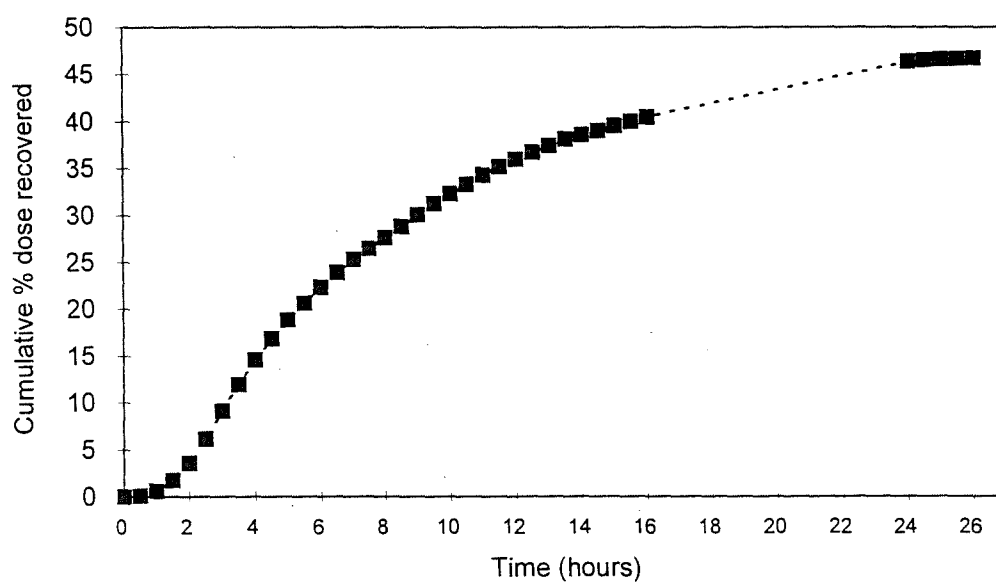
**Figure 5.7.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 4



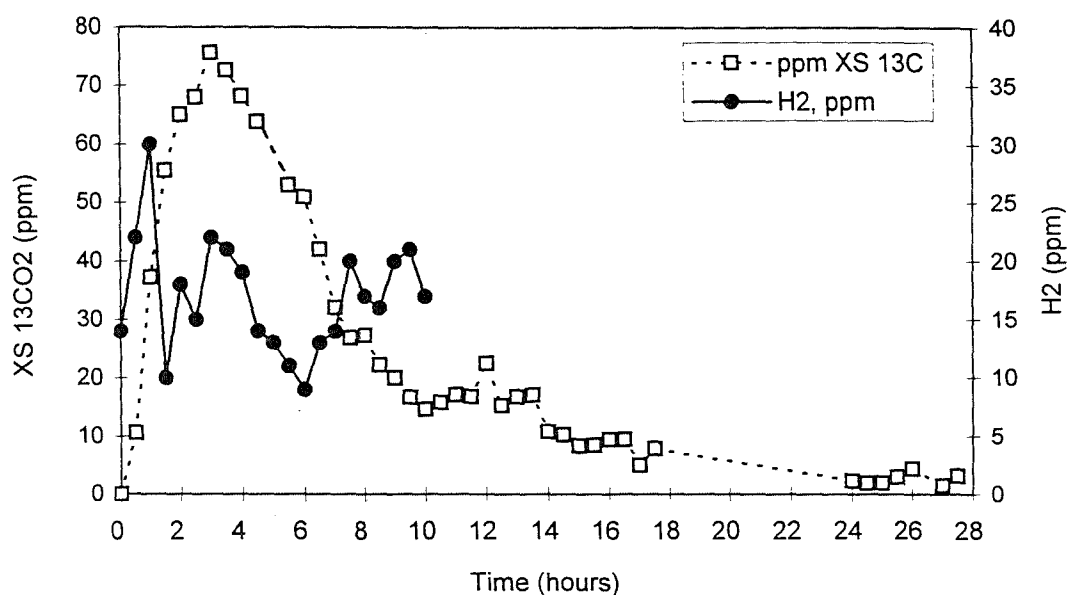
**Figure 5.8.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 4



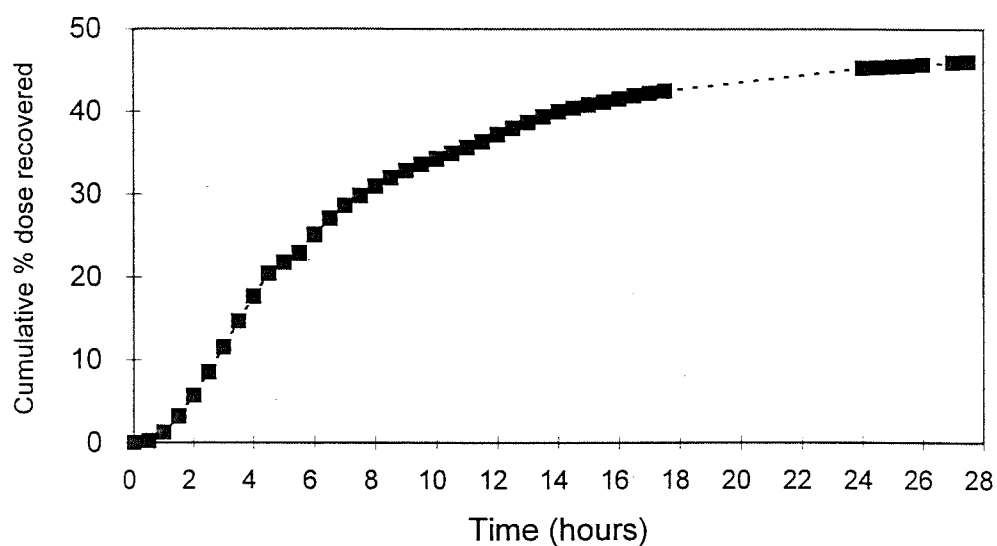
**Figure 5.9.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 5



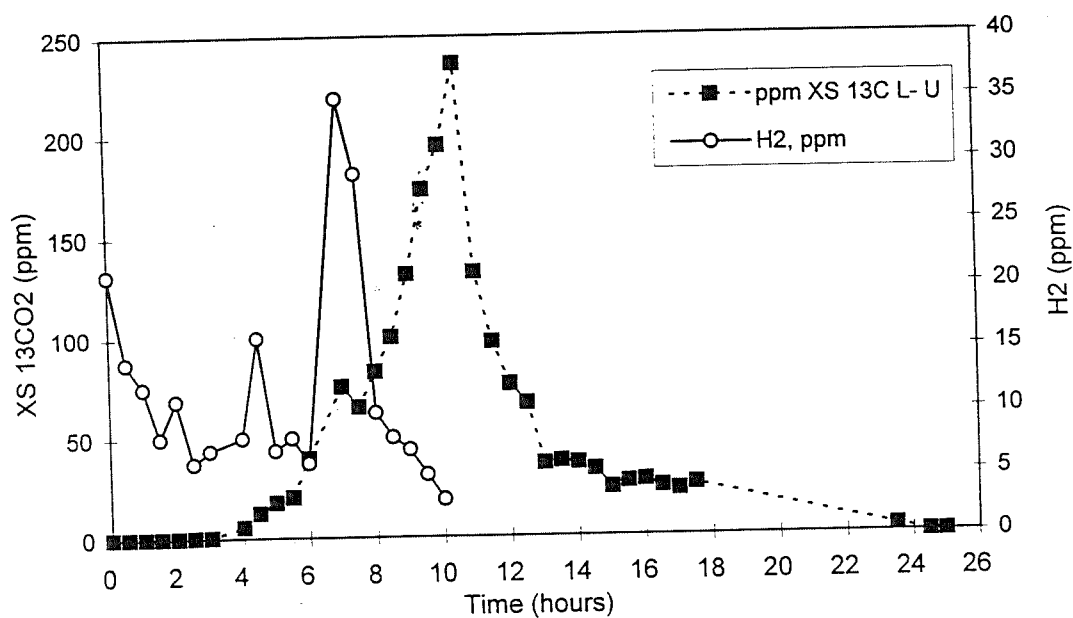
**Figure 5.10.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 5



**Figure 5.11.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300 mg labelled pea flour in subject 6

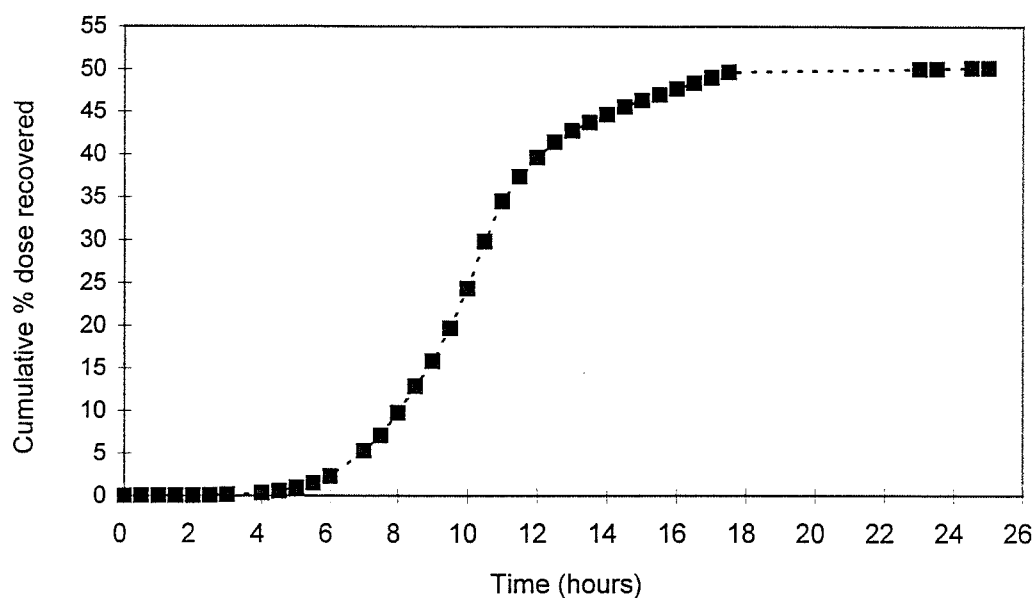


**Figure 5.12.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 6

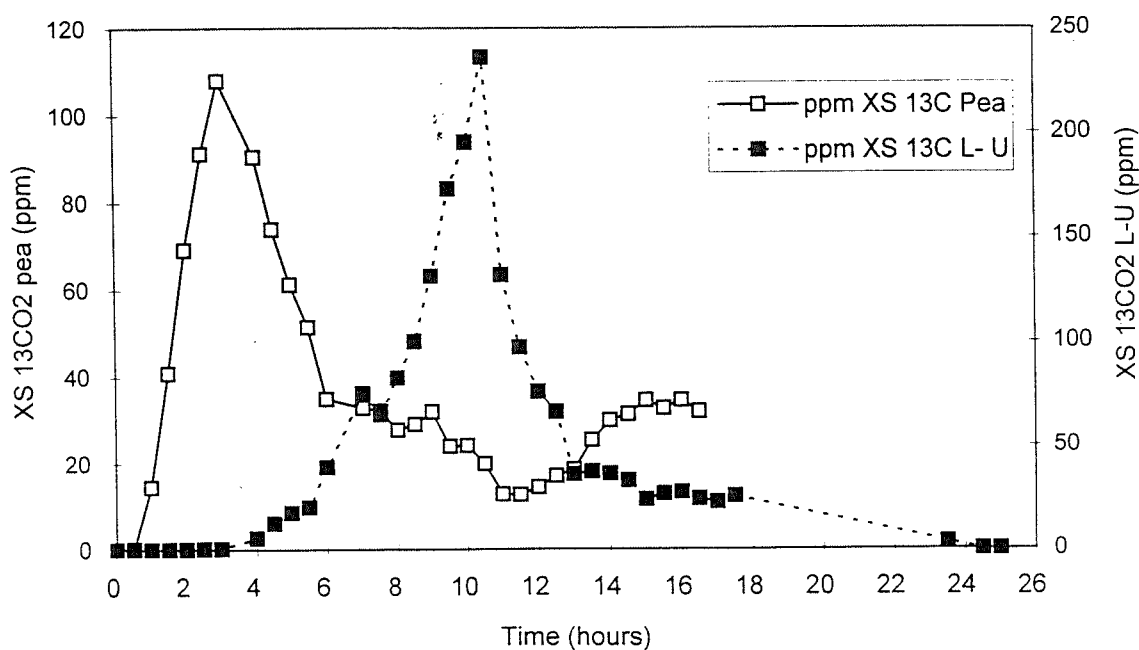


**Figure 5.13.** Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 500 mg lactose- $^{13}\text{C}$ ureide in subject 1





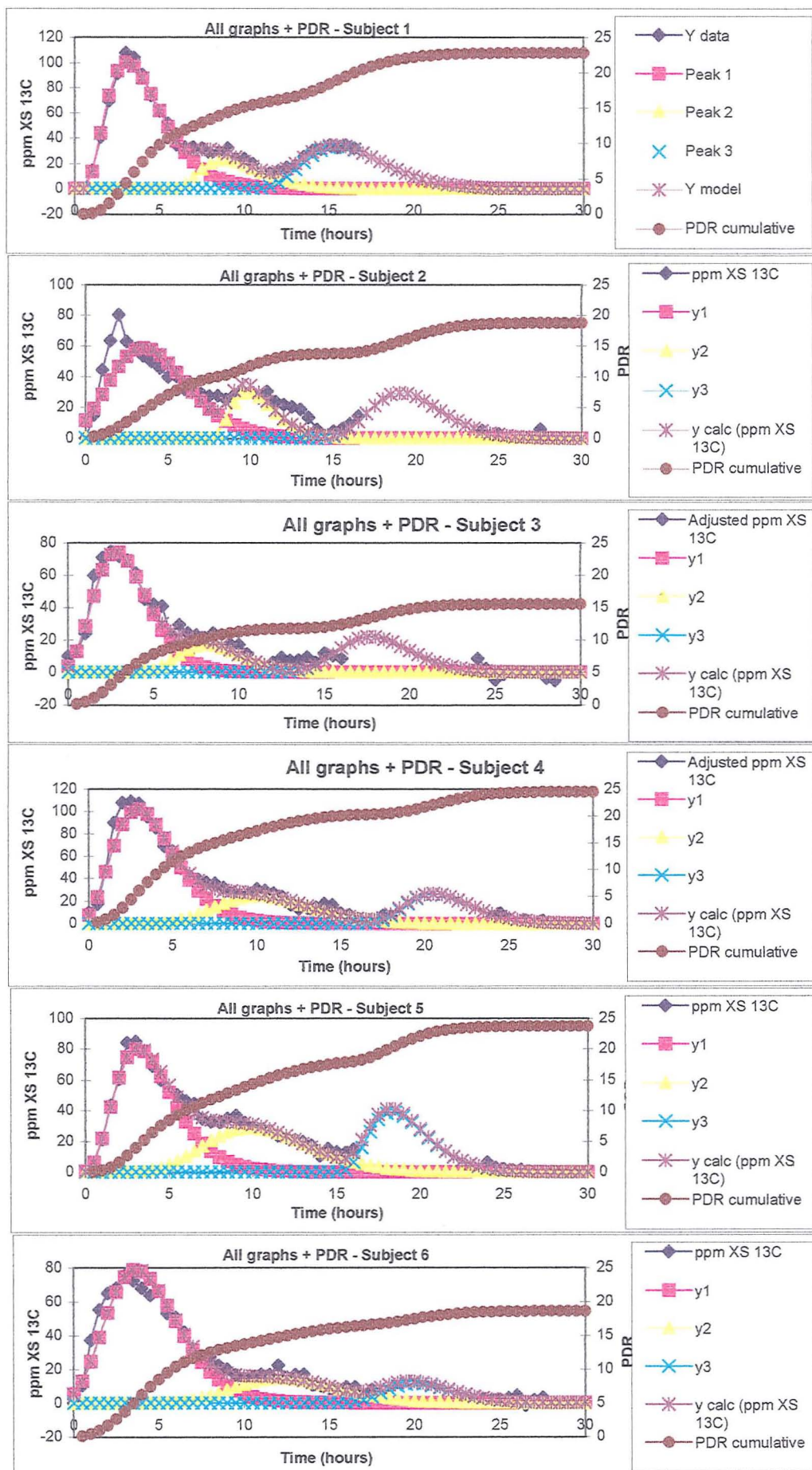
**Figure 5.14.** Cumulative percentage of administered dose of  $^{13}\text{C}$  recovered as breath  $^{13}\text{CO}_2$  in subject 1



**Figure 5.15.** Breath  $^{13}\text{CO}_2$  after ingestion of biscuits containing 500 mg lactose- $^{13}\text{C}$ ureide and biscuits containing 300 mg labelled pea flour in subject 1

**Table 5.1.** Area under the curve of breath  $^{13}\text{CO}_2$  (ppm XS  $^{13}\text{C.h}$ ) and percentage of total dose of  $^{13}\text{C}$  recovered with mathematical calculation\* (Morrison et al., 1998) using the equation  $y = a.(t-d)^b.e^{[-c(t-d)]}$  in breath after ingestion of 300 mg labelled pea flour

Subjects	1		2		3		4		5		6		<i>In vitro</i> measurement of starch in pea flour
	AUC $^{13}\text{CO}_2$	*PDR	AUC $^{13}\text{CO}_2$	*PDR	AUC $^{13}\text{CO}_2$	*PDR	AUC $^{13}\text{CO}_2$	*PDR	AUC $^{13}\text{CO}_2$	*PDR	AUC $^{13}\text{CO}_2$	*PDR	
	(ppm XS $^{13}\text{C.h}$ )	(%)	(ppm XS $^{13}\text{C.h}$ )	(%)	(ppm XS $^{13}\text{C.h}$ )	(%)	(ppm XS $^{13}\text{C.h}$ )	(%)	(ppm XS $^{13}\text{C.h}$ )	(%)	(ppm XS $^{13}\text{C.h}$ )	(%)	(g/100g) (%)
<b>Peak 1</b> (small intestine)	51.3	58.0	53.8	49.0	46.0	60.1	48.6	61.2	46.2	47.7	62.1	71.4	RDS + SDS 40.6 78.1
<b>Peak 2</b> ? (large intestine)	20.5	14.1	26.5	24.2	18.3	16.0	29.6	22.3	24.7	29.5	19.2	18.8	
<b>Peak 3</b> ? (large intestine)	28.2	27.9	19.7	26.8	35.7	23.9	21.8	16.5	29.1	22.8	18.7	9.8	RS 11.4 21.9
<b>Total</b>	100	100	100	100	100	100	100	100	100	100	100	100	52.0 100



**Figure 5.16.** Cumulative PDR calculated mathematically by Morrison et al., (1998) using the equation:  $y = a.(t-d)^b.e^{[-c(t-d)]}$

### 5.3. Discussion

Previous attempts to measure starch digestibility and colonic fermentability depended on ileostomy patients who may not be "normal", as they have adapted to compensate for the lack of a colon, which may result in changed transit and absorption rates as well as bacterial contamination of the end of the ileum. Studies of normal subjects who have swallowed multi-lumen tubes which reached the terminal ileum have also been used. It has been shown that the tube in this case may speed up transit and affect digestibility (Read et al., 1983). Our approach using stable isotopes to quantify starch digestion and fermentation, is the only method to allow use of non-invasive techniques. We have produced successfully high enrichment of  $^{13}\text{C}$ -labelled pea flour by photosynthetic incorporation of  $^{13}\text{CO}_2$  (explained in chapter 4) for use in human studies. It was clear that 300 mg of this labelled pea flour was sufficient to produce the measurable amount of  $^{13}\text{CO}_2$  in breath (explained in chapter 4).

In this chapter 300 mg labelled pea flour was ingested in five more healthy subjects and the results of  $^{13}\text{CO}_2$  in breath showed a complex of three peaks. The first peak occurred over the first 6 hours after ingestion of 300 mg labelled pea flour biscuits. This peak was a result of digestion and absorption of starch in the small intestine. Theoretically, this should represent the absorption of the

rapidly digestible starch; RDS: 14.4% and slowly digestible starch fractions ; SDS: 63.7% in the labelled pea flour. In addition, the labelled pea flour also contains 20% protein thus the first peak could show the small intestinal digestion of protein as well, if the protein was labelled. In chapter 4 we could not completely identify what was labelled but it did appear that some protein at least was  $^{13}\text{C}$  labelled. The timing of the first peak is in agreement with the observation of Harding et al., (1994), after ingestion of 400 mg  $^{13}\text{C}$ -enriched wheat flour biscuits in a healthy subject.

The second peak occurred between 7 and 11 hours and in the most of subjects this peak coincided with the rise in breath hydrogen, indicating the test meal had entered the colon and was being fermented. This peak appeared as a shoulder on peak one in most subjects. Confirmation that the second peak represented colonic fermentation, was obtained with lactose- $^{13}\text{C}$ ureide in one subject (subject one). Lactose- $^{13}\text{C}$ ureide resists breakdown in the small intestine, but is split by the colonic microflora to glucose and urea which is then absorbed and metabolised to  $^{13}\text{CO}_2$  (Merry et al., 1982). This  $^{13}\text{C}$  labelled ureide is given in only trace amounts and therefore, can be used without contribution to the volume of the test meal. However, in the present protocol as both  $^{13}\text{C}$  pea flour and lactose- $^{13}\text{C}$ ureide produce  $^{13}\text{CO}_2$  they must be studied on different days. Thus, in our present experiment  $\text{H}_2$  was a better marker for colonic transit. In addition it has been suggested that lactose

ureide needs induction of bacterial enzymes and use of a single dose may exhibit a lag phase between arrival in the colon and fermentation (Heine et al., 1995; Wutzke et al., 1997).

These results suggest that the second peak of  $^{13}\text{CO}_2$  after pea flour may represent easily fermentable carbohydrate in the colon. Fermentation of fibre (Levitt et al., 1987; Olesen et al., 1992) occurs earlier than that of resistant starch. The second peak may therefore result from fermentation of fibre in labelled pea flour or some of the slowly digestible starch and any easily fermented resistant starch.

The third peak of  $^{13}\text{CO}_2$  in breath happened much later between 12 and 20 hours in individual subjects. In most of the subjects this peak occurred when they were asleep so we could not get full information about this peak. The third peak may reflect fermentation of resistant starch (RS: 21.9%) in the colon and the late timing of this peak is in agreement with previous reports of breath hydrogen from resistant starch fermentation (Olesen et al., 1994).

*In vitro* fermentation of resistant starch in previous studies has shown that raw starches are fermented much more slowly than cooked starch (Edwards et al 1996b).

The fact that our *in vivo* data would estimate more resistant starch in the pea flour than the *in vitro* Englyst method (Englyst et al., 1992) suggests that the *in vitro* method overestimates the digestibility of some starches. The *in vitro* assay is designed to mimic small intestinal digestion of starch, but is a long and complicated assay with many steps requiring precise timing and shaking speeds. The method is particularly sensitive to variations in shaking speed and in this study it took several days to calibrate the shaking speed of water bath to give correct values for the standards provided. We do not know the equivalent of the shaking speed *in vivo* and there must be much variation from person to person and from day to day.

The digestibility of starch in our pea flour measured by the *in vitro* method (Englyst et al., 1992) was 78.1%. This is in contrast to the 57.9% PDR of  $^{13}\text{C}$  obtained by the first peak in our *in vivo* study. This first peak represents small intestinal digestion and absorption and should be equivalent to the sum of rapidly and slowly digestible starch in the *in vitro* assay. Moreover, the *in vitro* assay (Englyst et al., 1992), showed that pea flour contains 21.9% of RS fraction. The mean cumulative PDR of  $^{13}\text{C}$  in breath of all subjects from the second and third peaks showed 42.1%, which was nearly double the results of *in vitro* assay (21.9%). Therefore, the value of digestible starch fractions in our pea flour by Englyst method (Englyst et al., 1992), was overestimated, and the value of RS was underestimated. The Englyst method has been compared

previously with ileostomy models. While with some foods the *in vitro* and *in vivo* methods show similar results, with other foods there is some underestimation of RS (Table 5.2).

**Table 5.2.** *In vitro* and *in vivo* quantification of resistant starch (RS/100g)

Source of starch	<i>In vitro</i> (Englyst et al 1992)	<i>In vivo</i> (ileostomy)	References
Raw potato starch	54.1	67.9	Langkilde & Andersson, (1995)
Beans flakes	4.6	9.0	Schweizer et al., (1990)
Raw green banana	54.2	55.3	Langkilde & Andersson, (1995)

According to the results of area under the curve of breath  $^{13}\text{CO}_2$  in this study, in most of the subjects more than 40% of  $^{13}\text{C}$  from the labelled pea flour entered the colon (42.1% from mathematical calculation of PDR).

The higher than expected values for the second and third peaks could also be due to fibre fermentation. It is likely fibre contributed more to the second peak than the third. Peak 3 was not measured in all subjects but extrapolated from data points. This is most important for subjects 4, 5 and 6. If we consider only peak 2, this would still be equivalent to more RS than *in vitro*. In subjects 1, 2 and 3 a third peak is very evident so peak 2 is not the only  $^{13}\text{CO}_2$  produced from fermentation and given the timing is unlikely to be due to RS.



In this study, we did not recover all the  $^{13}\text{C}$  in our pea flour. In previous human studies with  $^{13}\text{C}$  labelled substrates (Harding et al., 1994), 400 mg of  $^{13}\text{C}$  labelled wheat flour was fed to one healthy subject and breath samples collected over 6 hours. PDR of  $^{13}\text{C}$  in breath was 23-27%, less than PDR we got in this study (41.6%), this is probably due to the longer collection period in our experiment. In previous animal studies with rats (Gray et al., 1993b), 4 mg of  $^{14}\text{C}$ -labelled plant cell wall from spinach was fed to three rats as a marker for plant cell wall metabolism. After 24 h, 22% of the  $^{14}\text{C}$  was recovered in the liver, adipose tissue and skin, 26% excreted as  $^{14}\text{CO}_2$  and up to 18% was excreted in the faeces. But this was labelled dietary fibre so in our study when starch, protein and fibre was potentially labelled we would expect more  $^{13}\text{CO}_2$  in breath and perhaps more to be accumulated in the body tissues with less appearing in faeces.

One of the problems with the  $^{13}\text{CO}_2$  breath test is that we assume constant  $\text{CO}_2$  out put in breath over the test day. The amount of  $\text{CO}_2$  produced per minute was measured on a Deltatrac indirect calorimeter. However, although this may be acceptable for short term studies when the subject is lying down quietly all day, in our study subjects were moving around and eating second and third meals. Thus, we have estimated  $\text{CO}_2$  production as  $\text{RMR} \times 1.4$  (PAL).

RMR = resting metabolic rate, PAL = physical activity level,

PAL 1.4 is equivalent to sedentary activity. This calculation, however, is still

very imprecise. Ideally the CO<sub>2</sub> production should be measured throughout the day either by indirect calorimetry (Deltatrac) which is impractical, by measuring respiration with strain gauge around the chest or by measuring physical activity for example with a questionnaire or heart rate monitor. None of these methods will give the exact CO<sub>2</sub> produced but may be more accurate than our assumption of constant CO<sub>2</sub> production.

In summary, in this study, there are several limitations in interpreting the data:

- a) Complete separation of starch from fibre and protein was not achieved, therefore it is difficult to determine from which component of labelled pea flour each of the <sup>13</sup>CO<sub>2</sub> breath peaks resulted.
- b) It is not possible to identify if the <sup>13</sup>CO<sub>2</sub> which was produced in the 2nd and 3rd peaks after ingestion of 300 mg labelled pea flour was directly from fermentation of <sup>13</sup>C enriched pea flour or from human cells oxidising labelled short chain fatty acids.
- c) The late timing of the third peak in breath <sup>13</sup>CO<sub>2</sub> prevented collection of the full information about the height and duration of the peak in subjects because they were asleep. This meant that mathematical modelling was necessary and extrapolation of data points may have overestimated fermentation. This modelling must be validated in further studies.

However, it may be concluded that pea flour can be successfully labelled with <sup>13</sup>C. Use of <sup>13</sup>C-breath tests following intake of <sup>13</sup>C-labelled starchy foods, can

give valuable information on the metabolism of these foods over a long period in man and may provide a better bio marker for fermentation than plasma acetate and breath hydrogen. Moreover, we achieved a new method in addition to previous *in vivo* models (ileostomy patients and intubation) for measurement of the digestion and fermentation of starchy foods by using the AUC of breath  $^{13}\text{CO}_2$  and estimated mathematical calculation of cumulated PDR from each peak of breath  $^{13}\text{CO}_2$ .

# **CHAPTER SIX**

## General discussion and conclusion

## 6. General discussion and conclusion

The importance of the fermentation of RS as a potential preventive agent against colonic disease is becoming increasingly evident. This has led in recent years, to manufacturers producing RS containing ingredients for incorporation into foods to increase the "dietary fibre" content. RS is easier to incorporate into foods without changing the physical structure and appearance than dietary fibre. However RS is not one substance but may be one of three types as discussed earlier and each varies in its fermentability and therefore action in the gastrointestinal tract. Moreover, incorporation of RS into processed foods may alter its properties and therefore its impact on gut function. It is therefore important that we can measure the potential physiological effects of these RS in foods.

The major impact of RS is in the colon where they are fermented by the colonic bacteria. Both *in vivo* and *in vitro* studies have shown that fermentation of starch increases the amount of butyrate formed compared with the other SCFA (Englyst et al., 1987*b*; Scheppach et al., 1988*b*). Butyrate is the major source of energy for the colonocytes (Roediger, 1982) and is thought to be essential in maintaining the health of the colonic mucosa.

Scheppach et al., (1992) and others (Steinhart et al., 1994) have shown that

butyrate enemas improve the conditions and symptoms of the colonic mucosa in patients with distal ulcerative colitis. Butyrate is also thought to play a key role in the prevention of colonic cancer. It prolongs doubling time and reduces the growth rate of human colorectal cancer cell lines (Whitehead et al., 1986) and *in vivo* (Weaver et al., 1988) and *in vitro* (Clausen et al., 1991) studies have shown a significantly lower ratio of butyrate to total SCFA in polyp-colon cancer patients than that in normal subjects.

Butyrate has two contrasting effects on colonic epithelium. In normal colonic epithelium it is used as the preferred source of energy and stimulates the growth of colonic mucosa, whereas, in colonic tumour cell lines it acts as a growth inhibitor and may induce differentiation and stimulate apoptosis (programmed cell death) (Hague & Paraskeva, 1995).

If RS is to be used in food or as a supplement to promote colonic health and prevent colonic cancer, we must be able to measure its fermentation to allow evaluation of new food sources.

The major problem with measuring *in vivo* fermentation of RS is the inaccessibility of proximal colon, where the fermentation occurs. The end products of RS fermentation are short chain fatty acids (SCFA), carbon dioxide, hydrogen and methane (Englyst & Macfarlane, 1986*b*). There is no

non-invasive way to measure RS fermentation in healthy human subjects. An alternative approach is to look at fermentation indirectly by measuring products of fermentation in the blood or excreted in breath and faeces. The main aim of this PhD thesis was to develop a method for measuring RS fermentation *in vivo*.

### **6.1. Plasma acetate and breath hydrogen**

Our initial approach was to measure breath H<sub>2</sub> and plasma acetate at the same time (chapter 3). This approach was based on the work of other authors (Muir et al., 1995b) who had measured single time point samples of plasma acetate, and the successful use of plasma acetate to measure fermentation of dietary fibres (Pomare et al., 1985). We developed the method to take serial plasma acetate measurements and compared them with breath hydrogen. This method, however, had several problems.

The main problems were:

- 1) Finding an appropriate standard for RS fermentation studies. In our initial study, we found that lactulose had a short transit time (TT: within 0.5-1 h) and rapid fermentation completely different to that of RS fermentation. Lactulose is not a suitable standard for fermentation studies with RS. We therefore chose guar gum as the standard. The fermentation rate was slower than lactulose, but guar gum still produced rises in breath hydrogen and acetate at 4

hours which was much earlier than the 11 hours taken with RS. There does not seem to be a really suitable standard for RS.

2) The very slow fermentation of RS caused problems of compliance and with the practicalities of blood collection. It was a very long study day during which the subjects had an indwelling intravenous catheter in their forearm. Although flushed with heparin the length of the study increased the rate of blockage of the cannula and necessitated medical supervision for many hours. Moreover subjects could not eat ad libitum and became bored and unsettled.

3) The long delay in RS fermentation made it difficult to capture the initial rise in  $H_2$  and acetate when we adjusted the protocol to minimise the above problems. When subjects ate the test meal the night before the study day in two subjects the  $H_2$  and plasma acetate had already risen before the first measurement although we did not anticipate a rise until after 10 hours.

4) The delayed fermentation of RS also caused problems in interpreting the plasma acetate curves. There was little correlation between breath hydrogen and plasma acetate after RS. The difference between the rises in the two measurements could be several hours. One reason for this could be the long time for fermentation to occur and the difficulty to distinguish between fed and fasted acetate ie that from fermentation and that from the liver. There were



long gaps between low calorie meals during our study. In the Pomare et al., (1985) study of plasma acetate after pectin ingestion over 24 hour, the mean values shown have a good match of plasma acetate and breath hydrogen. However individual data were not reported. In that study meals were given every four hours and drinks, which may have contained sugar, every 2 hours. Thus endogenous acetate production was suppressed. In our study with much longer fasting periods endogenous acetate production may have been more variable. In future studies small meals throughout the study day may improve this method.

One approach to this problem would be to use stable isotope labelled substrates. As part of the work for this thesis I explored the possibility of measuring  $^{13}\text{C}$  acetate in plasma. I was able to measure  $^{13}\text{C}$  acetate, propionate and butyrate from *in vitro* fermentation of  $^{13}\text{C}$  labelled wheat starch (which resulting in 5.7% atom % excess for acetate, 7.2% for propionate and 6.2% for butyrate) but did not have time (or machine availability) to develop the sensitive methods needed to determine enrichment of the low levels of acetate in plasma.

Our results did, however, confirm previous studies that RS fermentation is delayed with the first rise in breath hydrogen and acetate occurring about 11 hours after ingestion. *In vitro* studies of raw potato starch fermentation by

human faecal microflora by Edwards et al., (1996b) demonstrated that raw potato starch (RS<sub>2</sub>) has a slow rate of fermentation. Therefore, differences between RS and fibre in apparent TT and appearance peak of plasma acetate and breath hydrogen are likely to be related to greater resistance of RS to bacterial enzymes degradation in the colon than fibre. This slow fermentation of RS<sub>2</sub> in the human colon is in contrast to previous piglet studies (Heijnen et al., 1997b) which showed that RS<sub>2</sub> was fermented in the proximal colon. This may mean that the piglet is not a suitable model for RS fermentation in man. However, although it is established that in human RS fermentation is delayed we can not yet tell if fermentation of RS occurs in the proximal or the distal colon. It is not possible to determine this with a non-invasive method.

The area under the curve (AUC) of breath hydrogen after one week ingestion of raw potato starch was smaller in 4 out of 5 subjects than before adaptation period (although differences were not statistically significant) suggesting altered pathways for hydrogen disposal. Similar results have been reported from previous studies. After several days of ingestion of 20g lactulose (Florent et al., 1985) and 45g high amylose maize starch; RS<sub>2</sub> (Van Munster et al., 1994a) less breath hydrogen was produced than on the first day of ingestion. However apart from a higher fasting H<sub>2</sub> level reported by Olesen et al., (1994), there is very little data confirming an adaptation to prolonged feeding of RS. This would indicate that RS is likely to be slowly fermented in

the human colon even with constant ingestion which may make it a useful addition to the diet as a provider of slowly released SCFA.

This method showed great variation in breath hydrogen (ppm) and plasma acetate ( $\mu\text{mol/l}$ ) responses which made it impracticable as a repeatable method for monitoring RS fermentation. Therefore, better bio-markers for measurement of the digestibility and fermentability of starchy foods were needed. I had to choose between spending more time on developing the plasma acetate method with larger numbers of subjects and more frequent samples or to take a completely new approach. I therefore decided to explore the use of stable isotopes.

## **6.2. Stable isotope method**

A novel approach to measuring fermentation of carbohydrate *in vivo* is to use stable isotope labelled carbohydrate sources. These had been used previously to measure starch digestibility but ours was the first study to use stable isotope enriched starch to measure fermentation.

To develop this method (chapter 4) peas and potatoes were chosen because of their potential high RS content and faster rate of growth. The plants were enriched with  $^{13}\text{C}$  by photosynthetic incorporation of  $^{13}\text{CO}_2$  in enriched air with the plants growing in  $\text{CO}_2$  impermeable bags during the pod/tuber filling

stage.

We were not successful in labelling our potato (the mean atom % excess of  $^{13}\text{C}$  enrichment in our potato flour was 0.71%) because the potato plants could not tolerate the conditions in the polypropylene bags. After six days of incubation with 500 ml  $^{13}\text{CO}_2$ , most of the leaves turned yellow. When the bags were removed and potato plants rested for one week, it was clear that the potato plants could not be incubated with  $^{13}\text{CO}_2$  for a second time. A more sophisticated method for labelling potato plants is needed. Potato plants were chosen because of their high starch content and their low content of protein and fibre (most of the fibre is easily removed by peeling off the skin). For this reason, it would be very worthwhile improving the labelling process in potato plants for future studies. This could be achieved by growing more compact varieties and by using a bigger container for the enriched atmosphere. The disadvantage of this is that a greater volume of  $^{13}\text{CO}_2$  will be needed which will increase the costs substantially. It may be possible to place only one or two leaves in the  $^{13}\text{CO}_2$  atmosphere and still achieve sufficient labelling. The plants could also be placed in the atmosphere for a greater number of shorter periods (eg 1 day) with rest periods in between.

We were however highly successful in labelling the Pea plants. The mean atom % excess of  $^{13}\text{C}$  enrichment in our pea flour after twice labelling was

8.64. We also achieved 2.4% excess after only 6 days in the enriched atmosphere. This may be sufficient for most studies.

To fully investigate the fermentation of RS it is necessary to know what in the peas was labelled. However our attempts to separate the different components of the pea flour were not totally successful. Although we managed to remove some of the protein by enzymic and chemical digestion we could not remove all the protein. However, we were able to establish that it was not only the starch that was enriched with  $^{13}\text{C}$  but also the protein and fibre components. This makes the breath tests more difficult to interpret and this problem needs to be addressed in further studies. It is not clear why our separation techniques were unsuccessful but this must be related to the close integration of the molecular species within the plant material.

An initial pilot study, over 16½ hours (h) after ingestion of  $^{13}\text{C}$ -labelled pea flour in one subject demonstrated a complex of three apparent peaks. This confirmed that only a small quantity (300 mg) of our labelled-pea flour was needed to produce measurable  $^{13}\text{CO}_2$  excretion for breath test analyses.

The next step was to feed 300 mg labelled-pea flour to 5 more subjects and to collect breath samples for a longer period (chapter 5) to develop the method

using  $^{13}\text{CO}_2$  for assessing RS and for quantification of colonic fermentation.

The results of  $^{13}\text{CO}_2$  in breath confirmed the appearance of a complex of three peaks. The first peak represented the digestion and absorption of starch in the small intestine and corresponded with the peaks seen in previous studies with wheat flour and naturally enriched corn flour (Hiele et al., 1990; Harding et al., 1994). The second and third peak occurred after the rise in breath hydrogen was seen, suggesting they could result from colonic fermentation. Moreover, ingestion of lactose- $^{13}\text{C}$ ureide, a substrate which escapes digestion in the small intestine but is released by colonic microflora and metabolised to  $^{13}\text{CO}_2$ , confirmed that the second and third peak occurred after the meal had reached the colon.

To determine the contribution of small intestinal digestion and colonic fermentation to the total cumulative percentage of administered dose of  $^{13}\text{C}$  recovered (PDR), the three peaks were analysed in two ways. First the area under the curve (AUC) of breath  $^{13}\text{CO}_2$  (ppm XS  $^{13}\text{C.h}$ ) was calculated for each peak. The third peak was extrapolated from the data points available. Secondly, values of estimated cumulative PDR were calculated using a mathematical model:  $y = a.(t-d)^b.e^{[-c(t-d)]}$  (Morrison et al., 1998).

The AUC of breath  $^{13}\text{CO}_2$  (ppm XS  $^{13}\text{C.h}$ ) and using the mathematical

calculation (Morrison et al., 1998) suggested that over 40% of the  $^{13}\text{CO}_2$  was produced from fermentation or fermentation products.

The first peak should correspond to the amount of RDS and SDS fractions in our pea flour. The second and the third peaks should be equivalent to the amount of RS. Measurement of starch digestibility using *in vitro* models (Englyst et al., 1992) showed values of RDS and SDS equal to 78.1%, whereas, during the  $^{13}\text{CO}_2$  breath test the first peak accounted for only 57.9% of the accumulated PDR. Conversely, the second and third peaks corresponded to 42.1% and RS *in vitro* was only 21.9%.

The Englyst method for starch digestibility has been compared previously with ileostomy models, and although similar results for RS were obtained for some foods with other foods there was some underestimation of RS (**Table 5.2**). Our *in vivo* study suggests that the Englyst method underestimates the RS component of our pea flour perhaps by overestimating the slowly digestible starch fraction. This needs to be confirmed with pure starch sources but suggests that our non-invasive method may be a useful tool for estimating digestible and resistant starch.

Therefore, the value of digestible starch fractions in our pea flour appeared to be overestimated and the value of RS underestimated by Englyst method

(Englyst et al., 1992). Indeed as some of the label was known to be associated with protein we would have expected the RDS to be overestimated by the  $^{13}\text{CO}_2$  method.

The higher values for the second and third peaks could also be due to fibre fermentation, (which may have been enriched with  $^{13}\text{C}$ ). It is likely the fibre contributed more to the second peak than the third one. Peak 3 was not measured in all subjects but extrapolated from data points. If we consider only peak 2, this would be more equivalent to the values gained for RS *in vitro* but the early timing of peak 2 makes it unlikely to be due to RS fermentation which has been shown by us and others to occur after approx. 11 hours. Indeed the occurrence of a third peak after 11 hours provides circumstantial evidence for this being RS fermentation.

The breath  $^{13}\text{CO}_2$  tests make several assumptions which need further validation. Apart from the assumptions about constant rate of release of  $\text{CO}_2$  from the various bicarbonate pools in the body, we also assume constant  $\text{CO}_2$  output throughout the study day. This is obviously a great simplification and needs to be validated in further studies. The assumption may be acceptable for short term studies when the subject is lying down quietly all day. In our study subjects were moving around and eating second and third meals. Ideally the  $\text{CO}_2$  production should be measured throughout the day. This could be



achieved by *a*) constant monitoring with the indirect calorimeter, although this is not really practical for such long studies, *b*) by measuring activity with a heart rate monitor, but at low activity levels heart rate does not correlate well with energy expenditure or by measuring respiration rate as well as the CO<sub>2</sub> content of each breath sample. To allow for the activity occurring in our studies we have use 1.4 RMR to allow for a mild physical activity level. The choice of PAL varies between studies and is often not even given. The PAL can make more of a difference to the accumulative PDR than the actual measurement of enrichment. For example the total PDR using a PAL of 1 in our study would have been about 25% but with a PAL of 1.4 becomes 40-60%. Thus it is important to look at relative proportions of dose recovered for each peak rather than absolute amounts until we have established true CO<sub>2</sub> production by a more sophisticated method.

The length of our study makes it difficult to quantify the third peak without extrapolation from a few data points. In some subjects this was reasonable but in others the data available was very limited. The mathematical calculation of the peaks could help recover data where some is lost during sleep but it will be important to keep data loss to a minimum by allowing only very short sleep periods. This does mean that resistant starch studies in future will need to take place over 24 hours. This could be achieved by keeping subjects in a metabolic ward or training them to take samples at home.

From the results of several studies in this thesis, it is concluded that using  $^{13}\text{C}$  labelled starchy foods and the amount of  $^{13}\text{C}$  enrichment in breath  $^{13}\text{CO}_2$  ( $^{13}\text{C}$ -breath test) is a much more sensitive and accurate method for digestion and fermentation than using either breath hydrogen or plasma acetate alone or in combination. The  $^{13}\text{CO}_2$  breath test could also be used to measure RS content of foods, however, it would be necessary to validate the  $^{13}\text{CO}_2$  method against more established *in vivo* measurement of RS including the ileostomy and intubations models.

The use of  $^{13}\text{C}$  labelled starchy foods offers a safe, simple and non-invasive way of assessing metabolism of starchy foods in man. The expense of production  $^{13}\text{C}$  labelled foods is a major problem of this new technique. The  $^{13}\text{CO}_2$  gas is very expensive. However as more stable isotope work is performed these costs may decrease.

In the future stable isotope techniques may revolutionise human metabolic studies. The safe tracers produced will allow studies of individual nutrients *in vivo* in subjects of all age groups and medical conditions. For fermentation studies we should aim to produce stable isotope labelled forms of all types of starch, dietary fibres, both soluble and insoluble, fermentable and non-fermentable as well as oligosaccharides and other potential bacterial substrates. Then we can use these tools to investigate fermentation and the

fate of fermentation products in man. We can look at interactions of different fermentable carbohydrates by comparing labelled and non-labelled different fibres and we should be able to identify the factors determining the production of individual SCFA and other bacterial products. Eventually as new techniques to measure low levels of  $^{13}\text{C}$  enrichment of a variety of complex molecules are developed (including bacterial constituents, plant cell wall polysaccharides, bile acids and sterols, human cellular contents) we should be able to get a much better characterisation of the complex metabolism of the diverse ecosystem in the human colon. This will help us manipulate our own colonic flora to promote health and prevent disease.

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## Communications

### Abstract:

Pea flour digestibility and fermentability measured with  $^{13}\text{C}$  isotopes. (1998)

Edwards C.A., Zavoshy R. & Preston T. *Biochemical Society Transactions*, **26**, S185.

Morrison D.J., Zavoshy R., Edwards C.A., Dodson B., Preston T. & Weaver L.T. (1998)

Lactose- $^{13}\text{C}$ ureide as a marker for colonic fermentation and the deconvolution of a complex  $^{13}\text{CO}_2$  breath test curve. *Biochemical Society Transactions*, **26**, S184.

### Presentations:

Pea flour digestibility and fermentability measured with  $^{13}\text{C}$  isotopes.

Edwards C.A., Zavoshy R. & Preston T.. Biochemical Society & Nutrition Society, Winter meeting; 15-19 December 1997, University of Reading.

Morrison D.J., Zavoshy R., Edwards C.A., Dodson B., Preston T. & Weaver L.T. Lactose- $^{13}\text{C}$ ureide as a marker for colonic fermentation and the deconvolution of a complex  $^{13}\text{CO}_2$  breath test curve. Biochemical Society & Nutrition Society, Winter meeting; 15-19 December 1997, University of Reading.

# 119 Pea flour digestibility and fermentability measured with $^{13}\text{C}$ isotopes.

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It is well established that some starches are resistant to human digestive enzymes and enter the large intestine where they may be fermented by the colonic bacterial flora to short chain fatty acids and gases ( $\text{CO}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ) [1].

It is difficult to measure the digestibility and fermentability of starch foods in vivo, in normal man, and most studies have been performed with ileostomy patients or animal studies. Use of breath hydrogen to quantify the malabsorption of carbohydrate is not quantitative as hydrogen production can be influenced by many other factors than the carbohydrate source.

An alternative approach is to use  $^{13}\text{C}$  labelled starchy foods and to measure the  $^{13}\text{C}$  enrichment of breath  $\text{CO}_2$ . This is released by human cells from the metabolism of glucose, indicating small intestine digestion and absorption, or from bacterial fermentation of carbohydrate in the colon. Fermentation may release  $\text{CO}_2$  directly or indirectly by human metabolism of the short chain fatty acids produced.

Pea plants (Bacarra variety) were grown and as soon as pods began to form were placed in a  $^{13}\text{CO}_2$  enriched environment in polypropylene bags, (Propylene CD 35; ICI Welwyn Garden City UK) approx 154 x 30 x 45 cm to fit individual plants, and sealed air-tight. 250 mls of  $^{13}\text{CO}_2$  (99 atom %; Cambridge Isotope Laboratories, Welwyn Garden Cit

y) were added and the bags were then filled to capacity with room air using an air pump. The plants were incubated for 6 days on two occasions separated by 1 week. Peas were allowed to ripen under normal conditions and the peas harvested and dried to form pea flour, enriched by 8.64 atom %  $^{13}\text{C}$  excess.

After an overnight fast, six human subjects ingested 300mg of enriched pea flour contained in 175g biscuits. The digestibility of starch, measured in an in vitro model, was 7.5% rapidly digestible starch, 33.1% slowly digestible starch and 11.4% resistant starch.

Breath samples were taken every 30 mins for up to 24 hours, with a gap when subjects were asleep, and analysed for hydrogen and  $^{13}\text{CO}_2$  enrichment. Subjects were asked to avoid naturally  $^{13}\text{C}$  enriched foods but were allowed to eat a low fat/low fibre lunch and evening meal.

The profiles of enrichment of  $^{13}\text{CO}_2$  in breath showed a complex of three apparent peaks. Each peak was not easily separated by eye for every subject and the second peak usually formed a shoulder on the trailing end of the first peak. However

this second peak occurred as the breath hydrogen began to rise indicating the meal had entered the large intestine. Fig 1 shows a typical profile.

The third peak in  $^{13}\text{CO}_2$  enrichment occurred much later between 12 and 20 hours in individual subjects. In some subjects this occurred when they were asleep and so critical information about the height and duration of the peak was lost. The late timing of this peak is consistent with previous reports of breath hydrogen from fermentation of resistant starch [2].

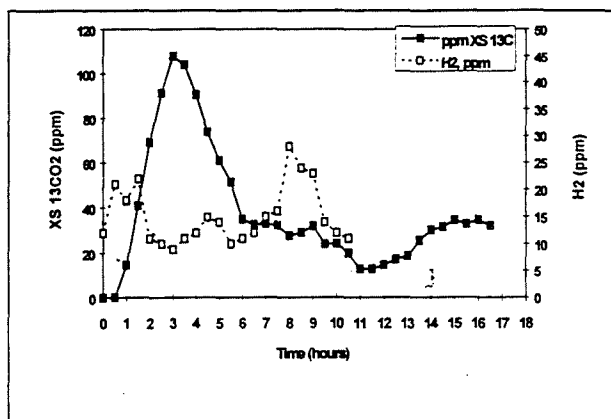


Figure 1 Breath  $^{13}\text{CO}_2$  and  $\text{H}_2$  after ingestion of biscuits containing 300mg labelled pea flour in one human volunteer

Mathematical modelling [3] and longer sampling periods, to separate small intestinal and colonic events, should allow this method to quantify digestibility and fermentability of starchy foods in vivo.

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# 118 Lactose [<sup>13</sup>C]Ureide as a marker for colonic fermentation and the deconvolution of a complex <sup>13</sup>CO<sub>2</sub> breath test curve.

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The digestion and fermentation by colonic microflora of dietary starch has been studied, in an adult subject, using a <sup>13</sup>CO<sub>2</sub> breath test, after ingestion of 300 mg <sup>13</sup>C enriched uncooked pea flour [1]. Modelling of the data, generated by IRMS analysis of breath, can yield information on the site and extent of <sup>13</sup>C recovery in the breath.

<sup>13</sup>CO<sub>2</sub> breath test data generated from gastric emptying studies have been routinely modelled by the equation  $y = a \cdot t^b \cdot e^{-ct}$ , where  $a$  is a scaling factor and variables  $b$  and  $c$  describe the shape of the curve [2]. In this study a new variable,  $d$ , has been introduced to facilitate the fitting of multiple peaks. The expression that describes a peak now becomes  $y = a \cdot (t-d)^b \cdot e^{-c(t-d)}$ . The model can be further simplified by assuming that all <sup>13</sup>CO<sub>2</sub> is excreted via a common body pool therefore the variable  $c$ , which describes the decay of a curve, will be the same for all curves. Non linear regression analysis was used to minimise the relative residual values between  $Y_{data}$  and  $Y_{model}$ , where  $Y_{data}$  is the measured experimental value and  $Y_{model} = a_1 \cdot (t-d_1)^{b_1} \cdot e^{-c(t-d_1)} + a_2 \cdot (t-d_2)^{b_2} \cdot e^{-c(t-d_2)} + \dots + a_n \cdot (t-d_n)^{b_n} \cdot e^{-c(t-d_n)}$ .

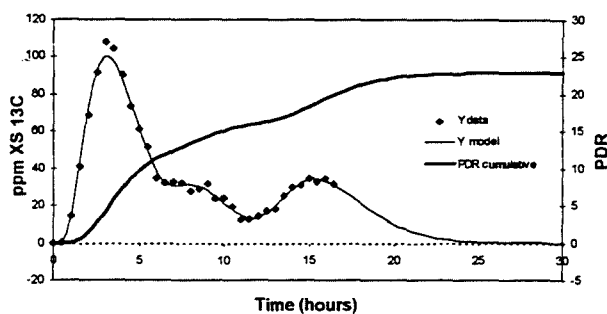


Figure 1. Non linear regression fit of  $Y_{model}$  to the experimental data with the cumulative PDR plotted on the secondary axis.

## Abbreviations used:

[ <sup>13</sup> C]LU	Lactose [ <sup>13</sup> C]Ureide
IRMS	Isotope Ratio Mass Spectrometry
PDR	Percentage dose recovered

Table 1. Results of non linear regression analysis

	a	b	c	d	t <sub>max</sub>	PDR
				(hours)	(hours)	%
peak 1	107.02	2.73	1.03	0.42	3.07	13.26
peak 2	13.97	3.24	1.03	5.63	8.78	3.22
peak 3	0.41	5.89	1.03	9.75	15.48	6.37

Using the SOLVER function of Microsoft Excel 5.0, this model was used to analyse the breath <sup>13</sup>CO<sub>2</sub> data. A three peak model produced the best fit to the pea flour data. The root mean square [3] value which is an index of goodness of fit was < 0.1. The parameter  $t_{max}$  ( $b/c+d$ ) was used to define the time of maximal <sup>13</sup>CO<sub>2</sub> excretion for a given peak [2].

In this set of data where  $Y_{data}$  values were still above baseline at 16.5 hours, the time course of the model data was extrapolated until  $Y_{model}$  returned to baseline (Figure 1). The cumulative PDR and the PDR for each peak were calculated (Table 1).

[<sup>13</sup>C]LU, a substrate that resists digestion in the small intestine, can be used as a marker for colonic fermentation [4]. A 500 mg dose of [<sup>13</sup>C]LU, given several days after the previous test, was used to indicate which peaks may have resulted from colonic fermentation. <sup>13</sup>C enrichment above baseline was observed at 5.5 hours suggesting peaks 2 and 3 were the result of colonic fermentation. [<sup>13</sup>C]LU was synthesised by the acid catalysed condensation of lactose with [<sup>13</sup>C]urea [5]. Recrystallisation yielded 65% product. IRMS analysis [6] showed chemical purity ≥ 99% and isotopic enrichment ≥ 99%.

This model permits mathematical analysis of complex <sup>13</sup>CO<sub>2</sub> breath test data and has been used to ascertain the contribution of each peak to the total PDR. Furthermore, [<sup>13</sup>C]LU breath test data can determine which peaks may have resulted from colonic processes.

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