Physiological effects of consumption of resistant starch

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Stellingen

1. Resistant starch is een voedingsvezel. *(o.a. dit proefschrift)*

2. Consumptie van ongekookt of geretrogradeerd onverteerbaar zetmeel in realistische hoeveelheden heeft bij gezonde mensen geen effect op het serum-cholesterolgehalte. *(dit proefschrift)*

3. Consumptie van ongekookt onverteerbaar zetmeel verhoogt de schijnbare absorptie van magnesium bij ratten. Dit wordt niet veroorzaakt door een toename van de oplosbaarheid van magnesium in de digesta via een door fermentatie geïnduceerde pH-verlaging. *(dit proefschrift)*

4. Er is geen experimentele onderbouwing voor de suggestie dat een toename van het aantal bifidobacteriën in het colon van de mens gunstig is voor diens gezondheid.

5. Het functional food-concept ondermijnt de voorlichtingsinspanningen om de consument te leren dat niet de afzonderlijke voedingsmiddelen, maar juist de totale voeding in meer of mindere mate als gezond kan worden beschouwd.

6. De hoeveelheid acetylsalicylaat in voeding is te laag om het risico op hart- en vaatziekten te beïnvloeden.

7. In laboratorium-toxiciteitsproeven, bedoeld om de ecotoxicologische normering van bodemverontreiniging te onderbouwen, is het gebruik van natuurlijke gronden te verkiezen boven het gebruik van OECD-grond.

8. Door de huidige Nederlandse normen en waarden over zorgtaken zijn de kansen op de arbeidsmarkt voor de vrouw kleiner dan voor de man.

9. De boventoon die economisch denken in Nederland voert, vormt een gevaar voor de inhoudelijke discussie.

10. Onderzoek doen naar onverteerbaar voedsel terwijl er mensen sterven van de honger is een teken van enorme weelde.

Stellingen behorend bij het proefschrift "Physiological effects of consumption of resistant starch". Marie-Louise Heijnen, Wageningen, 2 april 1997.

Aan iedereen die, op welke wijze dan ook,
een bijdrage heeft geleverd aan de
totstandkoming van dit proefschrift.

Abstract

Physiological effects of consumption of resistant starch

PhD thesis by Marie-Louise Heijnen, Department of Human Nutrition, Wageningen Agricultural University, Wageningen, the Netherlands. April 2, 1997.

Resistant starch (RS) is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals. Thus, RS enters the colon where it may be fermented. In this respect, RS resembles some types of dietary fibre. Three types of RS are being discerned: RS_1, physically entrapped starch; RS_2, uncooked starch granules; RS_3, retrograded starch. The estimated current mean *per capita* RS intake in the Netherlands is 5 g/d. The amount of RS in foods can be manipulated by the choice of raw products and food processing techniques. This is of potential interest if an increased RS consumption would be beneficial for human health. In this thesis several of the hypotheses concerning putative positive effects of RS consumption on human physiology are studied. Daily consumption of up to 32 g RS_2 or RS_3 was tolerated well by healthy individuals and increased colonic fermentative activity and stool weight. Replacement of 27 g digestible starch by RS_2 reduced diet-induced thermogenesis and postprandial glucose and insulin responses proportionally to the amount of indigestible carbohydrate consumed. When compared with an equivalent amount of glucose, daily supplementation of 30 g RS_2 or RS_3 for 3 wk did not affect serum lipid concentrations in healthy subjects, and daily supplementation with 32 g RS_2 or RS_3 for 1 wk did not affect putative risk factors for colon cancer, subjective feelings of hunger, faecal ammonia excretion and apparent absorption of magnesium, calcium and phosphorus in healthy individuals. No differences were observed between RS_2 and RS_3 in the parameters studied. In piglets, dietary RS_3, but not RS_2, shifted nitrogen excretion from urine to faeces, and RS_2 reduced apparent magnesium and calcium absorption. In rats, dietary RS_2, but not RS_3, increased apparent, but not true magnesium absorption. It was concluded that daily consumption of up to 32 g RS_2 or RS_3 is not unfavourable for healthy individuals, but it also does not have great beneficial effects on human physiology, at least for the parameters and time span studied in this thesis. Especially the significance for human health of increased activity and site of fermentation in the colon, and the possible role of the various types of RS in the prevention of colon cancer should be studied further.

Contents

1

General introduction

Introduction

Starches are an important component of the human diet. In the Netherlands, on average 45 percent of energy intake is derived from carbohydrate, *i.e.* 248 g/d. About half of the carbohydrate intake is provided by starches (126 g/d) and the other half by mono- and disaccharides (121 g/d) (Voorlichtingsbureau voor de Voeding 1993). The Dutch Health Council (1992) recommends to increase, at the expense of fat, the carbohydrate intake to 55 percent of energy intake, which would be 300 g/d on average, but to limit the intake of mono- and disaccharides to 15-25 percent of energy, which would be 82-136 g/d. These recommendations imply an increase of starch intake to 164-218 g/d on average.

Starch structure

Starches consist of amylose and amylopectin. Amylose is a straight-chain polymer of glucose linked by α-1,4 glucosidic bonds, with a molecular weight varying from 50,000 to 200,000 Dalton (Whistler & Daniel 1985). Amylopectin is a branched-chain polymer of glucose linked by α-1,4 and α-1,6 glucosidic bonds, with a molecular weight of one to several million Dalton (Whistler & Daniel 1985). The amylopectin:amylose ratio varies in different starch sources, but is normally about 80:20 (Whistler & Daniel 1985). However, *e.g.* maize varieties with an amylose content as low as 0% and as high as 70% do exist. Native starch occurs in granules, which vary widely in shape (from spheres to rods) and size (from 1 μm to over 100 μm). The size and form of such a granule are characteristic of the plant of origin (Whistler & Daniel 1985). The crystallinity of native starch granules varies from 15 to 45%. The rest of the granule has an amorphous structure (Asp *et al.* 1996). Three crystalline forms of starch granules have been identified with diffractometric spectra: *(i)* the A-pattern, displayed in general by cereal starches; *(ii)* the B-type, as in starch from potatoes and some tropical tubers; *(iii)* the C-form, displayed by most legumes. Some consider the C-type as a distinct crystallographic pattern, others as a mixture of the A- and B-forms.

On heating in an aqueous environment the starch granule absorbs water and swells, the crystalline structure of the amylopectin disintegrates, and the granule ruptures. The polysaccharide chains take up a random configuration, causing swelling of the starch and

thickening of the surrounding matrix (gelatinization). The extent of gelatinization depends on the temperature, the amount of water present and the duration of the heat treatment. Most heat-treated foods contain granular structures ranging from relatively intact granules to virtually destroyed granules (Whistler & Daniel 1985, British Nutrition Foundation 1990). When the granule ruptures, amylose leaks out. On cooling or drying, the linear amylose molecules readily associate and form hydrogen bonds. This is referred to as retrogradation. Amylopectin molecules associate less readily, but retrogradation occurs when linear side-chains align (Whistler & Daniel 1985, British Nutrition Foundation 1990).

Starch digestion and absorption

Starches have to be hydrolysed to their constituent monosaccharides because man can only absorb monosaccharides. In the alimentary tract, starches are mainly hydrolysed by substrate-specific enzymes. Starch hydrolysis starts in the mouth: saliva contains the enzyme α-amylase, which hydrolyses the α-1,4 glucosidic bonds at random, producing oligosaccharides and small amounts of glucose (Shils & Young 1988). Since the α-1,6 linkages of amylopectin are not affected by α-amylase, branched oligosaccharides, the so-called α-limit dextrins (5 to 10 glucose units) remain too. Salivary α-amylase continues its action in the stomach until it is destroyed by the stomach acid. Some acid hydrolysis of starch occurs in the stomach, though only to a minor extent (Shils & Young 1988). Pancreatic α-amylase continues the work of salivary α-amylase in the duodenum and jejunum before it is destroyed by trypsin in the lower intestine (Brand 1988, Shils & Young 1988). The remaining residues of dietary starch (oligosaccharides and α-limit dextrins) and the disaccharides saccharose and lactose are hydrolysed to monosaccharides by brush border enzymes in the upper and mid-jejunum: maltase, isomaltase (which can hydrolyse the α-1,6 linkage), saccharase and lactase (Shils & Young 1988).

Monosaccharides are transported across the intestinal mucosa into the splanchnic capillaries by several ways, either separately or combined: glucose and galactose by active absorption and passive diffusion, fructose by facilitated diffusion, and sugar alcohols by passive diffusion (Shils & Young 1988). After absorption, most of the glucose and other monosaccharides after conversion to glucose in the liver pass into the

circulation. They serve either as energy source for cells, or are stored as glycogen (mainly in the liver) or as fat (mainly in adipose tissue and the liver). Small quantities of monosaccharides are used by the gut wall for maintaining its own viability.

The key factor that influences the rate and extent of starch digestion and absorption is the accessibility of the digesta to the intestinal enzymes. This depends in turn on *e.g.* the nature of the starch, food processing, physical structure and particle size of the food, and the presence or absence of interactions with other nutrients such as protein and fat.

Resistant starch

Until about 15 years ago starch was supposed to be completely digested in the upper digestive tract. However, in 1982 Dr. Englyst introduced the expression "resistant starch" (RS) for starch that was not hydrolysed by incubation with α-amylase and pullulanase (enzyme that breaks up α-1,6 glycosidic bonds) during determination of non-starch polysaccharides (Englyst *et al.* 1982). At that time, Englyst was referring to retrograded starch that is resistant to digestion by α-amylase in the small intestine (Englyst & Cummings 1985). Later, he showed in experiments with ileostomists that retrograded starch is only a small proportion of the starch remaining undigested in the small intestine of man (Englyst & Cummings 1986, 1987).

Recently, resistant starch was the subject of a concerted action within the agroindustrial research programme of the Commission of the European Communities (FLAIR Concerted Action no. 11 'Physiological Implications of the Consumption of Resistant Starch in Man'). EURESTA is the acronym for the EUropean RESistant STArch research group that consisted of scientists from universities, research institutes and industry from 40 research groups in 11 European countries. The concerted action ran from July 1990 until June 1994.

At the beginning of EURESTA the following definition of resistant starch was agreed upon: ***'Resistant starch is the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals'*** (Asp 1992). Thus, RS enters the colon where it may be (partly) fermented.

Three types of resistant starch are being discerned (Englyst *et al.* 1992):

RS_1: starch that is physically inaccessible to α-amylase due to the partitioning by intact plant cell walls, *e.g.* starch in legumes, and in coarsely ground grains and seeds;

RS_2: uncooked B-type starch granules, *e.g.* as in unripe bananas, raw potato, and uncooked high-amylose maize starch;

RS_3: retrograded starch, mainly consisting of retrograded amylose, *e.g.* part of the starch in stale bread, and in cooked and cooled potatoes or pasta.

RS_1, RS_2 and RS_3 can coexist in the same food. RS as defined by EURESTA is composed *in vivo* (*i.e.* at the end of the small intestine) of three main fractions: *(i)* glucose and oligosaccharides, *(ii)* a crystalline fraction of intermediate molecular size, and *(iii)* a high molecular weight fraction containing residues of resistant granules and physically enclosed starch. The relative size of these fractions varies, depending on the origin and the treatment of the starch (Faisant *et al.* 1993, 1995*a*). The fraction with glucose and oligosaccarides indicates that some starch is partially hydrolysed in the small intestine without being totally hydrolysed and absorbed. This may be due to a lack of time and/or to the relatively low activity of the brush border enzymes and limited capacity of glucose transport (Asp *et al.* 1996).

Measurement of resistant starch

Various *in vitro* methods are being used to determine the RS content of foods. The four most commonly mentioned are the methods of Englyst *et al.* (1992), Muir and O'Dea (1992), Champ (1992), and Berry (1986).

In the method of Englyst *et al.* (1992) the various types of starch are measured by controlled enzymic hydrolysis and measurement of the released glucose using glucose oxidase. Chewing of the food is mimicked by mincing and standardized milling with glass balls, in the presence of guar gum to increase the viscosity. Total starch (TS) is determined as glucose released by enzymic hydrolysis with α-amylase and amyloglucosidase following gelatinization in boiling water and treatment with potassium hydroxide to disperse retrograded amylose. These two enzymes, α-amylase and amyloglucosidase, only hydrolyse α-1,4 glycosidic bonds and not β-1,4 bonds as in *e.g.* cellulose so that indeed only starch is being determined. TS is corrected for free glucose

but includes maltose and maltodextrins. Rapidly digestible starch (RDS) and slowly digestible starch (SDS) are measured after incubation with pancreatic amylase and amyloglucosidase at 37 °C for 20 min and a further 100 min, respectively. RS is the starch not hydrolysed after 120 min incubation. RS_1 is determined by comparing the glucose released by enzymic digestion of a homogenized food sample with that released from a non-homogenized food sample. RS_2 is determined by comparing the glucose released by enzymic digestion of a boiled, homogenized food sample with that of an uncooked, homogenized food sample. RS_3 is determined as the fraction that resists both dispersion by boiling and enzymic digestion, and that can only be dispersed to measure glucose with potassium hydroxide or dimethyl sulphoxide.

An advantage of the method of Englyst *et al.* is that it is validated in ileostomy patients for a limited number of experimental starchy products, foods, and mixed meals (Langkilde & Andersson 1995*a*, Silvester *et al.* 1995, Englyst *et al.* 1996). On average, very good agreement between RS measured *in vitro* and starch recovered in ileostomy effluent was found, but there was substantial variation between individuals. Therefore, Englyst *et al.* (1996) proposed to change the definition of RS slightly into: *'Resistant starch is the sum of starch and starch-degradation products that, on average, reach the human large intestine'*. A disadvantage of the Englyst method is that it is time-consuming.

The 2 h-incubation time in the method of Englyst *et al.* is much less than the time food remains in the small intestine *in vivo*. Muir and O'Dea (1992) initially used incubation times based on physiological transit times, *i.e.* 6 h for the small intestine. After preparation, the food samples were chewed normally to mimic the stage just before swallowing. This phase of the method is difficult to standardize. The method of Muir & O'Dea (1992) was validated against starch digestion in ileostomates. After the incubation time was increased from 6 h to 15 h good agreement was found both for single foods (Muir & O'Dea 1993) and for mixed meals (Muir *et al.* 1995).

In Berry's method (1986) only α-amylase and amyloglucosidase are used for enzymic breakdown of starch. Therefore, this method is not suitable for samples that contain protein or fat. Further, RS_1 is not measured by this method. Champ (1992) modified Berry's method to make it easier and quicker by eliminating the gelatinization step and the pullulanase hydrolysis. Minor modifications were proposed by Sauro-Calixto (*unpublished*) and Faisant (Faisant *et al.* 1995*b*).

Each method has its own advantages and disadvantages; in general they give comparable results for foods and semipure starchy reference materials, although differences do exist (Champ 1992, Dysseler & Hoffem 1995*b,c*). None of the methods takes into account the low-molecular weight fragments soluble in aqueous ethanol that are products of starch breakdown potentially digestible but found at the end of the small intestine of humans *in vivo* (Champ 1995).

In this thesis the method of Englyst *et al.* (1992) is used because *(i)* it is the only available method discerning the three types of RS, *(ii)* it measures RS in foods as eaten, and *(iii)* it is the method which is best validated with *in vivo* studies.

Intake of resistant starch

Within the framework of EURESTA, the *per capita* availability of RS was evaluated from data on the intake of starchy foods from ten European countries during the period 1992 to 1994. The RS content of the foods used in the calculations was determined by the method of Englyst *et al.* (1992) or by Berry's method as modified by Champ (1992). Different food consumption methods and balance sheets or disappearance statistics were used. The data from some countries were incomplete.

The mean RS intake in Europe is estimated to be 4.1 g dry matter/d (Table 1.1; Dysseler & Hoffem 1995*a*). RS intake ranges from 3.2 g/d in Norway to 5.7 g/d in Spain. In the Netherlands mean RS intake is estimated to be 5.3 g/d. Bread and potatoes are the major sources of RS in most countries, providing together 60% to 90% of the total RS intake. The contribution of other sources of RS fluctuates considerably between the countries. The differences between the countries may be due to differences in dietary habits, but also to under- or overestimation in the analysis of some starchy foods.

These data should be regarded as indicative only, because they differ with respect to their origin and completeness and because different analytical methods were used. In any case, the current RS intake in Europe seems low.

Table 1.1 Mean intake of resistant starch in Europe (g dry matter/d)

	Switzerland	Netherlands	England	Germany	Sweden	Spain	France	Norway	Belgium	Denmark	European
METHOD USED	Berry Mod.	Englyst	Englyst, Berry	Englyst	Englyst	Berry Mod.	*	*	Berry Mod.	Englyst	Mean
PRODUCTS											
White bread	0.52	0.41	0.6	0.59		2.6	1.7	0.31	0.52	0.7	
Special bread	0.92	0.75	0.2	0.11				0.51	0.63	1.6	
Wholemeal bread	0.35	1.3	0.3	0.75	1.05		0.13	0.14	0.28	0.5	
Potatoes boiled eaten warm	0.89	0.95	0.4	0.55	1.9	0.2	0.59	0.91	1.08	0.3	
Potatoes boiled eaten cold			0.7							0.04	
Fried fresh potatoes		0.22	0.2	0.42		0.8					
Fried frozen potatoes	0.14		0.2						0.1	0.2	
Bisctuis		0.36	0.33	1.3		0.6	0.13	0.17	0.43		
Breakfast cereals	0.2		0.4		0.24		0.18	0.55	0.1	0.16	
Pasta	0.54	0.29	0.04	0.03	0.17	0.3	0.11	0.03	0.14	0.01	
Rice	0.17	0.09				0.2	0.12		0.15	0.16	
Banana	0.65	0.5	0.6			0.04	0.64	0.6	0.56		
Rusk		0.06					0.13				
Vegetables		0.36				1					
Total RS intake (1993-1994)	4.38	5.29	3.97	3.75	3.36	5.74	3.73	3.22	3.99	3.67	4.11

* Values calculated

With permission from Dysseler & Hoffem 1995*a*.

Rationale of this thesis

An average *per capita* intake of about 5 g RS per day in the Netherlands is only 4% of the mean total starch intake of 126 g/d (Voorlichtingsbureau voor de Voeding 1993). However, the intake of RS can be varied at the individual level by the choice of foods and preparation method. Further, in food industry, the amount of all three types of RS in foods can be manipulated (Muir *et al.* 1995). This can be achieved through the choice of raw materials (*e.g.* high- or low-amylose variants of cereals), and through food processing techniques and conditions (*e.g.* extent of milling of grains, amount of water present during cooking, or repeated heating and cooling cycles to promote retrogradation).

The possibility to manipulate the RS content of foods to increase RS intake is of potential interest if an increased RS consumption would be beneficial for human health. Because RS is by definition not absorbed in the small intestine, it enters the colon where it may be (partly) fermented. In this respect, RS resembles some types of dietary fibre. Therefore, some of the hypotheses about putative beneficial effects of dietary fibre on human health may be applicable to RS as well. The research carried out within the scope of this thesis aimed at studying several of the hypotheses concerning putative positive effects of RS consumption on human physiology.

Physiological effects of resistant starch consumption

The putative beneficial effects of RS consumption on human physiology that are studied in the experiments described in this thesis are introduced below. Different types of RS may have different effects on human physiology. Therefore, in most of the studies reported in this thesis, uncooked resistant starch (RS_2) was compared with retrograded resistant starch (RS_3). These two types of RS were chosen because some studies suggest that RS_2 is better fermentable than RS_3 (Schulz *et al.* 1993, Olesen *et al.* 1994, Cummings *et al.* 1995, Champ *et al. unpublished results*), and because it is relatively easy to prepare well-defined experimental foods that contain RS_2 and RS_3, in contrast to RS_1. Glucose was used as control because it is fully digestible. In each study, subjects also were asked to report any discomforts perceived to be due to RS consumption.

Glucose and insulin

Because RS is by definition not digested and absorbed as monosaccharides in the small intestine of man, the blood glucose concentration is expected not to rise after consumption of RS, in contrast to after consumption of digestible starch. When less glucose is available in the blood for uptake by the body cells, less insulin will be secreted by the pancreas as insulin is the principal hormone facilitating glucose uptake by cells. More attenuated concentrations of glucose and insulin in blood may be beneficial for diabetes patients, but also for healthy individuals.

In the randomized multiple cross-over study described in ***Chapter 2*** postprandial glucose and insulin concentrations in blood were measured in 10 healthy men after consumption of RS_2 from uncooked potato starch or digestible starch from pregelatinized potato starch. In the randomized multiple cross-over study reported in ***Chapter 3*** 24-h insulin secretion was measured in 24 healthy men after consumption of a daily supplement containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose. Each supplement was consumed for 1 wk by every subject in random order.

Diet-induced thermogenesis

Consumption of RS instead of digestible starch implies a reduction in net energy intake because RS is not absorbed in the small intestine. The energy value of RS is not zero as RS is fermented in the colon, and estimated to be 8 kJ/g (Livesey 1995) whereas the energy value of digestible starch is 17 kJ/g. Thus, consumption of RS instead of digestible starch may be of benefit in weight-reducing diets if no compensation occurs. The obligatory energy costs of ingesting, digesting, absorbing and metabolising the food consumed makes up 60-70% of the diet-induced thermogenesis (DIT). The rest of the DIT can be ascribed to a facultative thermogenic effect of the food (Himms-Hagen 1989). The facultative part of the DIT may be increased by insulin via stimulation of the sympathetic nervous system (Landsberg & Young 1983). If consumption of RS induces a lower blood insulin concentration than consumption of digestible starch, RS may reduce the DIT. This would counteract the above mentioned advantage of consumption of RS instead of digestible starch in weight-reducing diets.

In the randomized multiple cross-over study described in ***Chapter 2*** the DIT was measured in 10 healthy subjects after consumption of RS_2 from uncooked potato starch

and after consumption of digestible starch from pregelatinized potato starch. To assess the impact of fermentation products on the DIT, lactulose (an indigestible disaccharide that is rapidly fermented in the colon) was studied as well. H_2 and CH_4 excretion in breath were determined as a semi-quantitative measure for colonic fermentation (Rumessen 1992).

Feelings of hunger and food intake

Some dietary fibres have satiating power that may be due to delaying gastric emptying (Roberfroid 1993, Truswell 1993) or to attenuating the blood glucose concentration (Leathwood & Pollet 1988, Holt *et al.* 1992). Since RS resembles dietary fibre in several aspects, RS may have a satiating effect, too. If so, consumption of RS would be helpful in weight-reducing diets also by suppressing feelings of hunger. Alternatively, RS may reduce satiety when it replaces digestible starch because RS provides less net energy (Livesey 1995).

The effect of RS on feelings of hunger and food intake was studied in 24 healthy men during consumption of a daily supplement containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose as reported in ***Chapter 3***. Each subject consumed every supplement for 1 wk in random order in this multiple cross-over study.

Colon cancer risk

Colon cancer is the second most common cause of cancer deaths in both males and females in Western, affluent, societies. Genetic predisposition accounts for probably a minority of the colon cancer cases. Apart from a possible inherited susceptibility to colon cancer, international incidence and migrant studies indicate that environmental factors, especially dietary factors, play an important role in the aetiology of sporadic (acquired rather than inherited) colon cancer (Lapré 1992, Govers 1993). By analogy with dietary fibre, RS is hypothesised to reduce the risk for colon cancer in several ways:

(i) By 'dilution' of the intestinal contents and reduction of intestinal transit time, thus reducing the contact of carcinogens with the colonic mucosa (Burkitt 1971). This may happen if consumption of RS increases stool mass.

(ii) By the production of butyrate. Colonic fermentation of RS may lead to the production of short-chain fatty acids (SCFA; Muir *et al.* 1994, Cummings *et al.* 1995,

Nordgaard *et al.* 1995). Some studies indicate that fermentation of RS leads specifically to an increase in butyrate (Scheppach *et al.* 1988*b*, van Munster *et al.* 1994*b*, Phillips *et al.* 1995). Butyrate is a putative protective factor towards colon cancer (Roediger 1982, Gamet *et al.* 1992, Scheppach *et al.* 1995, Csordas 1996).

(iii) By reduction of the cytotoxicity of faecal water. The SCFA produced during colonic fermentation of RS induce a decrease in colonic pH resulting in reduced solubility of bile acids (Bruce 1987). Further, the initial, irreversible step in bacterial conversion of primary into secondary bile acids (7α-dehydroxylation) is inhibited at pH below 6.5 (MacDonald *et al.* 1978, Nagengast *et al.* 1988*a*). The amount of soluble long-chain fatty acids and soluble bile acids (Rafter *et al.* 1986), the secondary bile acids in particular (van der Meer *et al.* 1991), affect the cytotoxicity (*i.e.* the cell-damaging properties) of faecal water. Faecal water is the fraction of faeces which contains the water-soluble, not-bound components of the faeces (Lapré 1992) that are in contact with the colonic mucosal cells (Bruce 1987, Geltner Allinger *et al.* 1989, van Munster & Nagengast 1991). There are indications, but not yet conclusive evidence, that a higher cytotoxicity of faecal water is associated with higher colonic cell proliferation in rat (Lapré & van der Meer 1992) and man (Stadler *et al.* 1988). Hyperproliferation of colonic epithelial cells is suggested to be an important biomarker of increased susceptibility to colon cancer (Lipkin 1988).

(iv) By reduction of the amount of deoxycholic and lithocholic acid. Rat studies indicated that the secondary bile acids deoxycholic and lithocholic acid may be colon tumour promoters (Narisawa *et al.* 1974, Bull *et al.* 1983, Summerton *et al.* 1985).

The effect of daily consumption of a supplement containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose on putative risk factors for colon cancer as explained above was studied in 24 healthy men in a randomized multiple cross-over study described in ***Chapter 4***. Each subject consumed every type of supplement for 1 wk in random order.

Nitrogen excretion

When RS enters the colon, more fermentable substrate becomes available for the bacteria. Hence, more energy is available for bacterial growth. Bacterial growth also requires nitrogen. Nitrogen is derived from ammonia (NH_3) produced by bacteria from dietary protein that escapes digestion, endogenous proteins such as pancreatic and intestinal secretions and sloughed epithelial cells (Mason 1984), and urea that diffuses from blood

to the colon (Rémésy & Demigné 1989, Younes *et al.* 1995*a*). Ammonia can be used for bacterial protein synthesis, thus trapping nitrogen for excretion in the faeces. Further, the conversion of ammonia into ammonium (NH_4^+) is enhanced by a reduction in pH due to colonic fermentation of RS. Ammonium is not well absorbed from the colon and will be excreted in the faeces. The usual route of excretion of ammonia is via urine after conversion into urea. Thus, increased colonic fermentation of RS may shift nitrogen excretion from urine (urea) to faeces (bacteria, ammonium). This may be of interest for the dietary management of chronic renal disease, such as may occur in diabetic patients (Rampton *et al.* 1984, Parillo *et al.* 1988). Also, reduction of the return of ammonia from the gut to the body, thereby decreasing detoxification of ammonia to urea in the liver, may lessen the workload for the liver, which is of interest for cirrhotic patients (Wolpert *et al.* 1971, Weber *et al.* 1985).

In the randomized multiple cross-over study described in Chapter 4, in which 24 healthy men consumed a daily supplement containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose, each for 1 wk, faecal ammonia and urinary urea excretion were measured, too, as reported in ***Chapter 5***. To measure nitrogen absorption by the colon, nitrogen metabolism was studied in piglets with a cannula at the end of the ileum as described in ***Chapter 6***. The piglets consumed a diet containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose. The pig is the animal closest to man in terms of anatomy and physiology of the digestive tract (Bach Knudsen *et al.* 1993, Rowan *et al.* 1994).

Mineral absorption

Minerals play an essential role in vertebrate animals, including man. For example, magnesium plays a key role in many fundamental biological processes such as muscle contraction, enzyme activation and neural excitability (Ryan 1991), and calcium is the most prominent mineral in the skeleton and is essential for *e.g.* several enzyme activities, transmission of nerve impulses, and second messenger functions (Nordin 1988). Some dietary fibres may reduce mineral absorption by binding or complexing minerals in the gut (Rossander *et al.* 1991). RS has no binding or complexing capacities as it is devoid of uronic acids (Younes *et al.* 1996). The effect of RS on mineral absorption is thought to be a consequence of its fermentation in the colon. Several hypotheses have been

proposed. Mineral absorption may be stimulated by fermentable RS by increasing the soluble pool of the mineral (only solubilised minerals can be absorbed) through acidification of the gut contents (Heijnen *et al.* 1993, Schulz *et al.* 1993, Hara *et al.* 1996, Younes *et al.* 1996) and/or by hypertrophy of the colonic wall, *i.e.* by increasing the surface area for absorption (Younes *et al.* 1996). It has been proposed also that the SCFA produced during RS fermentation in the gut may stimulate colonic cell proliferation (Lupton & Kurtz 1993) which would increase the mineral absorption capacity. Further, SCFA may enhance magnesium absorption by a Mg^{2+}/H^{+} exchanger located in the apical membrane of the epithelium in the distal colon (Scharrer & Lutz 1992). These hypotheses imply that fermentable RS would enhance not only the absorption of dietary (exogenous) minerals but also that of endogenous minerals, thus leading to an increase in true mineral absorption. This can only be valid if the excretion of endogenous minerals is not affected.

The apparent absorption of magnesium, calcium and phosphorus was studied in man and pigs consuming RS_2, RS_3 or glucose as reported in ***Chapter 7***. The piglets were cannulated at the end of the ileum to study the contribution of the small and the large intestine to mineral absorption. The results were compared with those of the rat study described in ***Chapter 8***. In all species studied, the same RS_2 and RS_3 preparations from high-amylose maize starch were used. To test whether RS enhances true magnesium absorption, the retention of orally and intraperitoneally administered ^{28}Mg (van den Berg *et al.* 1995) was measured in rats fed RS_2 or RS_3, as described in ***Chapter 8***. The results were compared with those from rats fed either glucose or lactulose.

Serum cholesterol concentration

Consumption of soluble dietary fibres decreases serum cholesterol concentration both in normo- and in hyperlipidaemic subjects (Topping 1991, Truswell 1995). A decrease in serum cholesterol concentration is associated with a reduction of the risk for coronary heart disease. The proposed mechanisms whereby soluble fibres exert their cholesterol-lowering effect include *(i)* inhibition of cholesterol absorption by enhanced viscosity of the intestinal contents, *(ii)* enhanced bile acid excretion and *(iii)* inhibition of hepatic cholesterogenesis and lipoprotein synthesis by propionate produced by bacterial fermentation of dietary fibre in the colon. The latter two mechanisms may be valid for RS, too. However, it has been noted that the physiological concentration of propionate may be too low to inhibit cholesterogenesis in the liver (Topping 1991, Lin *et al.* 1995).

Furthermore, it is not obvious why soluble fibres and RS would increase bile acid excretion since specific binding of bile acids is not likely (Topping 1991). Nevertheless, interruption of the enterohepatic circulation of bile acids may lead to a smaller bile acid pool, which, in turn, impairs cholesterol and fat absorption by reduced micellar solubilisation (Färkkilä & Miettinen 1990). Further, bile acid malabsorption stimulates bile acid synthesis from cholesterol, ultimately causing enhanced cholesterol synthesis and (through upregulated hepatic LDL receptors) a decrease in serum cholesterol concentration (Färkkilä & Miettinen 1990).

To study the effect of consumption of RS_2 and RS_3 on serum cholesterol concentrations in man, the randomized multiple cross-over study described in ***Chapter 9*** was conducted. Fifty-seven healthy men and women consumed a daily supplement containing RS_2 from uncooked high-amylose maize starch, RS_3 from retrograded high-amylose maize starch or glucose. Each subject consumed every supplement for 3 wk in random order.

The following chapters (2-9) contain the reports of the experiments that were conducted to study the above explained possible effects of RS consumption on human physiology. In ***Chapter 10***, the conclusions from these experiments are discussed and recommendations for further research are made.

2

Replacement of digestible by resistant starch lowers diet-induced thermogenesis in healthy men

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Abstract

This study describes the effect of replacement of digestible starch by resistant starch (RS) on dietary-induced thermogenesis (DIT), postprandial glucose and insulin responses, and colonic fermentation. Ten healthy males consumed three test meals, consisting of diluted, artificially-sweetened fruit syrup and either 50 g raw potato starch (550 g RS/kg), or 50 g pregelatinized potato starch (0 g RS/kg) or 30 g pregelatinized potato starch plus 20 g lactulose (670 g indigestible disaccharide/kg). The meals were served in the morning after an overnight fast. Each volunteer consumed each meal twice on six separate days in random order. Metabolic rate was measured by indirect calorimetry in the fasting state for 15 min and postprandially for 5 h. Shortly before and hourly up to 7 h after consumption of the test meal, end-expiratory breath samples were obtained for H_2 and CH_4 analysis. Shortly before and 30, 60, 180, and 300 min postprandially, blood samples were taken for glucose and insulin analyses. Postprandial increases in glucose and insulin levels were proportional to the amount of digestible carbohydrate in the meal. Breath H_2 and CH_4 concentrations indicated that the pregelatinized starch was not fermented and that lactulose was fermented rapidly. Fermentation of the raw starch started only 6 to 7 h after consumption, resulting in a rise in breath H_2 but not in CH_4. The replacement of 27 g digestible starch by RS in a single meal lowered DIT by on average 90 kJ/5 h, as could also be calculated by assuming that RS does not contribute to DIT. The ingestion of lactulose resulted in a substantial rise in DIT which was most likely caused by its fermentation.

Introduction

Dietary-induced thermogenesis (DIT) depends on the composition of the meal, including the type of carbohydrate (Sharief & MacDonald 1982, Schwarz *et al.* 1989). About 60-70% of DIT comprises the obligatory energy costs of ingesting, digesting, absorbing and metabolizing the food consumed. The rest of the DIT can be ascribed to a facultative thermogenic effect of the food (Himms-Hagen 1989). A meal with a high content of rapidly digestible and absorbable carbohydrate, resulting in relatively high postprandial blood glucose concentrations and hence relatively high insulin responses, may increase the facultative part of the DIT via stimulation of the sympathetic nervous system (Landsberg & Young 1983).

Resistant starch (RS) is not absorbed in the small intestine of healthy humans (Englyst *et al.* 1992). Therefore, RS consumption represents a lower net energy intake compared with an identical amount of digestible starch. This might be of benefit for the obese. Furthermore, because of its indigestible character, RS consumption may result in lower postprandial glucose and insulin responses compared to digestible starch consumption which in turn could lower the DIT. A lower DIT after ingestion of RS would counteract the advantage for obese people when consuming RS instead of digestible starch.

Since RS is not absorbed in the small intestine it enters the colon where it can be fermented, resulting in the production of H_2, short-chain fatty acids (SCFA), CO_2 and in some people also CH_4 (Cummings & MacFarlane 1991). Parts of these products are absorbed from the colon, and H_2, CH_4 and CO_2 are partly excreted in breath. The percentage of people producing CH_4 (as measured by CH_4 in end-expiratory air) varies from 20 to 70% in different studies (Pitt *et al.* 1980, Bjørneklett & Jenssen 1982, McNamara *et al.* 1986, Segal *et al.* 1988, Gibson *et al.* 1990). It could be suggested that the absorption and metabolism of fermentation products contribute to the DIT.

In this study the effect of replacing digestible starch with resistant starch on DIT, postprandial glucose and insulin responses, and colonic fermentation (as measured by H_2 and CH_4 in end-expiratory air) was investigated. To assess the impact of fermentation products generated from RS, lactulose, an indigestible disaccharide that is rapidly fermented in the colon, was studied as well.

Subjects and methods

The study was carried out at the Department of Human Nutrition, Wageningen Agricultural University.

Subjects

Ten apparently healthy (as assessed by a medical questionnaire) male students, aged 24 (SD 2, range 20-26) y, mean body weight (in bathing trunks) 72.2 (SD 8.6, range 61.0-91.1) kg, mean height 1.83 (SD 0.08, range 1.72-1.99) m, mean body mass index 21.5 (SD 1.5, range 19.4-23.5) kg/m^2, with no history of gastrointestinal diseases or diabetes mellitus and not using a special diet or medication, participated in the study.

Before entering the study, the volunteers were screened for CH_4 in their breath on three separate days. A subject was classified as a CH_4 producer when at least two of three end-expiratory breath samples had a CH_4 concentration ≥ 3 ppm after subtracting the CH_4 concentration of the ambient air (McNamara *et al.* 1986, Nagengast *et al.* 1988*b*, Rumessen 1992). Three out of the ten subjects were found to be CH_4 producers.

Experimental meals

Table 2.1 shows the composition of the experimental meals. For meal A, 50 g raw potato starch (supplied by the Institut National de la Recherche Agronomique, Nantes, France) containing 550 g RS/kg (type 2, *i.e.* raw starch granules, RS_2) as measured *in vitro* according to the procedure of Englyst *et al.* (1992) was used. For the control meal B, 50 g pregelatinized potato starch (Institut National de la Recherche Agronomique, Nantes, France) containing 0 g RS/kg (Englyst *et al.* 1992) was used. As a reference (meal C) we used lactulose (Sirupus Lactulosi; Pharmachemie B.V., Haarlem, the Netherlands), an indigestible disaccharide (4-O-β-D-galactopyranosyl-D-fructose, $C_{12}H_{22}O_{11}$) that is rapidly and extensively fermented in the colon. However, because of its laxative effect only 20 g lactulose was served in a meal. Therefore we added 30 g pregelatinized potato starch to meal C to have equal amounts of 50 g carbohydrates in all meals. The raw and pregelatinized potato starch were both in a dry powdered form and lactulose was available as a syrup, containing 667 g lactulose, 110 g galactose, 10 g glucose, 10 g fructose and 60 g lactose/l. The starches and lactulose syrup were added to 125 ml concentrated fruit syrup (Irma A/S, Rødovre, Denmark) containing 23 g glucose, 35 g fructose and 9 g

Table 2.1 *Composition of the experimental meals*

	Meal		
	A Raw potato starch	B Pregelatinized potato starch	C Lactulose plus pregelatinized potato starch
Total starch (g)	40.7[a]	46.5[b]	27.9[b]
Rapidly digestible starch (g)	3.0[a]	44.0[b]	26.4[b]
Slowly digestible starch (g)	10.6[a]	2.6[b]	1.5[b]
Resistant starch (g)	27.1[a]	-	-
Total mono- + disaccharides (g)	8.4[c]	8.4[c]	34.1[c,d]
Digestible mono- + disaccharides (g)	8.4[c]	8.4[c]	14.1[c,d]
Lactulose (resistant disaccharide) (g)	-	-	20.0[d]
Total carbohydrate (g)	49.1	54.9	62.0
Digestible carbohydrate (g)	22.0	54.9	42.0
Resistant carbohydrate (g)	27.1	-	20.0
Gross energy[e] (kJ)	830	930	1008

[a] From raw potato starch.
[b] From pregelatinized potato starch.
[c] From concentrated fruit syrup.
[d] From lactulose syrup.
[e] Calculated as total starch (g) x 17.2 kJ plus total mono- + disaccharides (g) x 15.5 kJ (Passmore & Eastwood 1986).

sucrose/l. Tap water was added to reach a final volume of 500 ml. The raw and pregelatinized potato starches differed in water content (166 and 49 g/kg respectively) so that meal A and B differed slightly in total starch content (Table 2.1).

The meals were prepared freshly every day just before consumption. Meal A was a liquid suspension in which the raw starch tended to sink to the bottom of the glass. Meal B was viscous and had to be eaten with a spoon. Meal C was less viscous than B and could be drunk as meal A. Because of these clear differences in viscosity the subjects were able to distinguish between the meals although they did not know which viscosity corresponded to which type of meal.

Experimental design

On a measurement day, subjects were picked up at home by car after an overnight fast. After voiding and weighing, the subjects rested on a bed in a semi-supine position and after a period of about 15 min in which the metabolic rate stabilized, resting metabolic rate (RMR) was measured for 45 min. Then the subjects consumed one of the experimental meals within 10 min and postprandial energy expenditure (PEE) was measured for 5 h. During the metabolic rate measurements the subjects watched video movies. They were allowed to go to the toilet if necessary. All urine produced during the metabolic rate measurements was collected for N determination, necessary to estimate the amount of protein oxidation. Shortly before and 30, 60, 180 and 240 min after consumption of the meal, blood samples were taken by venepuncture for determination of glucose and insulin concentrations. Immediately after the meal the subjects judged the palatability of the meals using visual analogue scales. Shortly before and every hour for 7 h after consuming the meals a questionnaire asking for gastrointestinal complaints was filled in and end-expiratory breath samples were taken for measuring H_2 and CH_4 concentrations. Every time a breath sample was taken a sample of the ambient air was taken as well. After the metabolic rate measurements were completed (after 5 h) the subjects were served a standardized lunch consisting of two white rolls and one roll with raisins, together with 20 g margarine, 24 g raw (*i.e.* salted, dried and smoked) ham, 20 g cooked ham, 250 ml partly skimmed milk and coffee or tea (total meal: 3.11 MJ; carbohydrate 43% energy intake, fat 41% energy intake, protein 16% energy intake, and 3.9 g dietary fibre). Two more breath samples were collected at 6 and 7 h after the test meal. Figure 2.1 shows the flow diagram of an experimental day. On most measurement days, two subjects were measured simultaneously.

Each subject consumed every type of meal twice. Since a period of at least 6 d separated each two successive measurement days of a subject, carry-over effects were not expected nor was an effect of the order of the meals. To be sure, the order of the meals was random but different for every subject. All measurements were completed within 2.5 months. On the 3 d before the measurements a standardized diet consisting of ordinary foods and containing carbohydrate 60% energy intake, fat 28% energy intake, protein 12% energy intake, and 3.5 g dietary fibre/MJ was provided. The amount of food was attuned to individual energy needs as based on World Health Organization energy requirement formulas (1985); a suitable activity factor was assessed by asking the

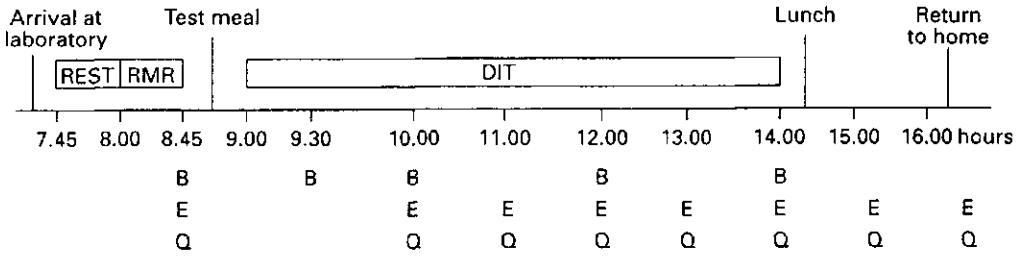

Figure 2.1 *Time schedule for a measurement day. RMR, resting metabolic rate; DIT, diet-induced thermogenesis; B, blood sample; E, end-expiratory breath sample; Q, questionnaire on gastrointestinal complaints.*

subjects about their sporting activities. At 2 d before the measurements the subjects abstained from strenuous physical activities. Throughout the experimental period the subjects kept a diary in which they noted any deviations from the study protocol, gastrointestinal complaints, illnesses and medication taken.

Methods

RMR and DIT were measured by indirect calorimetry with a ventilated-hood system as described in detail elsewhere (Weststrate 1993). Metabolic rate was calculated using the formula of Jéquier *et al.* (1987). DIT was calculated by subtracting RMR from postprandial energy expenditure (PEE).

Plasma glucose was measured enzymically by the combined activities of hexokinase (*EC* 2.7.1.1) and glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) (Abbott Spectrum High Performance Diagnostic System; Abbott Laboratories, North Chicago, IL, USA). Serum insulin was measured by immunoassay with a commercial test combination (Boehringer Mannheim GmbH, Germany).

Urinary N was determined by the Kjeldahl method with a Kjeltic autosampler system 1035 analyser (Tecator, Sweden).

End-expiratory breath samples were collected in plastic syringes of 60 ml (Plastipak, Becton Dickinson, Dublin, Ireland). Within 2 h after collection, a 20 ml portion of each sample was used to measure H_2 with an electrochemical measurement cell (Exhaled Hydrogen Monitor; Gas Measurement Instruments Ltd., Renfrew, Scotland). The measurement cell was calibrated twice a day with 105 ppm H_2 in N_2 gas (Intermar B.V.,

Breda, the Netherlands). The remaining portion of the breath sample was used for a duplicate CH_4 determination by gas chromatography (Hewlett Packard, model 427, Stimadzu, Chromatopac C-R3A). For calibrating (once daily), 5 ppm and 29 ppm CH_4 in N_2 gases were used (Intermar B.V., Breda, the Netherlands).

Statistical analysis

Results are expressed as means with their standard errors. First the means of the duplicates per subject per type of meal were calculated, then the mean and standard error per type of meal. The significance of the differences between the three meals regarding glucose, insulin and DIT was assessed by analysis of variance with type of meal as fixed factor and subject as random factor (thus taking the intrinsic individual levels into account). In general, adding the order of the meals to the model did not contribute to the model. In the case of a statistically significant effect (P value < 0.05) in the analysis of variance, group means were compared by pairwise Student's t tests. Because of the small numbers of CH_4 ($n=3$) and non-CH_4 producers ($n=7$) the differences between the meals regarding H_2 and CH_4 excretion in breath were only described and not statistically evaluated. The statistical analysis package SAS, release 6.07 (Statistical Analysis Systems Inc., Cary, NC, USA) was used to perform the statistical analyses.

Ethical considerations

The experimental design and possible discomforts were explained to the subjects before written informed consent was obtained. The experimental protocol was approved by the Medical-Ethical Committee of the Department of Human Nutrition of the Wageningen Agricultural University.

Results

On one of the measurement days, lactulose was added wrongly to one meal and omitted wrongly from another. Inspection of the diaries revealed that one subject was taking antibiotics on his last measurement day. The results of these volunteers on those three experimental days were excluded from the statistical analysis.

The meals were acceptable to the volunteers but in general palatability was regarded as suboptimal. With increasing viscosity (raw starch < lactulose < pregelatinized starch) the meals were less appreciated.

Few gastrointestinal complaints were reported after consumption of the raw and pregelatinized starch meals. One subject reported abdominal complaints starting in the evening of a measurement day on which he had consumed a raw starch meal. Consumption of the lactulose meal resulted in increased flatulence and intestinal rumbling in most subjects, indicative of fermentation of lactulose by the intestinal bacteria. The discomfort caused by the reported intestinal complaints was described as light in most cases and sometimes as moderate.

Postprandial plasma glucose rose to a peak value 30 min after consumption of the meals and returned to baseline levels within 3 h. The peak values differed significantly between the meals ($P < 0.0001$): the mean changes from baseline were 3.9 (SE 0.5), 2.5 (SE 0.5) and 0.5 (SE 0.2) mmol/l for the pregelatinized starch, lactulose and raw starch meals respectively. At 1 h after consumption the plasma glucose concentration was 1.5 (SE 0.4) mmol/l higher after the pregelatinized meal than after the raw starch meal and 1.3 (SE 0.3) mmol/l higher than after the lactulose meal ($P < 0.0001$). At 3 h after consumption plasma glucose concentration was 0.4 (SE 0.2) mmol/l higher after the raw starch meal than after the pregelatinized meal and 0.3 (SE 0.2) mmol/l higher than after the lactulose meal ($P < 0.001$).

Postprandial serum insulin rose to a peak value 30 min after consumption of the meals and returned to baseline levels within 3 h. The peak value after consumption of the raw starch meal was lower ($P < 0.01$) than after the pregelatinized starch meal (19 mU/l) and the lactulose meal (8 mU/l), the changes from baseline being 37 (SE 18), 36 (SE 7) and 7 (SE 3) mU/l for the pregelatinized starch, lactulose and raw starch meals respectively. At 1 h after consumption the serum insulin concentration was 36 (SE 13) mU/l higher after the pregelatinized meal than after the raw starch meal and 27 (SE 8) mU/l higher than after the lactulose meal ($P < 0.0001$).

After consumption of lactulose the H_2 concentration in end-expiratory air rose rapidly to a peak value 3 h after consumption of the meal, both in non-CH_4 and CH_4 producers. The total area below the H_2 curve, as a measure for the total amount of H_2 expired during the experimental period, was larger after consumption of the lactulose meal than after consumption of the other two meals, both for CH_4 and non-CH_4 producers (Table 2.2).

However, the amount of H_2 produced by CH_4 producers after consumption of lactulose was about half of the amount produced by non-CH_4 producers. In CH_4 producers breath CH_4 concentration also tended to increase after lactulose consumption. Pregelatinized potato starch seemed to be fully absorbed and not fermented since the H_2 and CH_4 concentrations in end-expiratory air up to 7 h after consumption of the meal were not different from the fasting values, both in CH_4 and non-CH_4 producers. Up to 5 h after ingestion of the raw starch meal in non-CH_4 producers, and up to 6 h in CH_4 producers, breath H_2 concentration did not differ from that after consumption of the pregelatinized starch meal. At 6 to 7 h after consumption of the raw starch meal, however, breath H_2 concentration started to rise: mean difference 7 (SE 2) ppm at 6 h and 12 (SE 5) ppm at 7 h in non-CH_4 producers, and 5 (SE 1) ppm at 7 h in CH_4 producers. No difference was found in breath CH_4 excretion after consumption of the pregelatinized and raw starch meals (Table 2.2).

Table 2.2 *Amount of H_2 expired during 7 h after consumption of a meal containing 50 g raw potato starch, 50 pregelatinized potato starch or 20 g lactulose plus 30 g pregelatinized potato starch by CH_4 ($n=3$) and non-CH_4 producers ($n=7$)*[a]

	Area below the H_2 curve (ppm x 7 h)		Area below the CH_4 curve (ppm x 7 h)
	Non-CH_4 producers	CH_4 producers	CH_4 producers
Meal:			
Raw starch (A)	63 ±12	23 ±6	449 ±186
Pregelatinized starch (B)	43 ±10	28 ±7	400 ±164
Lactulose (C)	511 ±57	271 ±140	592 ±256
Mean difference[b]			
A - B	20 ±14	-5 ±1	49 ±51
C - B	468 ±60	243 ±134	192 ±101
A - C	-448 ±53	-248 ±135	-143 ±71

[a] Mean values with their standard errors. First the means of the duplicates per subject were calculated, then the mean and SE per meal.

[b] Values were calculated from the differences on each individual separately for each pair of diets.

RMR was similar before every meal: 5.0 (SE 0.2), 5.0 (SE 0.2) and 4.8 (SE 0.2) kJ/min for the raw starch, pregelatinized starch and lactulose meals respectively.

DIT rose rapidly after consumption of the meals, started to decrease within 1 h and levelled off after 2 h. Mean DIT, total DIT and DIT as percentage of RMR after the pregelatinized and lactulose meals were similar; DIT after the raw starch meal was significantly lower, both in terms of magnitude and duration (Table 2.3).

Table 2.3 *Effects of meals containing 50 g raw potato starch, 50 g pregelatinized potato starch or 20 g lactulose plus 30 g pregelatinized potato starch on diet-induced thermogenesis (DIT; calculated as postprandial energy expenditure minus resting metabolic rate (RMR)) in young men (n=10)*[a]

	DIT		
	kJ/min	kJ	% RMR
Meal:			
Raw starch (A)	0.1 ±0.1[b]	36 ±23[b]	2.6 ±1.5[b]
Pregelatinized starch (B)	0.4 ±0.1	125 ±16	8.6 ±1.2
Lactulose (C)	0.5 ±0.1	164 ±18	11.5 ±1.4
Analysis of variance:			
Meal	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Subject	NS	NS	NS
Mean difference[c]			
A - B	-0.3 ±0.1	-90 ±30	-6.0 ±2.0
C - B	0.1 ±0.1	39 ±22	2.9 ±1.6
A - C	-0.4 ±0.1	-128 ±20	-8.9 ±1.4

NS, not significant.

[a] First the means of the duplicates per subject were calculated, then the mean and SE per meal.

[b] Mean values were significantly different from the pregelatinized starch and lactulose meals, $P < 0.05$ (Student's t test).

[c] Values were calculated from the differences on each individual separately for each pair of diets.

Discussion

To our knowledge the influence of RS consumption on DIT has not been reported before. We found a DIT of 125 (SE 16) kJ in 5 h after consumption of a meal containing 1000 g digestible carbohydrates/kg (B), *i.e.* 14% of the gross energy content of the meal. Based on the fact that the raw starch meal (A) contained 550 g RS/kg and 450 g digestible carbohydrate/kg, an expected theoretical value of DIT of about 50 kJ in 5 h can be calculated. The observed value of 36 (SE 23) kJ was well within the 95% confidence limits. The somewhat lower value could be due to a lower digestibility *in vivo* compared with the digestibility determined *in vitro* by the Englyst method (Englyst *et al.* 1992). DIT after 2 h was 83 (SE 6) kJ for the pregelatinized starch, whereas 35 (SE 8) kJ was found for the raw starch. The latter value compares well with the expected theoretical value of 33 kJ. It can be concluded that consumption of a meal in which part of the digestible starch is replaced by RS lowers DIT during the first 5 h after the meal to the extent expected based on the amount of indigestible carbohydrate in the meal.

Possibly the DIT after consumption of RS is postponed: SCFA produced upon colonic fermentation of RS could add to the DIT. However, since the fermentation of the type of RS used (type 2, RS_2, raw starch granules) started only 6 to 7 h after consumption, as indicated by the H_2 concentration in end-expiratory air (a finding that is in accordance with other studies; Olesen et al. 1992) we cannot confirm this with our current experimental design in which DIT was measured for the first 5 h after the meal. Since RS fermentation most probably occurs gradually the possible 'SCFA-effect' will be too small to be measured by indirect calorimetry. The delayed RS_2 fermentation compared with lactulose could mean that RS_2 is a less suitable substrate for colonic fermentation, or that RS_2 is fermented at a different site in the colon compared with lactulose. In addition, the time to pass through the digestive tract could be longer for RS_2 than for lactulose. It can be calculated that an intake of 27 g RS/d would reduce the daily energy expenditure of these subjects by approximately 0.7%.

Although the difference was not statistically significant, it is striking that DIT after consumption of 20 g (indigestible) lactulose plus 30 g pregelatinized (digestible) starch was larger than after consumption of 50 g pregelatinized starch. Based on the amount of digestible starch a DIT of 97 kJ in 5 h was expected. However, 164 (SE 18) kJ was found. Thus 66 kJ of the observed DIT was caused by the lactulose. Of this 3.3 kJ/g

lactulose, 1.2 kJ can be ascribed to heat of fermentation (Livesey 1992) and 1.8 kJ to the metabolism of the rapidly absorbed (McNeil *et al.* 1978, Ruppin *et al.* 1980, Pomare *et al.* 1985, Scheppach *et al.* 1991, Peters *et al.* 1992) SCFA (Smith and Bryant 1979, Stryer 1988) formed during the rapid fermentation of lactulose, as found in this and other studies (Bjørneklett & Jenssen 1982, Florent *et al.* 1985, Würsch *et al.* 1989, Cloarec *et al.* 1990, Rumessen *et al.* 1990). The combustion of part of the H_2 and CH_4 produced upon lactulose fermentation might account at least partly for the remaining gap of 0.3 kJ, although the efficiency of the conversion of fermentable carbohydrate to the combustible gases H_2 and CH_4 might be as low as the equivalent of 0.02 kJ gas (breath + flatus) per kJ carbohydrate fermented (Ruppin *et al.* 1980). Although these calculations seem to fit the data nicely, it should be kept in mind that large variations were found in DIT and, in view of the breath H_2 concentrations, probably not all lactulose was fermented within the 5 h period after consumption.

Ritz *et al.* (1993) found a significant increase in CO_2 production when 20 g lactulose was added to a standardized glucose load of 50 g. This excess CO_2 probably arose from colonic fermentation of lactulose and from addition to the fuel mix of SCFA, especially acetate, produced during lactulose fermentation. To be precise, the calculation of energy expenditure based on indirect calorimetry should be corrected for the CO_2 produced during colonic fermentation. However, for several reasons it is very hazardous to estimate the proportion of exhaled CO_2 that arises from lactulose fermentation. First, the end products of colonic fermentation depend on the composition of the bacterial flora, so that there are several fermentation equations possible. Second, it is not known which proportion of the CO_2 produced during fermentation is absorbed, and third, it is not known which proportion of the absorbed CO_2 can eventually be measured in breath. It can be calculated that energy expenditure is overestimated by a maximum of 0.9% when no correction is made for the amount of CO_2 produced during fermentation of lactulose. Since this is only a small error, especially in view of the large variation inherent in the ventilated-hood method, that would not alter the conclusion, no such correction was made when presenting the results of this experiment.

Not surprisingly, postprandial plasma glucose and serum insulin levels were proportional to the amount of digestible carbohydrate in the meals, as found by others as well (Collings *et al.* 1981, Jenkins *et al.* 1987*a*, Holm *et al.* 1988, 1989, Bornet *et al.* 1989, Holm & Björck 1992). However, plasma glucose and serum insulin responses after

the raw starch meal were smaller than expected from the amount of rapidly digestible carbohydrate (RDS) as was found by Raben *et al.* (1994) as well. Raben *et al.* (1994) suggested that the lack of increase in gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) that they found after a raw starch meal might explain the difference in insulin response between the meals, beyond what can be expected from the glucose-stimulated insulin secretion (GIP and GLP-1 are both potent stimulators of insulin secretion). Possibly the decrease in insulin response after consumption of the raw starch meal explains to a substantial extent the reduction in DIT found.

In conclusion, consumption of a meal in which part of the digestible starch is replaced by resistant starch was found to lower DIT during the first 5 h after the meal to the extent that would be expected based on the amount of indigestible carbohydrate in the meal. This outcome might be explained by the observation that the resistant starch used was not fermented within 5 h after consumption as evidenced by unchanged H_2 and CH_4 concentrations in end-expiratory air.

Acknowledgements

We are indebted to the volunteers for their cooperation. We thank Linda Grievink for her help in conducting the experiment. We thank Martine Champ from the Institut Nationale de la Recherche Agronomique, Nantes, France for kindly providing the experimental starches, and Ms. R. Brand and Ms. A. Wortel from Iglo Ola B.V., Utrecht, the Netherlands, for kindly providing frozen dinners.

3

Resistant starch has little effect on appetite, food intake and insulin secretion of healthy young men

de Roos N, Heijnen M-L, de Graaf C, Woestenenk G, Hobbel E.
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Abstract

This study investigated whether resistant starch types 2 and 3 are more satiating than glucose. During 4 wk 24 healthy male volunteers consumed a daily supplement with either glucose or high-amylose maize starch (RS_2) or extruded and retrograded high-amylose maize starch (RS_3) in a crossover, single-blind, randomized and balanced study design. Each type of supplement was consumed for 1 wk. In the first wk each subject consumed the glucose supplement. The RS_2 and RS_3 supplements provided for 30 g resistant starch/d. At the end of wk 2, 3 and 4, subjects rated their appetite each whole hour on a visual analogue scale. Food intake was measured 1 d/wk using the 24-h recall method. Subjects collected 24-h urine during the last two days of wk 2, 3 and 4 to determine C-peptide excretion as a measure for the 24-h insulin secretion. Supplementation with RS_2 caused significantly ($P < 0.05$) lower appetite scores than supplementation with RS_3 and glucose, though subjects paradoxically felt less full while consuming RS_2. The cyclic pattern of appetite during the day did not change with the supplements. Energy and macronutrient intake was similar in the three supplementation periods. When consuming RS_3, subjects had a significantly ($P < 0.0012$) lower urinary C-peptide excretion than when consuming RS_2 or glucose: 3.74 $\pm$1.42 nmol/d for RS_3, 4.39 $\pm$1.52 nmol/d for RS_2 and 4.71 $\pm$1.73 nmol/d for glucose. The mechanism for this lower insulin secretion is yet unclear. Consumption of 30 g/d RS_2 and RS_3 had little influence on appetite and food intake, but RS_3 reduced the insulin secretion.

Introduction

Resistant starch (RS) is the sum of starch and products of starch hydrolysis that passes undigested into the colon of healthy subjects (Asp 1992). This is estimated to be approximately 10% (2-20%) of the amount of starch consumed in the Western diet (Stephen *et al.* 1983, McNeil 1984, National Institute of Nutrition 1990, Stephen 1991). In the Netherlands the average RS intake is estimated to be 5 g/d (Dysseler & Hoffem 1995*a*). In the colon the undigested starch is partly fermented, producing short-chain fatty acids, CO_2, H_2 and in some subjects CH_4 (Cummings & Englyst 1991).

Three forms of RS are currently distinguished (Asp *et al.* 1993): RS_1 is starch that is included in a plant cell wall, and thus physically inaccessible to α-amylase. RS_2 is native starch, included in granula, that can be made accessible to the enzyme by gelatinization. RS_2 is almost totally fermented in the colon (Flourie *et al.* 1986). RS_3 is retrograded starch that forms after cooling of gelatinized starch (Colonna *et al.* 1992). Malabsorption of RS_3 is about 50% (Molis *et al.* 1992). RS_3 is fermented in the colon (Faulks *et al.* 1989, Molis *et al.* 1992) but less well than RS_2 (Gee *et al.* 1992).

RS is thought to have physiological effects comparable with soluble dietary fibres (Faulks *et al.* 1989, National Institute of Nutrition 1990, Stephen 1991, Siemensma 1991, de Deckere *et al.* 1992). For this reason, several positive effects on human health have been attributed to RS. It is possibly protective against colon cancer, mainly by decreasing the conversion of primary bile acids in secondary bile acids; it can be beneficial for diabetics by lowering blood glucose and insulin levels; and it can be helpful in the therapy of obesity by increased feeling of fullness and because of the reduced energy intake when RS replaces digestible carbohydrate (National Institute of Nutrition 1990, Nishimune *et al.* 1991, Ritz *et al.* 1991, Rasmussen 1993). This article focuses on the possible influence of RS on food intake, appetite and insulin secretion.

While assuming that RS can be compared with soluble fibres, several mechanisms are proposed by which RS might suppress appetite and food intake. Consumption of soluble fibres delays the emptying of the stomach (Dreher 1987, Torsdottir 1989, Roberfroid 1993, Truswell 1993). This is caused by the water-binding capacities of the fibre, thus binding the fluid contents of the stomach. A delayed gastric emptying can cause an extended feeling of fullness. This has been shown by the correlation between echographic gastric emptying and appetite measured on a visual analogue scale (VAS) (Bergmann *et*

al. 1992). A slower emptying rate means a delayed digestion and absorption of nutrients (Jenkins *et al.* 1978*b*, Ritz *et al.* 1991, Truswell 1992, Roberfroid 1993). Postprandial glucose levels in the blood will thus be lower than after consumption of digestible carbohydrates (Grossman 1986, Leathwood & Pollet 1988, Holt *et al.* 1992, Dowse *et al.* 1993, Moffett *et al.* 1993, Raben *et al.* 1994). Some investigators suggest that this lower, but longer-lasting, glucose peak is associated with an extended feeling of satiety and delayed return of appetite (Grossman 1986, Leathwood & Pollet 1988, Holt *et al.* 1992). This effect is not always found (Krishnamachar & Mickelsen 1987, Sepple & Read 1989, Raben *et al.* 1994). The digestion and absorption of glucose is also delayed by the increased viscosity that hinders the contact between enzyme and substrate (Stephen 1991). Soluble, viscous fibres also increase the water-stirred layer in the gut, thus again delaying the diffusion of sugars from the lumen into the blood (Jenkins *et al.* 1987*b*, Roberfroid 1993, Truswell 1993).

The aim of this study was to answer the following questions:

1. Does RS have a more and longer-lasting satiating effect then digestible carbohydrate?
2. Does the average food intake decrease when consuming RS instead of digestible carbohydrate?
3. Does RS decrease the 24-h excretion of insulin compared with digestible carbohydrate?
4. Is there a difference between native starch (RS_2) and retrograded starch (RS_3) with respect to questions 1, 2 and 3?

Materials and methods

Subjects

Twenty-four healthy male volunteers participated in the study. Table 3.1 shows some characteristics of the subjects at the beginning of the study. Selection criteria were: age (>18 y), stable weight (reported fluctuation during last 3 months $< \pm 2.5$ kg), no diabetes mellitus, no kidney diseases, no stomach or bowel surgeries, and a good appetite. All subjects were non-CH_4 producers (breath CH_4 concentrations <3 ppm above the concentration in ambiant air on three different days). Subjects gave their written informed consent. The study protocol was approved of by the Medical-Ethical Committee

of the Department of Human Nutrition in Wageningen. Subjects kept a diary during the experiment in which illness, medications, deviations from normal daily physical activities and the time-points of consumption of the supplements were reported.

Table 3.1 *Characteristics of the 24 male subjects in the run-in period (wk 1)*

Characteristic	Mean ±SD	Range
Age (y)	23 ±1.8	20 - 27
Height (m)	1.84 ±0.07	1.70 - 1.99
Weight (kg)	76.9 ±7.5	63.0 - 94.9
BMI (kg/m^2)	22.7 ±1.8	19.4 - 26.0

Study design

Subjects consumed a daily supplement in three portions per day during 4 wk in a single-blind, randomized, balanced cross over study. In the first wk (run-in period) every subject consumed the supplement with glucose (control supplement). In the next 3 wk each of the three types of supplement (glucose, RS_2, RS_3) were consumed for 1 wk. Appetite was measured on day 6 of wk 2, 3 and 4. Subjects collected their 24-h urine during days 6 and 7 in wk 2, 3 and 4. Body weight was measured twice a wk and every wk each subject was interviewed by a dietitian to measure food intake (energy and macronutrient intake). Body weight and energy and macronutrient intake had already been measured in the screening.

Supplements

Table 3.2 shows that the supplements consisted of a mixture of skim yogurt (Coberco), skim milk (Coberco), mashed canned fruit, glucose (glucose monohydrate (CL 02001; Cerestar, Vilvoorde, Belgium), Amaizo-7 (maize starch with 70% amylose; Cerestar, Vilvoorde, Belgium) or extruded, retrograded Amaizo-7 (Cerestar, Vilvoorde, Belgium). Amaizo-7 contains 63.3 g RS/100 g; extruded, retrograded Amaizo-7 contains 29.9 g (*in vitro* measurements; Englyst *et al.* 1992). The three different supplements are indicated by the terms glucose, RS_2 and RS_3. The supplements were made three times a wk, and the subjects took them home. The subjects consumed three portions of about 200 g each

day, together with their regular meals. They were instructed not to eat pulses and unripe bananas during the experiment, because of their relatively high RS-content.

Table 3.2 *Recipe for each supplement/d. The levels of ingredients were set in order to obtain 30 g resistant starch in the RS_2 and RS_3 supplements. The three supplements contain equal amounts of carbohydrate.*

Ingredient	Glucose	RS_2	RS_3
Skim yogurt (g)	144	144	144
Skim milk (g)	216	216	216
Glucose (g)	110.4	58.5	0
Amaizo-7 (g)	0	47.4	0
Treated[a] Amaizo-7 (g)	0	0	100.2
Mashed fruit (g)	123-183[b]	123-183[b]	123-183[b]
Mean weight (g)	606	603	579

[a] Extruded retrograded.

[b] Depending on the type of fruit.

Table 3.3 shows the macronutrient composition of the supplements. The daily dose RS given, 30 g/d, is about six times the average intake of RS in the Netherlands (Dysseler & Hoffem 1995*a*). Earlier experiments with 30 g RS/d did not lead to serious gastro-intestinal complaints, though some subjects reported increased flatulence (Heijnen *et al.* 1995, van Munster *et al.* 1994*a*).

Compliance

To check whether the subjects really consumed the supplements, 80 μmol lithium was added per supplement portion (Sanchez-Castillo *et al.* 1987). This amount is 100 times more than the amount that is found in food, and 100 times less than the therapeutic dose. Lithium is almost totally (>95%) excreted in urine. By determining the amount of lithium in 24-h urine the consumption of the supplements could be checked. When in a wk lithium recovery from a subject was less then 80%, data from this subject for that wk were excluded from statistical analysis.

Table 3.3 *Composition of the supplements/d, computed from the Dutch food table (NEVO Foundation 1987)*

	Glucose	RS_2	RS_3
Energy (MJ)	2.46	1.98[a]	1.95[a]
Water (g)	454.5	452.4	441.9
Protein (g)	12	12	12
Fat (g)	0	0	0
Carbohydrate (g)	138	136	136
Mono- + disaccharides (g)	132	90	42
Polysaccharides (g)	0	12	60
Dietary fibre (g)	2	2	2
Resistant starch[b] (g)	4	32	32

[a] Assumed that RS is totally undigestible, not taking fermentation into consideration.
[b] As determined *in vitro* by the procedure of Englyst *et al.* (1992).

Measurements

Appetite

Subjects were instructed to rate their appetite at each whole hour of the day. The first rating had to be done just after waking up, and the last rating just before going to bed. Appetite was measured according to six dimensions:

- appetite for a meal
- appetite for something sweet
- appetite for something savoury
- satiety (fullness)
- feeble, weak with hunger
- appetite for a snack.

Each appetite dimension was represented by a 150 mm VAS. The left- and right-hand sides of the VAS were anchored with the terms 'weak' and 'strong' respectively. The subjects were instructed that 'appetite for a meal' referred to appetite for a whole meal, either a hot meal or a sandwich meal; 'appetite for something sweet' referred to appetite for a cookie, chocolate, candy bar, sweet pie or a sweet dessert; 'appetite for something savoury' referred to appetite for peanuts, cheese, cocktail nuts, french fries or a savoury dessert; 'satiety (fullness)' referred to a feeling of having eaten too much; and 'feeble,

weak with hunger' referred to a strong urge to eat, with clear physical symptoms. The ratings on the 150 mm VAS were read automatically by an Optical Mark Reader and were converted into scores from 1 to 25. A score of 1 corresponded with the left side of the scale (weak), a score of 25 corresponded with the right side of the scale (strong). Ratings that were given half an hour before or a quarter of an hour after a whole hour were regarded as if they had been given on that whole hour.

Pleasantness

Subjects rated the pleasantness of the three supplements on a 150 mm visual analogue scale. The left- and right-hand sides of the scale were anchored by the terms 'very bad' and 'very good'. The ratings were converted into scores from 1 to 25 by hand. This measurement was done in wk 2, 3 and 4, and subjects rated the supplement that they had had the last three days. Differences in pleasantness because of the type of fruit were eliminated in this way.

Energy and macronutrient intake

Every wk, preferably on different days, subjects were interviewed by one of three dietitians to get a 24-h food recall. Throughout the study each subject was interviewed by the same dietitcian, who also coded the food intake. Subjects were also asked some questions on how they included the supplements into their habitual diet. Food intake was computed using a computerized food composition table (NEVO Foundation 1987). Energetic compensation (EC) for the supplements was computed using the following formula: $EC = (E_{screening}-E)/E_{suppl} \times 100\%$, in which $E_{screening}$ is the mean energy intake during the screening, E is the mean energy intake during one supplement period, and E_{suppl} is the energy content of the supplement. An EC of 100% would mean that subjects reduced their energy intake with the energy content of the supplement. Energy and macronutrient intake during the screening and wk 2, 3 and 4 are discussed in this article.

C-peptide in 24-h urine

Subjects collected 24-h urine on days 6 and 7 of wk 2, 3 and 4. They stored their urine in plastic bottles with an antibacterial agent. Completely collected urine of both days was pooled. Incompletely collected urine was not used. C-peptide concentration was measured using a radioimmunoassay (Biodata C-peptide kit, code 10282).

Statistical analysis

Distribution of the data was first tested for normality using the Wilk-Shapiro test (Snedecor & Cochran 1980). Visual judgement showed that residual variances were homogeneous. Differences between the three supplements were tested by analysis of variance, using the procedure GLM (general linear model) of the SAS software package (SAS Institute Inc., 1989). Testing started with an extensive model with 'group' (six subjects who received the supplements in the same order) and 'supplement' as main effects. To correct for possible carry-over effects, 'subject' nested in 'group' was added. 'Group x supplement' was included as interaction term. If possible, the model was simplified to only 'subject' and 'supplement' as main effects. Supplements were two-by-two compared by defining them as contrasts in the GLM procedure.

Results

Compliance

Lithium recovery was $<80\%$ for three subjects in wk 3. In wk 3 one subject reported that he had forgotten to eat one of the three portions of supplement on a measurement day. Because of illness, data of one subject in wk 2 are eliminated. Data of these subjects in these wk were eliminated from statistical analysis. According to the diaries 99% of the supplements was consumed.

Pleasantness

The pleasantness of the supplements as measured on a VAS from 0 (very bad) to 25 (very good) was 17.3 $\pm$5.3 for glucose, 12.6 $\pm$5.5 for RS_2 and 4.7 $\pm$4.9 for RS_3. These ratings were significantly different ($P < 0.002$). Despite differences in taste, subjects did not know when they consumed which supplement.

Appetite ratings averaged over the day

Figure 3.1 shows the appetite ratings, averaged over the day (0800-2300 h). Differences between the scores for the three supplements had normal distributions and the variances were homogeneous. Average scores were fitted in a model with 'subject' and 'supplement' as main effects. Consumption of RS_2 caused significantly lower scores than

either the glucose or the RS_3 supplement for all appetite dimensions except for appetite for something savoury. This means that subjects consuming RS_2 had less appetite, but paradoxically were feeling less full.

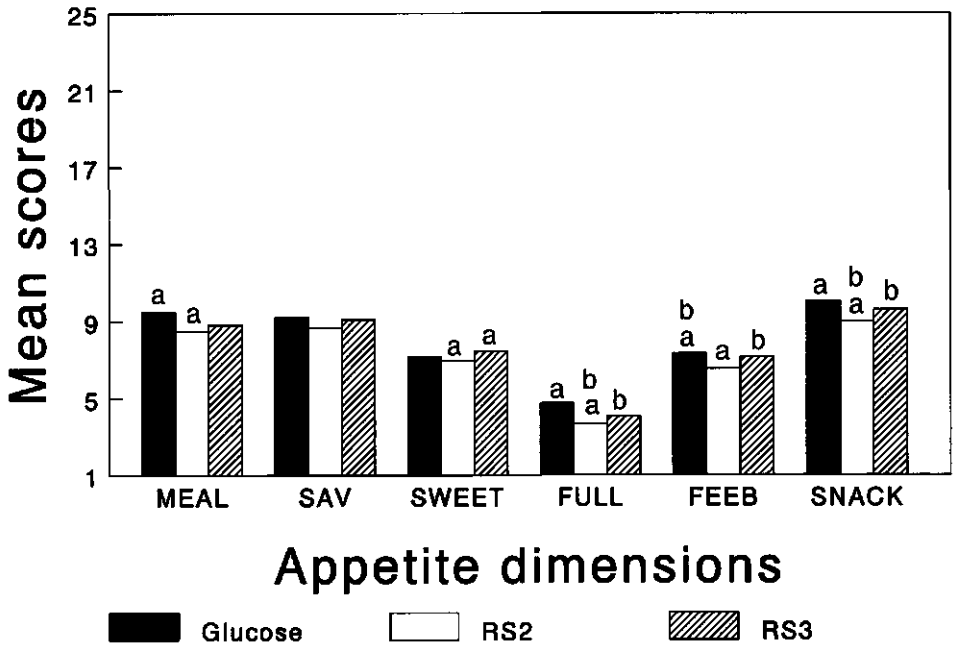

Figure 3.1 *Mean appetite scores averaged over the day, measured with a 150 mm visual analogue scale. A score of 1 means 'weak'; a score of 25 means 'strong'. Each mean score was computed from 351-364 scores. Abbreviations on the x-axis mean: MEAL, appetite for a meal; SAV, appetite for something savoury; SWEET, appetite for something sweet; FULL, satiety, fullness; FEEB, feeble and weak with hunger; SNACK, appetite for a snack.* ^{a,b}*Same characters indicate significant differences* ($P < 0.05$).

Appetite ratings during the day

Mean appetite scores for each supplement were also plotted against time of the day. The course during the day of responses for appetite for a meal, appetite for something

savoury, and feeble and weak with hunger were almost identical, while the course of responses for satiety (fullness) was the reverse. Therefore, only responses for appetite for a meal, appetite for something sweet and appetite for a snack are shown in Figure 3.2. Only responses between 0800 and 2300 h are shown, because very few responses were given outside this time frame.

Figure 3.2A shows that appetite for a meal peaked at the usual Dutch meals: breakfast (0800 h), lunch (1200 h), dinner (1800 h) and, less distinct, evening snack (2200 h). Appetite for something sweet (Figure 3.2B) did not show such distinct peaks, but was highest at lunchtime, dinnertime and in the late evening. Figure 3.2C shows three peaks in appetite for a snack: at 1200 h, 1600-1800 h, and after 2200 h. Differences between supplements had a normal distribution, and residual variances were homogeneous. Differences were tested using a model with 'subject' and 'supplement' as main effects.

Figure 3.2A shows that resistant starch seemed to cause less appetite for a meal between 1000 and 1300 and after 2000 h. Appetite for something sweet (Figure 3.2B), tended to be higher when consuming RS_3 between 1500 and 1800 h. Figure 3.2C shows that glucose tended to give a higher appetite for a snack between 1000 and 1500 h, and in the evening after 2000 h. RS_2 tended to decrease the appetite for a snack. Appetite for something savoury was not affected by the type of the supplement. Subjects felt less feeble and weak with hunger at dinnertime when consuming RS_2 than when consuming glucose. The type of supplement did not have a significant effect on satiety (fullness).

Intake of energy and macronutrients

Table 3.4 shows the average daily intake of energy and macronutrients during screening, and in wk 2, 3 and 4 as computed without the supplements. Energy intake was not significantly affected by the different supplements. Carbohydrate, fat and protein intake, as percentage of total energy intake, did not differ between the supplements. There was a small, but not significant, shift in the ratio of mono- and disaccharides to poly-saccharides as percentage of total energy intake. Subjects tended to eat more dietary fibre when consuming the glucose supplement. Energetic compensation when consuming the glucose supplement was 24%, when consuming RS_2 -5% (*i.e.* an increase in energy intake) and when consuming RS_3 15%. Subjects sometimes replaced habitual food items for the supplements. This happened mostly at breakfast, and instead of bread or milk products. Body weight of the subjects remained constant during the study.

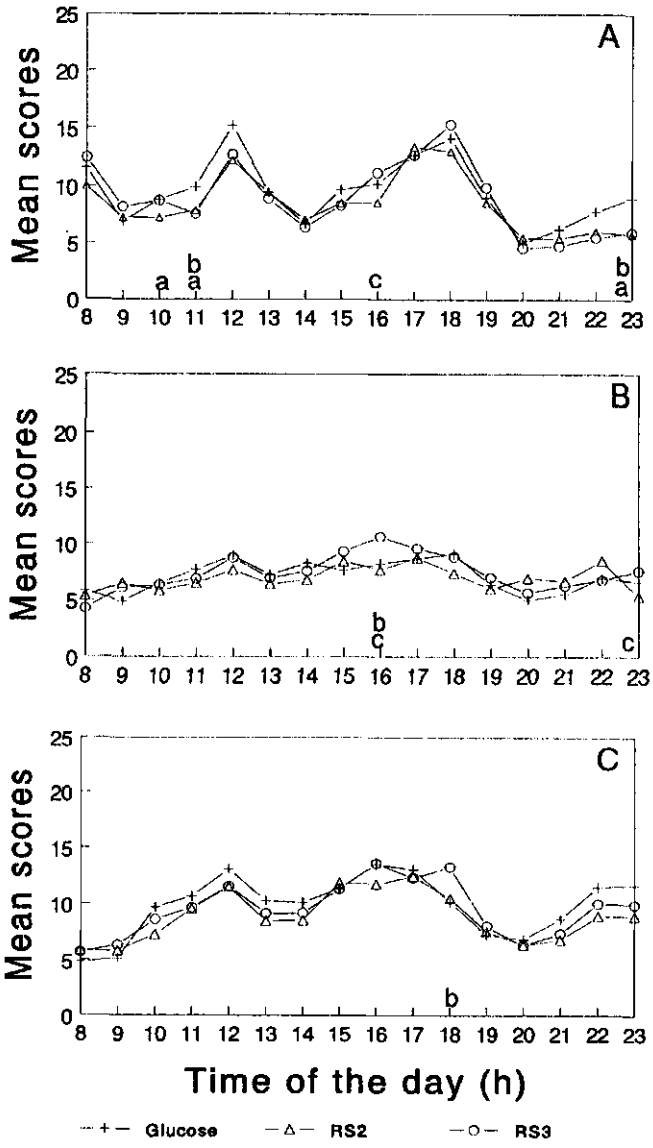

Figure 3.2 *Average scores during the day for (A) appetite for a meal, (B) appetite for something sweet and (C) appetite for a snack. Each mean score was computed from 18-24 scores. Significant differences are indicated as follows: a = glucose significantly different from RS_2; b = glucose significantly different from RS_3; c = RS_2 significantly different from RS_3 ($P < 0.05$).*

Table 3.4 *Daily intake of energy and nutrients during screening and wk 2, 3 and 4 of the experiment, computed without the supplements. Results are expressed as mean ±SD.*

	Screening	Glucose	RS_2	RS_3
Energy (MJ)	13.3 ±3.7	12.7 ±4.2	13.4 ±4.3	13.0 ±3.0
Protein (% of energy intake)	13 ±3	13 ±5	13 ±5	14 ±3
Fat (% of energy intake)	34 ±8	37 ±16	35 ±14	37 ±13
Carbohydrate (% of energy intake)	50 ±9	46 ±9	46 ±9	46 ±10
Mono- + disaccharides (% of energy intake)	20 ±11	18 ±7	19 ±7	21 ±9
Polysaccharides (% of energy intake)	30 ±11	27 ±7	26 ±8	25 ±7
Alcohol (% of energy intake)	3 ±5	5 ±8	7 ±11	4 ±7
Dietary fibre (g/MJ)	3.4 ±1.1	3.0 ±1.1	2.7 ±1.0	2.7 ±1.1

Excretion of C-peptide in 24-h urine

Excretion of C-peptide was highest while consuming the glucose supplement: 4.71 ±1.73 nmol/d. When consuming RS_2 the excretion was 4.39 ±1.46 nmol/d, and when consuming RS_3 3.74 ±1.42 nmol/d. The difference between glucose and RS_3 was significant ($P = 0.0001$), as was the difference between RS_2 and RS_3 ($P = 0.0012$).

Discussion

This study did not show a marked effect of RS on appetite and energy intake. Hourly ratings for the six appetite dimensions on a 150 mm VAS did not show a more satiating effect for RS compared to glucose. Averaged over the day RS_2 tended to be more satiating, but the differences between the supplements were of little practical significance. The biggest difference was 1.1, which is relatively small on a scale from 1 to 25. Paradoxically, when consuming RS, energetic compensation tended to be smaller than when consuming glucose; in other words RS tended to be less satiating. Presumably, no effect on appetite was found because in this study, subjects were allowed to eat and drink *ad libitum*. It could be expected that they adjusted their energy intake to the supplements so that their habitual pattern of appetite dimensions did not change. Energy intake though,

computed from the 24-h food recalls, remained constant during the three supplement periods and as high as before the experiment (Table 3.4). A preload-testmeal study design, as described by Delarghy *et al.* (1993), would give a better insight in the relationship between appetite and energy intake. In the study of Delarghy, dietary fibre was given in a breakfast, and subjects fasted until lunchtime. Energy intake during lunch and the rest of the day was recorded. Subjects rated their appetite on a visual analogue scale. Energy intake during lunch (and during the whole day) can be an indication for the satiating effect of the fibre.

When analysing the results of energy intake, the big within-subject variance attracted attention. With 23 subjects, a within-subject variance of 3.27 MJ (five 24-h food recalls per person) was found. When the level of significance is set at 5% and the desired power is 90% a reduction in energy intake of at least 1.73 MJ would be classified as significant. This is about 13% of the daily energy intake. In future experiments the within-subject variance could be minimized by providing for all the meals.

Appetite during the day did not change when consuming indigestible starch. Subjects maintained their regular pattern of food intake. This was also shown in a weight reduction study of de Graaf *et al.* (1993). The three peaks during the day for appetite for a meal and for something savoury are in agreement with a statement of Read that people are batch feeders (Read 1992). This means that they take discrete meals, with 3 to 4 h in between, in which they eat little or no food.

Consumption of the RS_3 supplement tended to cause a higher consumption of mono- and disaccharides and a lower consumption of polysaccharides compared to the glucose supplement. This can be explained by the bad taste of RS_3: to make this supplement more palatable to eat, subjects added sugar and fruit syrup. Subjects also tended to exchange bread for the RS_3 supplement at breakfast.

This study showed that consumption of RS decreased 24-h insulin excretion. RS_3 had the biggest effect: a decrease of 20%. The excretion of C-peptide was relatively low compared to a study of Jenkins *et al.* (1987*b*) where diets with high and low glycaemic index (GI) index were compared. In Jenkins' study excretion of C-peptide was 12.2 nmol/d for the low-GI diet. Consumption of carbohydrates was 38 g/MJ. The subjects of our study consumed 32 g carbohydrate/MJ when consuming the glucose supplement. This difference is too small to explain the seemingly low excretion of C-peptide in our study as compared to Jenkins' study.

C-peptide excretion when consuming glucose was highest, but this is caused by one very high excretion (11.2 nmol/d). Without this number, C-peptide excretion was in the range of 3.15-7.04 nmol/d. Mean excretion becomes 4.39 $\pm$0.92 nmol/d. This is still significantly higher than when consuming RS_3 and similar to RS_2.

The reason why RS_3 had more influence on the C-peptide excretion than RS_2 is yet unclear. It is possible that RS_3 delays the emptying of the stomach more than RS_2 does, so less glucose enters the blood at one time. It is also possible that RS_3 is better fermented in the colon. Fermentation products could decrease the hepatic output of glucose (Thorburn *et al.* 1993). However, other studies indicate that RS_3 is less well fermented than RS_2.

There were small differences in effect between RS_2 and RS_3 with the variables measured. RS_2 tended to be more satiating than RS_3. The C-peptide excretion was more decreased by RS_3. It is too early to decide whether RS_2 and/or RS_3 can be used in the therapy of obesity and diabetes. Further insight in the fermentation of resistant starch in healthy people is necessary. Beside this, efforts should be made to make resistant starch more palatable, because the RS used in this study was not fit for household use.

4

Limited effect of consumption of uncooked (RS_2) or retrograded (RS_3) resistant starch on putative risk factors for colon cancer in healthy men

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Submitted for publication.

Abstract

To investigate whether resistant starch (RS) affects putative risk factors for colon cancer, 24 healthy men consumed for 4 wk a daily supplement in addition to their habitual diet in a single-blind randomized balanced multiple cross-over trial. During the first week, all subjects consumed the control supplement containing glucose. Subsequently, each subject consumed in random order a supplement with RS_2 (uncooked high-amylose maize starch), RS_3 (extruded and retrograded high-amylose maize starch), and glucose, each for 1 wk. The RS_2 and RS_3 supplements provided 32 g RS/d and the glucose supplement 4 g RS/d. To measure compliance lithium was added to the supplements. Weekly, faeces, 24-h urine and breath samples, and a 24-h food-consumption recall were obtained from each subject. Compliance as measured by urinary lithium recovery was satisfactory. The mean composition of the background diet did not differ between the various supplementation periods. Breath H_2 excretion, stool weight and faecal starch excretion were significantly higher during RS than during glucose supplementation, and did not differ between RS_2 and RS_3. There were no significant differences between the three supplements in faecal dry weight, pH and short-chain fatty acid concentrations, nor in the pH, bile acid concentrations, cytotoxicity and osmolality of faecal water. It is concluded that in healthy men supplementating the habitual diet for 1 wk with 32 g/d RS compared with glucose has no effect on putative risk factors for colon cancer, except for increasing stool weight and colonic fermentative activity. There were no significant differences between RS_2 and RS_3 in the parameters studied.

Introduction

As resistant starch (RS) is not absorbed in the small intestine of healthy individuals (Asp 1992) but is (partly) fermented in the colon, it may have positive effects on putative risk factors for colon cancer, by analogy with dietary fibre. Colonic fermentation of RS may lead to the production of short-chain fatty acids (SCFA; Muir *et al.* 1994, Cummings *et al.* 1995, Nordgaard *et al.* 1995). Some studies indicate that fermentation of RS leads specifically to an increase in butyrate (Scheppach *et al.* 1988*b*, van Munster *et al.* 1994*b*, Phillips *et al.* 1995). Butyrate is a putative protective factor towards colon cancer (Roediger 1982, Gamet *et al.* 1992, Scheppach *et al.* 1995, Csordas 1996). SCFA, which are physiologically active in the large intestine, induce a decrease in colonic pH resulting in reduced solubility of bile acids (Bruce 1987). Further, the initial, irreversible step in bacterial conversion of primary into secondary bile acids (7α-dehydroxylation) is inhibited at pH below 6.5 (MacDonald *et al.* 1978, Nagengast *et al.* 1988*a*). The amount of soluble long-chain fatty acids and soluble bile acids (Rafter *et al.* 1986), the secondary bile acids in particular (van der Meer *et al.* 1991), may affect the cytotoxicity, *i.e.* the cell-damaging properties, of faecal water. Faecal water is the fraction of faeces which contains the water-soluble, not-bound components of the faeces (Lapré 1992) that are in contact with the colonic mucosal cells (Bruce 1987, Geltner Allinger *et al.* 1989, van Munster & Nagengast 1991). A diet-induced increase of the bile acid concentration in faecal water resulted in a higher cytotoxicity of faecal water in man (Rafter *et al.* 1987) and rat (Lapré *et al.* 1991) and was associated with higher colonic cell proliferation in rats (Lapré & van der Meer 1992). In healthy volunteers, proliferation of mucosal cells from a rectal biopsy was increased and associated with an increase in the concentrations of total and secondary bile acid in faecal water after consumption of fat as a bolus (Stadler *et al.* 1988). However, there is no conclusive evidence for a causal relationship between cytotoxicity of faecal water and mucosal proliferation. Hyperproliferation of colonic epithelial cells is suggested to be an important biomarker of increased susceptibility to colon cancer (Lipkin 1988). However, in rats colonic epithelial proliferation was found not to be a reliable predictor of tumor formation (Young *et al.* 1996). In addition, rat studies indicated that the secondary bile acids deoxycholic and lithocholic acid may be colon tumor promoters (Narisawa *et al.* 1974, Bull *et al.* 1983, Summerton *et al.* 1985).

The hypothesis that RS consumption may be protective towards colon cancer is supported by some epidemiological and experimental studies. In an ecological study, strong inverse associations were found between large bowel cancer incidence and consumption of starch or of non-starch polysaccharides (NSP) in combination with RS which was estimated to be 5% of total starch intake (Cassidy *et al.* 1994). Supplementation with 28 g RS/d for 2 wk resulted in an increase of breath H_2 excretion (a semi-quantitative measure of colonic fermentation; Rumessen 1992) and faecal SCFA excretion, in a decrease of secondary bile acid concentration and cytotoxicity of faecal water, and in a decrease of colonic mucosal proliferation in rectal biopsies (van Munster *et al.* 1994*b*). However, this study lacked a control group and only RS derived from uncooked granular starch (*i.e.* RS_2; Englyst *et al.* 1992) was studied. Cummings *et al.* (1996) found that RS_2 gave greater proportions of acetate in faeces but RS_3 (*i.e.* retrograded resistant starch; Englyst *et al.* 1992) more propionate. However, the amounts of RS consumed in their experiment were different between the various types of RS studied, and faecal bile acids, cytotoxicity of faecal water and mucosal proliferation were not measured. These parameters were also not measured in a study with a diet containing 39 g/d of a mixture of RS_1 (*i.e.* starch physically inaccessible to α-amylase; Englyst *et al.* 1992), RS_2 and RS_3 (Phillips *et al.* 1995). This high-RS diet induced a lower faecal pH and increased faecal concentration and excretion of butyrate and acetate compared with a diet containing only 5 g RS/d. Recently, three studies were published that investigated the effect of dietary RS on chemically-induced colon cancer in rats (Caderni *et al.* 1994, Sakamoto *et al.* 1996, Young *et al.* 1996). However, in these studies the amount and/or the type of RS used is unclear.

Because the studies mentioned above are not conclusive, we investigated whether supplementating the habitual diet with 32 g/d of either RS_2 or RS_3 compared with an equivalent amount of glucose would affect positively putative risk factors for colon cancer in 24 healthy men in a randomized balanced multiple cross-over trial. Because it has been suggested that RS_2 is better fermentable than RS_3 (Cummings *et al.* 1995, Schulz *et al.* 1993, Champ *et al. unpublished results*) we hypothesized also that consumption of RS_2 compared with RS_3 would increase breath H_2 excretion and faecal SCFA concentrations, and would decrease faecal pH, and secondary bile acid concentrations and cytotoxicity of faecal water.

Methods

Subjects

Twenty-four apparently healthy men were recruited by advertisements in local newspapers and posters mounted in public buildings in Wageningen. The inclusion criteria were as follows: age >18 y; weight fluctuation during the previous 3 mo not more than 2.5 kg; no diseases of the kidneys or the gastrointestinal tract; no diabetes mellitus; no stomach or bowel surgeries other than removal of the appendix; no complaints of diarrhea, obstipation or abdominal pain; no use of antibiotics or laxatives during the previous 3 mo; and preferably a good appetite and daily stools. One subject took antibiotics in week 2: his data were excluded from statistical analysis.

Characteristics of the subjects were (mean ±SD): age, 23 ±2 y; height, 1.84 ±0.07 m; body weight, 76.9 ±7.5 kg; body mass index (BMI), 22.7 ±1.8 kg/m^2. The experimental design of the study and possible discomforts of the consumption of the supplements were explained to the subjects before they gave their written informed consent. The study protocol was approved by the Medical-Ethical Committee of the Department of Human Nutrition of the Wageningen Agricultural University. Subjects were paid for their participation after they had completed the experiment.

Study design

The subjects consumed for 4 wk a daily supplement in three portions/d in addition to their habitual diet in a single-blind randomized balanced multiple cross-over trial using an orthogonal Latin-square design for three treatments. During the first week (run-in period) each subject consumed the control supplement containing glucose. Subsequently, each subject consumed a supplement containing RS_2, RS_3 or glucose; each type of supplement was consumed for 1 wk. The 24 subjects were randomly divided into six groups before the experiment started. Each group consumed the supplements in one of the six possible sequences to eliminate variation due to residual effects of the previous supplement or to drift of variables over time (Snedecor & Cochran 1980). The groups were not different with respect to age, height, body weight and BMI (*data not shown*).

During d 5-7 of wk 2, 3 and 4 the subjects defaecated twice at the Department of Human Nutrition. The faecal samples were weighed and frozen immediately at -20 °C. With each supplement portion 10 radioopaque barium-sulfate impregnated, polyethylene

rings (TD Medical B.V., Eindhoven, the Netherlands) were swallowed (*i.e.* 30/d) to serve as a marker for faeces collection. All stools were X-rayed before sampling to count the polyethylene rings in each frozen stool. At d 6 and 7 of wk 2, 3 and 4 the subjects collected 24-h urine for determination of lithium (*see* below). Weekly, a 24-h food-consumption recall was obtained from each subject to check whether the amount and composition of the habitual diet had remained constant. Subjects were weighed twice a week while wearing light indoor clothes with empty pockets and without shoes.

Supplements

The supplements consisted of a mixture of 144 g/d skim yogurt, 216 g/d skim milk, 123-183 g/d mashed canned fruit (the amount depending on the type of fruit used), and either one of the carbohydrate preparations (obtained from Cerestar Vilvoorde, Belgium). The carbohydrate added was 110.4 g/d glucose (glucose monohydrate, dry weight 91.6%) for the glucose supplement, 58.5 g glucose plus 47.4 g/d uncooked high-amylose maize starch (Amaizo-7, dry weight 90.3%, 63.3% RS by wt as measured *in vitro* (Englyst *et al.* 1992)) for the RS_2 supplement and 100.2 g/d retrograded high-amylose maize starch (extruded and retrograded Amaizo-7, dry weight 90.8%, 29.9% RS by wt as measured *in vitro* (Englyst *et al.* 1992)) for the RS_3 supplement. The amounts of ingredients were set to obtain 30 g RS in the RS_2 and the RS_3 supplements and equal amounts of glucose units in the supplements. Corrections were made for the different water contents of the carbohydrate preparations and for the water excluded during formation of glycosidic bonds. The supplements had identical nutrient compositions except for the type of carbohydrate (Table 4.1). *In vitro* analysis (Englyst *et al.* 1992) confirmed that the control supplement contained mostly digestible carbohydrate, whereas the RS_2 and the RS_3 supplements contained 32 g RS/d (Table 4.1). To measure compliance, 80 μmol lithium chloride was added to each supplement portion and lithium recovery in 24-h urine was measured by atomic absorption spectrophotometry as described before (Heijnen *et al.* 1996*a*). We did not try to equalize the gross energy content of the supplements because there is no accurate estimate of the amount of energy that RS supplies. At most, the glucose supplement contained 500 kJ (about 4% of total energy intake in this group of subjects) more than the RS supplements assuming that RS supplies no energy at all.

The supplements were prepared in the kitchen of the Department of Human Nutrition three times a week and the subjects took them home for consumption. Supplements were

described elsewhere (Glatz *et al.* 1985, Grundy *et al.* 1965) with minor modifications. The bile acid derivates were analyzed on a capillary fused silica column (length 25 m, internal diameter 0.20 mm) coated with 0.11 μm HP Ultra-1 phase (Hewlett-Packard Co, Polo Alto, CA) by using a Hewlett Packard gas chromatograph (model 5890 series II) equipped with a liquid sampler (model HP-7673) and a mass selective detector (model HP-5971) operating in electron impact (70 eV) and selected ion mode (SIM). One μl was injected in split mode (split ratio 1:100). Helium gas was applied as the carrier gas with a constant flow rate of 0.7 ml/min. The oven temperature which was initially 150 °C, was gradually increased to 275 °C and maintained at this temperature for 30 min. The temperature of the injection port was 325 °C and of the direct MS-interface 275 °C. Instrument control and data acquisition were performed with HP MS-ChemStation version C.03.00 software on a HP Vectra 486/25T computer. The amount of each bile acid was calculated from area response by using the internal standard method with a 5-points multilevel braqueting calibration with pure standards.

H_2 in breath

Assuming that the amount of H_2 in breath is directly related to the extent of colonic fermentation *in vivo* (Rumessen 1992), end-expiratory breath samples were obtained to test the hypothesis that RS_2 is better fermentable than RS_3. Unfortunately, due to unforeseen technical problems a considerable number of the breath samples could not be analyzed. Therefore, another experiment was carried out with 15 apparently healthy non-smoking men with the same characteristics as those in the initial study, and using exactly the same supplements, study design, and inclusion and exclusion criteria as in the first study, but omitting the run-in period. To avoid excess H_2 being channelled into CH_4 production, only subjects that were non-CH_4 excreters were enrolled. A subject was classified as non-CH_4 excreter when on three different days before the study started the CH_4 concentration in his breath was <3 ppm above the concentration in ambient air (Nagengast *et al.* 1988*b*, Rumessen 1992). All subjects completed the study successfully. They reported in their diaries that 98% of the supplements were consumed. The frequency of defaecation, the rated consistency of the faeces, and the number and severeness of gastrointestinal discomforts were very similar to those in the main experiment (*data not shown*). Body weight remained constant throughout the study.

To investigate whether an adaptation to RS as substrate for colonic fermentation took

place, end-expiratory breath samples were collected both on d 2 and 7 of each supplementation period. On each sampling day, one sample was collected between 0730-0830 h, one between 1200-1300 h, one between 1630-1730 h, one between 2000-2100 h, and one the next morning between 0730-0830 h. Breath samples were taken and stored in plastic syringes of 60 ml equipped with a cap (Plastipak, Becton Dickinson, Dublin, Ireland). Immediately after all samples at one time-point had been collected, their H_2 content was measured with an electrochemical measurement cell (Exhaled Hydrogen Monitor; Gas Measurements Ltd., Renfrew, Scotland). The measurement cell was calibrated before each run with ambient air and 100 ppm H_2-in-air gas (Intermar BV, Breda, the Netherlands). The 24-h integrated breath H_2 excretion was estimated by calculating geometrically the area under the curve of breath H_2 content versus time (Wolever & Jenkins 1986).

Statistical analysis

Differences between group means for each variable were evaluated by two-way analysis of variance with the GLM (General Linear Models) procedure of SAS (release 6.09; Statistical Analysis Systems Institute Inc, Cary, NC). The model contained 'subject' as the random factor, thus taking the intrinsic individual levels into account, and 'supplement' as the fixed factor. When the analysis of variance indicated a significant effect of supplement ($P < 0.05$), Tukey's Studentized range test was used for pair-wise comparison of the group means for each variable as induced by the three supplements. This method encompasses a downward adjustment of the significance limit for multiple testing. The 24-h integrated breath H_2 excretion on d 2 of a supplementation period was compared with d 7 by using a paired t test.

Results

Compliance

According to the diaries 0.4% of the glucose supplements, 1.4% of the RS_2 supplements, and 1.9% of the RS_3 supplements was not consumed. Mean (±SEM) urinary lithium recovery was 96 ±3% during glucose supplementation, 100 ±3% during RS_2 supplementation and 92 ±2% during RS_3 supplementation.

Food consumption and body weight

No significant differences were found in reported energy and nutrient intakes when the various supplements were given (Table 4.2). Body weight remained constant throughout the study (ANOVA, $P = 0.06$).

Table 4.2 *Energy and nutrient intakes during daily supplementation of the habitual diet with either 32 g glucose, RS_2, or RS_3 for 1 wk*[1]

Nutrient	Dietary supplement		
	Glucose[2]	RS_2	RS_3[2]
Energy (MJ/d)	13.0 ±1.0	13.6 ±1.0	13.3 ±0.6
Protein (g/d)	97 ±7	105 ±9	106 ±5
Fat (g/d)	126 ±12	123 ±11	132 ±8
Carbohydrate (g/d)	355 ±26	366 ±28	357 ±18
Alcohol (g/d)	22 ±7	36 ±11	18 ±7
Dietary fibre (g/d)	39 ±3	34 ±3	34 ±2

[1] Mean ±SEM. Supplements are not included in the calculations (n=23). Calculated by using a computerized food composition table (NEVO Foundation 1987). There were no significant differences by ANOVA. Amounts of RS as measured *in vitro* (Englyst *et al.* 1992).

[2] One subject reported to have consumed two supplement portions only on the day of the 24-h food-consumption recall; his data were excluded from analysis.

H_2 in breath

Supplementation with RS_2 and RS_3 led to significantly more H_2 excretion in breath than supplementation with glucose, both on d 2 ($P < 0.01$) and d 7 ($P < 0.05$, Table 4.3). Breath H_2 excretion during RS_2 and RS_3 supplementation was similar. For each supplement H_2 excretion was similar on d 2 and d 7.

Frequency and consistency of faeces

During supplementation with RS_2 more defaecations (mean ±SEM: 10.2 ±0.7 stools/wk) were reported than during supplementation with glucose (8.9 ±0.5 stools/wk; $P < 0.05$). The number of stools during RS_3 supplementation (9.6 ±0.5 stools/wk) did not differ

significantly from either glucose or RS_2 supplementation. The mean rated consistency of the faeces (scored on a scale of 1 to 8) did not differ during glucose (5.6 ±0.2), RS_2 (5.4 ±0.2), and RS_3 supplementation (5.5 ±0.2). This agrees with the lack of difference in percentage dry weight of the faeces (*see* below).

Table 4.3 *Area under the 24-h curve of H_2 concentration in end-expiratory breath versus time during daily supplementation of the habitual diet with either 32 g glucose, RS_2, or RS_3 for 1 wk*[1]

	Dietary supplement		
	Glucose[2]	RS_2	RS_3[2]
Area on day 2 (ppm.h)	420 ±39[a]	683 ±73[b]	660 ±71[b]
Area on day 7 (ppm.h)	454 ±58[a]	634 ±64[b]	656 ±67[b]
Area, mean of days 2 and 7 (ppm.h)	432 ±43[a]	658 ±61[b]	662 ±64[b]

[1] Mean ±SEM; $n=15$. Values in the same row with different superscript letters are significantly different ($P < 0.05$). Amounts of RS as measured *in vitro* (Englyst *et al.* 1992).

[2] $n=14$ due to missing values.

Faecal output

Stool weight was higher during RS supplementation than during glucose supplementation (Table 4.4). If the data of two subjects with relatively very high stool weights were omitted, mean (±SEM) stool weight during RS_3 supplementation changed from 301 ±29 g/d to 267 ±15 g/d which is similar to the stool weight after RS_2 supplementation (277 ±20 g/d). Faecal dry weight and pH did not differ significantly between the three supplementation periods (Table 4.4). After RS supplementation more starch was found in faeces than after glucose supplementation, the highest amount being 18% of the amount supplemented (RS_3 in this case). The pH, cytotoxicity and osmolality of faecal water were not different during the three supplementation periods (Table 4.4).

Table 4.4 *Faecal parameters after daily supplementation of the habitual diet with either 32 g glucose, RS_2, or RS_3 for 1 wk*[1]

	Dietary supplement		
	Glucose[2]	RS_2	RS_3
Mixed wet faeces			
Wet weight (g/d)	232 ±19[a]	277 ±20[a,b]	301 ±29[b]
Dry weight (g/d)	55 ±3	66 ±5	66 ±4
(%)	24.9 ±0.9	24.3 ±0.7	23.2 ±0.9
Starch[3] (g/d)	1.1 ±0.4[a]	4.5 ±1.7[a,b]	5.6 ±2.0[b]
pH	6.8 ±0.1	6.7 ±0.1	6.6 ±0.1
Faecal water			
pH	6.7 ±0.1	6.6 ±0.1	6.5 ±0.2
Cytotoxicity[4] (%)	53 ±8	59 ±9	49 ±9
Osmolality (mosmol/kg)	553 ±24	528 ±17	528 ±19

[1] Mean ±SEM; $n=23$. Values in the same row with different superscript letters are significantly different ($P < 0.05$). Amounts of RS as measured *in vitro* (Englyst *et al.* 1992).

[2] $n=22$ due to missing values.

[3] Measured as starch and its degradation products (corrected for free glucose) according to the procedure of Björck *et al.* (1987).

[4] Measured as release of potassium from erythrocytes due to lysis (Govers *et al.* 1996).

SCFA in faeces

Total SCFA concentration in faeces did not differ significantly between the three supplementation periods (Table 4.5). The molar ratio of acetate:propionate:butyrate (the main SCFA) was approximately 4:1:1 during the three supplementation periods. The sum of isobutyric, valeric, isovaleric and caproic acid comprised only 7.9 ±0.6% (mean ±SEM) of the total amount of faecal SCFA during glucose supplementation and 7.5 ±0.6% during RS_2 and RS_3 supplementation (not significantly different). Isovalerate comprised a significantly lower ($P < 0.05$) percentage of total SCFA during RS_3 than during glucose supplementation and caproate a significantly higher ($P < 0.05$) percentage.

Table 4.5 *Short-chain fatty acids (SCFA) in faeces after daily supplementation of the habitual diet with either 32 g glucose, RS_2, or RS_3 for 1 wk*[1]

	Dietary supplement		
	Glucose[2]	RS_2[3]	RS_3[3]
Total (μmol/g wet feces)	106.5 ±6.2	115.6 ±6.9	109.0 ±7.4
Acetate (% of total)	59.4 ±0.9	59.8 ±1.4	59.5 ±1.7
Propionate (% of total)	16.2 ±0.7	15.1 ±0.6	15.4 ±1.3
Butyrate (% of total)	16.5 ±0.5	17.7 ±1.2	17.6 ±0.8
Isobutyrate (% of total)	1.7 ±0.1	1.6 ±0.1	1.4 ±0.2
Valerate (% of total)	2.4 ±0.1	2.2 ±0.2	2.2 ±0.2
Isovalerate (% of total)	2.6 ±0.3[b]	2.3 ±0.2[a,b]	2.1 ±0.3[a]
Caproate (% of total)	1.3 ±0.2[a]	1.4 ±0.2[a,b]	1.8 ±0.4[b]

[1] Mean ±SEM; $n=23$. Values in the same row with different superscript letters are significantly different ($P < 0.05$). Amounts of RS as measured *in vitro* (Englyst *et al.* 1992).

[2] $n=22$ due to missing values.

[3] $n=21$ due to missing values.

Bile acids in faecal water

The concentration of total, primary (sum of cholic and chenodeoxycholic acid), secondary (sum of deoxycholic acid, isodeoxycholic acid, lithocholic acid, isolithocholic acid, 12-ketolithocholic acid, 12-keto-isolithocholic acid, ursodeoxycholic acid, and 7-ketodeoxycholic acid), and individual bile acid concentrations in faecal water did not differ significantly between the three supplementation periods (Table 4.6). However, compared with glucose, supplementation with RS_2 and RS_3 tended to increase total bile acid concentration, mainly due to an increase in secondary bile acids, especially lithocholic and isolithocholic acid. Secondary bile acids comprised about 85% of total bile acids in all supplementation periods. Deoxycholic acid was by far the most abundant bile acid, followed by lithocholic and isodeoxycholic acid.

Gastrointestinal discomforts

During RS_3 supplementation 91% of the subjects and during RS_2 supplementation 82% of the subjects reported flatulence, compared with 55% of the subjects during glucose supplementation. Of those mentioning flatulence, half reported flatulence on 4 d/wk or

Table 4.6 *Concentration of bile acids in faecal water after daily supplementation of the habitual diet with either 32 g glucose, RS_2, or RS_3 for 1 wk*[1]

	Dietary supplement		
	Glucose[2]	RS_2[2]	RS_3[3]
Total bile acids (μmol/l)	384 ±61	485 ±131	480 ±189
Total primary bile acids (μmol/l)	34 ±14	47 ±8	40 ±12
Cholic acid (μmol/l)	24 ±11	31 ±6	28 ±9
Cheno-deoxycholic acid (μmol/l)	11 ±3	16 ±2	12 ±3
Total secondary bile acids (μmol/l)	350 ±62	439 ±131	441 ±181
Deoxycholic acid (μmol/l)	196 ±34	226 ±60	224 ±93
Isodeoxycholic acid (μmol/l)	65 ±12	54 ±12	60 ±17
Lithocholic acid (μmol/l)	47 ±13	101 ±38	92 ±41
Isolithocholic acid (μmol/l)	19 ±8	37 ±19	42 ±27
12-Ketolithocholic acid (μmol/l)	15 ±3	11 ±3	13 ±5
12-Keto-isolithocholic acid (μmol/l)	5 ±2	6 ±2	6 ±4
Ursodeoxycholic acid (μmol/l)	2 ±0.5	3 ±0.6	3 ±0.8
7-Ketodeoxycholic acid (μmol/l)	1 ±0.3	2 ±0.3	2 ±0.5
Secondary bile acids (% of total)	89 ±3	84 ±3	87 ±3

[1] Mean ±SEM; $n=23$. There were no significant differences by ANOVA. Amounts of RS as measured *in vitro* (Englyst *et al.* 1992).

[2] $n=21$ due to missing values.

[3] $n=19$ due to missing values.

more during RS_2 and RS_3 supplementation, and only 18% during glucose supplementation. Bloated feelings were reported by 41% of the subjects during RS_3 supplementation, by 28% of the subjects during RS_2 supplementation and by 9% of the subjects during glucose supplementation. During glucose and RS_2 supplementation subjects reported bloated feelings for only 1 d/wk, while during RS_3 supplementation 33% of the subjects who suffered from bloated feelings reported them 4 d/wk or more. Very few other gastrointestinal discomforts were reported: only on 1 d/wk by 5% of the subjects during glucose supplementation, by 18% of the subjects during RS_2 supplementation and by 14% of the subjects during RS_3 supplementation. Severe side effects were not reported.

Awareness of the nature of the supplements

The subjects were not informed about the sequence in which they would receive their supplements until they completed the experiment. At the end of the study the participants were asked to indicate the supplement sequence as perceived. The sequence was perceived right by 2 of the subjects (8%); 10 subjects (42%) were able to discern the glucose from the RS supplements but could not discern between the RS_2 and RS_3 supplements. The other 12 subjects (50%) perceived the sequence of their supplements totally wrong.

Discussion

This study showed that in healthy men 1 wk supplementation with 32 g/d RS_2 or RS_3 compared with glucose increased H_2 excretion in breath, faecal starch excretion and stool mass, but had no effect on faecal pH and SCFA concentrations, nor on the bile acid concentrations and cytotoxicity of faecal water. No differences were found between RS_2 and RS_3. Others have shown that a dietary change for 1 wk is sufficiently long to detect changes in faecal pH (Govers *et al.* 1996), faecal bile acids (Rafter *et al.* 1987, Stadler *et al.* 1988, Govers *et al.* 1996), cytotoxicity of faecal water (Rafter *et al.* 1987, Govers *et al.* 1996) and mucosal proliferation (Stadler *et al.* 1988). In our study, both reported compliance and compliance as assessed by urinary lithium recovery were >90%. Lithium recovery during RS_3 supplementation was slightly lower than during glucose and RS_2 supplementation, possibly because most subjects appreciated the RS_3 supplement as the least palatable (de Roos *et al.* 1995). Despite differences in palatability, only half of the subjects was able to discern the glucose from the RS supplements. It is unlikely that awareness of the nature of the supplements or the small difference in lithium recovery could have affected the outcome of the study with regard to faecal composition and breath H_2 excretion. As in other studies with comparable supplements (van Munster *et al.* 1994*a*,*b*, Heijnen *et al.* 1996*a*) no changes were observed in the composition of the background diet during the study. Therefore, it may be assumed that differences in the outcome variables are caused by consumption of the supplements. The supplementation dose of 32 g RS/d is estimated to be about six times the current average intake of RS in

the Netherlands (Dysseler & Hoffem 1995*a*). This dose was tolerated well as only some flatulence and bloated feelings were reported.

As found by others too (Muir *et al.* 1994, van Munster *et al.* 1994*a,b*) breath H_2 excretion was increased during consumption of RS. Based on this semi-quantitative measure (Rumessen 1992), there did not seem to be a difference in fermentability between RS_2 and RS_3. However, the existence of a difference between RS_2 and RS_3 in the rate of fermentation and/or location of fermentation in the colon cannot be excluded on the basis of the results of this study (MacFarlane *et al.* 1992). The latter may have important consequences for colon cancer risk (Csordas 1996). We may not have measured the optimal fermentability of RS if a plateau level of H_2 excretion was reached *e.g.* because the amount of bacterial enzymes was limiting. Our results seem to disagree with *in vitro* data (Cummings *et al.* 1995), a rat study (Schulz *et al.* 1993), and two human studies (Olesen *et al.* 1994, Champ *et al. unpublished results*) that suggest that RS_2 is better and/or quicker fermentable than RS_3. However, the *in vitro* and human studies are difficult to interpret because not only the type of RS but also the amount of RS differed. Furthermore, *in vitro* studies may show inconsistent results depending on the inocula used: some subjects fermented one kind of RS well and another type poorly, implying that different colonic flora ferment various RS sources differently (Cummings *et al.* 1996). In the rat study (Schulz *et al.* 1993), the RS dose provided per kg metabolic body weight was about 5 times larger than the maximum dose that is tolerated well in human studies. Thus, the specific type and amount of RS and factors relating to the subject seem to determine whether or not and to which extent the amount of H_2 in breath increases after consumption of RS. During consumption of the glucose supplements there was also a significant H_2 excretion in breath, probably because the background diet provided a considerable amount of dietary fibre.

Compared with glucose, RS supplementation increased stool weight by 1.4 g/g RS_2 and by 2.2 g/g RS_3. As others reported similar results (van Munster *et al.* 1994*a*, Phillips *et al.* 1995, Cummings *et al.* 1996) it can be concluded that RS_2 and RS_3 have a mild laxative effect. This may be positive for human health because an inverse relationship has been reported between stool weight and colon cancer incidence (Cummings *et al.* 1992). Burkitt (1971) proposed a protective mechanism by dilution of the intestinal contents and reduction of intestinal transit time, thus reducing the contact of carcinogens with the colonic mucosa. The increase in stool weight can be explained for 7% only by the

increase of starch in the faeces. The increase in faecal mass cannot be explained by an increase in water content as, in agreement with others (Scheppach *et al.* 1988*a*, Phillips *et al.* 1995, Cummings *et al.* 1996), no differences in percentage dry matter of the faeces were found. Therefore, most likely stool weight was increased mainly by an increase in bacterial mass, which is supported by the reported increase in faecal nitrogen excretion after RS consumption (Birkett *et al.* 1996, Cummings *et al.* 1996). Further support is provided by a study in which starch malabsorption was induced by the α-amylase inhibitor acarbose which caused an increase in faecal bacterial mass and nitrogen excretion, and in bacterial nitrogen and diaminopimelic acid in faeces (Scheppach *et al.* 1988*a*).

Bacterial mass and colonic fermentation may be increased due to the availability of more substrate (RS) in the colon. This is confirmed by the increase in breath H_2 excretion and the small amount of starch in the faeces representing only 15-18% of the RS supplemented. Others (Phillips *et al.* 1995, Cummings *et al.* 1996) also reported an overall RS digestibility of 80-90%, and some found almost 100% (van Munster *et al.* 1994*b*). No significant differences between RS_2 and RS_3 were found with respect to faecal starch excretion which is largely in agreement with the findings of Cummings *et al.* (1996). The likely increase in colonic fermentation was not reflected in a drop of faecal pH which agrees with some studies (van Munster *et al.* 1994*b*) but not with others (Phillips *et al.* 1995). The pH of the colonic contents has been shown to rise gradually during the passage from caecum to sigmoid (Cummings *et al.* 1987, Fallingborg *et al.* 1989, MacFarlane *et al.* 1992), which is probably due to the rapid absorption of SCFA by the colonic epithelium (Cummings 1981, Cummings *et al.* 1987, MacFarlane *et al.* 1992). Thus, faecal pH is not necessarily a good indicator for fermentation and acidity in the proximal colon, and this may be the explanation for the absence of a change in faecal pH.

The rapid absorption of SCFA from the colon is probably also the explanation for the lack of differences between the supplements in faecal SCFA concentrations in this study and as reported by others (van Munster *et al.* 1994*b*, Phillips *et al.* 1995, Cummings *et al.* 1996), too. In contrast, starch malabsorption induced by acarbose resulted in an increased faecal SCFA excretion (Scheppach *et al.* 1988*b*). The total amount of SCFA excreted tended to be increased after RS supplementation both in this study and the one by Cummings *et al.* (1996) and was significantly increased after RS consumption in two

other studies (van Munster *et al.* 1994*b*, Phillips *et al.* 1995). This was probably related to the higher stool mass after RS consumption. The molar ratios of acetate, propionate and butyrate in this study, approximately 0.60, 0.15 and 0.17 are in the same range as reported before (Cummings 1981) and did not differ between the three supplements. The present study did not show a clear increase in faecal butyrate excretion after RS consumption, while some others did (Scheppach *et al.* 1988*b*, van Munster *et al.* 1994*b*, Phillips *et al.* 1995). Cummings *et al.* (1996) found a significant higher molar ratio of butyrate, but only after consumption of RS_2 from potatoes. Failure to measure an increase of faecal butyrate, however, does not exclude the possibility that *in vivo* fermentation of RS specifically increases butyrate production. Obviously, it is very difficult to measure SCFA production in the human colonic contents *in vivo*.

In the present study, bile acid concentrations in faecal water did not differ between RS and glucose supplementation, nor between RS_2 and RS_3. This is in accordance with the lack of a difference in cytotoxicity of faecal water. However, the faecal sampling method used may not have been optimal (Setchell *et al.* 1987) and the within-subject variability was very large, which may explain why the tendency of RS_2 and RS_3 to increase the concentration of total and secondary bile acids in faecal water was not statistically significant. In contrast, van Munster *et al.* (1994*b*) reported a decrease in total bile acid concentration in faecal water, mainly due to a decrease in deoxycholic acid concentration after consumption of 28 g/d RS_2 for 2 wk. The primary bile acid concentration in freeze-dried faeces rose significantly, and the secondary bile acid concentration tended to decrease in their study. Concomitantly, cytotoxicity of faecal water and mucosal proliferation in rectal biopsies decreased significantly. Unfortunately, however, there was no control group in this study. Starch malabsorption as induced by acarbose significantly decreased secondary bile acid concentration and excretion in faeces, and significantly increased the primary bile acid concentration and excretion (Bartram *et al.* 1991).

In conclusion, the present findings do not support the initial hypothesis that 1 wk supplementation of the habitual diet with 32 g/d of either RS_2 or RS_3 from maize starch compared with an equivalent amount of glucose positively affects putative risk factors for colon cancer in healthy men. No differences were found between RS_2 and RS_3. Neither does this study support a difference in fermentability between RS_2 and RS_3 as evaluated by breath H_2 excretion. No information could be obtained about the colonic location of fermentation of RS_2 and RS_3 nor on the magnitude of butyrate production in situ. Both

factors may be important regarding colon cancer risk. In addition, since consumption of RS_2 and RS_3 increased stool mass, a protective but perhaps limited effect of (longer term) resistant starch consumption towards colon cancer is still feasible.

Acknowledgements

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5

Retrograded (RS_3) but not uncooked (RS_2) resistant starch lowers faecal ammonia concentrations in healthy men

Heijnen MLA, Deurenberg P, van Amelsvoort JMM, Beynen AC.
American Journal of Clinical Nutrition 1997;65:167-168 (letter to the editor).

Dear Sir:

Birkett *et al.* (1996) reported that consumption of 39 g resistant starch (RS)/d but not 5 g/d, for 3 wk, lowered faecal concentrations of ammonia in healthy subjects. The RS used was a mixture of the three major types that occur naturally in the human diet (Englyst *et al.* 1992), namely, physically entrapped starch (RS_1), uncooked starch granules (RS_2), and retrograded starch (RS_3). We wish to extend the interesting findings of Birkett *et al.* (1996) by reporting here for the first time the results of an experiment that compared RS_2 and RS_3 as derived from well-defined maize starches.

Healthy men consumed a supplement each day in addition to their habitual diet in a single-blind, randomized 3 x 3 Latin-square experiment (de Roos *et al.* 1995). During the first week (run-in period) all subjects consumed the control supplement containing glucose. Subsequently, each subject consumed for 1 wk a supplement with RS_2 (Hylon VII; *i.e.* uncooked high-amylose maize starch), RS_3 (extruded, retrograded Hylon VII), and glucose. The 24 subjects were randomly divided into six groups before the start of the run-in period. Each group consumed the supplements in one of the six possible sequences so as to eliminate variation due to residual effects of the previous supplement or to drift of variables over time. The daily supplements provided 2 MJ and consisted of a mixture of skim yogurt, skim milk, mashed canned fruit, lithium, and either glucose, RS_2, or RS_3 (de Roos *et al.* 1995). The dietary variables were added to the supplements as identical amounts of glucose units (101 g glucose units/d). Radioopaque, barium-sulphate impregnated, polyethylene rings were swallowed with each supplement portion to serve as a marker for faeces collection. The RS_2- and RS_3-containing supplements each provided 32 g RS/d and the supplement with glucose contained 4 g RS/d as determined *in vitro* by the Englyst method (1992). Compliance, as measured by urinary recovery of lithium, was satisfactory and comparable in the three supplementation periods (de Roos *et al.* 1995). Weekly 24-h food consumption recalls showed that the amount and composition of the background diet were similar for all dietary periods (de Roos *et al.* 1995). Body weight remained constant throughout the study. On the last 2 d of each period, the subjects collected 24-h urine. Urinary urea and creatinine were measured by using commercial test combinations (no. 1688-05 and no. 1694-06; Abbott Laboratories, Irving, TX). During the last 3 d of each period, the subjects defaecated twice at the Department of Human Nutrition. The faeces were weighed and frozen immediately at

-20 °C. Ammonia was extracted from homogenized faeces with perchloric acid and measured with the use of a commercial test combination (Ammonia UV-method, cat. no. 1112732; Boehringer Mannheim GmbH, Mannheim, Germany).

One subject took antibiotics during the experiment; his data were excluded from statistical analysis. Glucose and RS_2 consumption produced similar faecal ammonia concentrations, whereas RS_3 had a significant lowering effect (Table 5.1). Thus, the finding of Birkett *et al.* (1996) that a diet rich in a mixture of RS types lowered faecal ammonia concentrations may have been caused specifically by the RS_3 component. Birkett *et al.* (1996, Pillips *et al.* 1995) and ourselves (Table 5.1) found that consumption of RS increased faecal output which explains the decrease in faecal ammonia concentration because the absolute ammonia excretion was only slightly affected (Table 5.1).

Table 5.1 *Faeces production, faecal ammonia excretion, and urinary urea excretion after 1 wk of daily supplementation of the habitual diet with 32 g of either glucose, uncooked resistant starch (RS_2), or retrograded resistant starch (RS_3) in 23 healthy men*[1]

	Dietary supplement		
	Glucose	RS_2	RS_3
Faeces			
Wet weight (g/d)	232 ±19[a]	277 ±20[a,b]	301 ±29[b]
Ammonia			
(μg/g wet faeces)	648 ±41[b]	595 ±39[b]	481 ±36[a]
(mg/d)	143 ±10	157 ±10	134 ±11
Urinary urea			
(g/d)	26.2 ±1.1	27.4 ±1.1	24.9 ±1.1
(g/g creatinine)	15.7 ±0.7	15.7 ±0.5	15.3 ±0.6

[1] Mean ±SEM; values in the same row with different superscript letters are significantly different, $P < 0.05$, as assessed by ANOVA with 'subject' as the random factor and 'supplement' as the fixed factor, followed by Tukey's studentized range test.

It is difficult to see that RS_3 lowers faecal ammonia concentrations by a specific mechanism rather than by raising the bulk of faeces. Birkett *et al.* (1996) suggested that RS fermentation in the colon stimulates bacterial growth and thereby ammonia

incorporation into bacterial protein. This mechanism should be associated with less absolute excretion of faecal ammonia, and with less urinary urea excretion, which was not observed either by Birkett *et al.* (1996) or by ourselves (Table 5.1). It would also imply that RS_3 is more fermentable than RS_2, for which there is no evidence (Olesen *et al.* 1992, Schulz *et al.* 1993, Cummings *et al.* 1995).

This study shows that RS_3, but not RS_2, significantly lowers faecal ammonia concentrations in healthy men, which might be advantageous in the protection against colon cancer. In further research on RS in health and disease, discrimination between the various types of RS would appear to be relevant.

6

Consumption of retrograded (RS_3) but not of uncooked (RS_2) resistant starch shifts nitrogen excretion from urine to faeces in cannulated piglets

Heijnen MLA, Beynen AC.
Submitted for publication.

Abstract

To study the effect of resistant starch (RS) on N excretion, three groups of six cannulated piglets each were fed a diet containing either uncooked resistant starch (RS_2), retrograded resistant starch (RS_3), or glucose. The use of piglets with a cannula at the end of the ileum allowed measurement of the contribution of colonic N to urinary N. We hypothesized that RS_2 would lower colonic absorption of N when compared with RS_3, because RS_2 may be more fermentable than RS_3, thus trapping more N in bacteria. Ileal digesta, urine and faeces were collected quantitatively. Replacement of glucose by either RS_2 or RS_3 did not affect N retention but increased faecal N excretion. The latter was probably due to the combination of a decrease in ileal N absorption and an increase in N trapping by bacteria. Bacterial growth probably was enhanced as a result of fermentation of RS_2 and RS_3 because virtually no starch was recovered in faeces and faecal mass had increased. RS_3, but not RS_2, reduced urinary N excretion, mainly in the form of urea, which can be explained by the observed reduced colonic N absorption. This difference between RS_2 and RS_3 may relate to the greater ileal fermentation of RS_2, as indicated by less starch in the ileal digesta after RS_2 feeding. Thus, more fermentable substrate was available in the colon of the RS_3-fed piglets so that more N could be trapped in bacteria in the colon after RS_3 instead of RS_2 consumption. If these results can be extrapolated to man, consumption of RS_3 rather than digestible carbohydrate may lower the workload for the kidneys and the liver and may therefore be beneficial for patients with kidney or liver malfunction.

Introduction

In rats, dietary uncooked resistant starch (RS_2) led to a shift of N excretion from urine to faeces (Younes *et al.* 1995*a*). Dietary retrograded resistant starch (RS_3) increased faecal N excretion without affecting urinary N excretion in rats (Brunsgaard *et al.* 1995). In pigs, RS_2 in the form of raw potato products (Wünsche *et al.* 1987) and starch infusions into the terminal ileum (Gargallo & Zimmerman 1981, Misir & Sauer 1982, Mosenthin *et al.* 1992) also increased faecal N excretion which was balanced by a reduction in urinary N output. A high-RS diet was found to increase faecal N excretion in man without a concomitant decrease in urinary N excretion (Birkett *et al.* 1996). If a shift of N excretion from urine to faeces occurs after consumption of RS, it can be explained by increased bacterial protein synthesis and a subsequent decrease in colonic absorption of N in the form of ammonia. The indigestible, fermentable carbohydrates that reach the colon supply energy for bacterial growth for which N also is required. N is derived from ammonia produced by bacteria from dietary protein that escapes digestion, endogenous proteins such as pancreatic and intestinal secretions and sloughed epithelial cells (Mason 1984), and blood urea after its diffusion into the gut (Rémésy & Demigné 1989, Younes *et al.* 1995*a,b*). It is reported that 60-90% of faecal N is bacterial N (Stephen & Cummings 1980, Mason 1984, Ahrens & Kaufmann 1985, Wünsche *et al.* 1987, Mosenthin *et al.* 1992).

When higher amounts of bacterial fermentable substrates are included in pig feed, the amount of soluble N in both faeces and urine can be lowered, so that nitrate and ammonia generation from manure is also lowered which in turn reduces environmental pollution (Hegedüs 1993, Kirchgessner & Roth 1993). In man, an increase in faecal N excretion at the expense of renal excretion may be of interest for the dietary management of chronic renal disease, such as may occur in diabetic patients (Rampton *et al.* 1984, Rivellese *et al.* 1985, Parillo *et al.* 1988). Also, reduction of the absorption of ammonia from the gut, thereby decreasing urea production in the liver, may lessen the workload for the liver, which is beneficial for cirrhotic patients (Weber 1979, Weber *et al.* 1985).

The aim of this experiment was to study the effect of dietary RS_2 and RS_3 on N excretion in cannulated piglets. The use of piglets with a cannula at the end of the ileum allowed measurement of the contribution of colonic N to urinary N. We hypothesized that RS_2 would lower colonic absorption of N when compared with RS_3, because RS_2 may be

more fermentable than RS_3 (Schulz *et al.* 1993, Olesen *et al.* 1994, Cummings *et al.* 1995, Champ *et al. unpublished data*) thus trapping more N in bacteria. We chose the pig as experimental animal because the pig is generally accepted as the species that is closest to man in terms of anatomy and physiology of the digestive tract (Fleming & Arce 1986, Graham & Åman 1986, Bach Knudsen *et al.* 1993, Rowan *et al.* 1994).

Materials and methods

The experimental protocol was approved by the Animal Ethical Committee of the Wageningen Agricultural University. The experiment was carried out at the Institute for Animal Nutrition and Physiology ILOB/TNO, Wageningen.

Animals and housing

Eighteen crossbred castrates (FL*NL)*GY aged 10 weeks and with an average body weight of 16 kg were used. At the age of 6 wk they had been fitted with a post-valve T-caecum cannula (PVTC), as described by van Leeuwen *et al.* (1991). In PVTC pigs, the caecum is partially removed and the cannula is joined with the remnants of the caecum directly opposite the ileo-cecal valve. When the cannula is open, the ileo-cecal valve, which normally protrudes into the caecum, protrudes into the cannula, allowing quantitative collection of the ileal digesta (van Leeuwen *et al.* 1991). The piglets were housed individually in stainless-steel metabolic crates in a temperature-controlled (25 °C) barn. Lights were on from 0800 to 1600 h except on the days that digesta were collected, when they were on from 0800 to 2100 h.

Experimental diets

Three diets were used, containing either glucose, RS_2 or RS_3 (Table 6.1). The amount of glucose equivalents was equal for the three diets. The RS_2 and RS_3 diets each contained 168 g RS per kg diet. Corrections were made for the different water contents of the carbohydrate preparations and for the water excluded during formation of glycosidic bonds. We did not try to equal the energy content of the diets because there is no accurate estimate of the amount of energy that RS supplies; in any event this energy is unlikely to greatly exceed 8.4 kJ/g (Livesey 1990). As the piglets in the RS_2 and RS_3

groups ate 114 g RS/d as determined by the *in vitro* method of Englyst *et al.* (1992) the energy intake may have been approximately 958 kJ less in the RS groups than in the glucose group (*i.e.* 9% of the energy content of the glucose diet). The powdered diets were stored at 4 °C until used for feeding.

Table 6.1 *Composition of the diets*

	Diet		
	Glucose	RS_2	RS_3
Ingredients			
Glucose[1] (g)	680.5	376.9	-
RS_2 preparation[2] (g)	-	275.1	-
RS_3 preparation[3] (g)	-	-	613.1
Constant components[4] (g)	320.0	320.0	320.0
Demineralized water (g)	-	28.5	67.4
Carbohydrates			
Glucose equivalents (g/kg)	619	619	619
Resistant starch (g/kg)	-	169	168

[1] Meritose, Cerestar, Vilvoorde, Belgium; dry weight 90.9%.

[2] Uncooked high-amylose maize starch, Cerestar, Vilvoorde, Belgium; dry weight 90.3%; 61.4 g resistant starch/100 g according to the procedure of Englyst *et al.* (1992).

[3] Retrograded high-amylose maize starch, Cerestar, Vilvoorde, Belgium; dry weight 90.8%; 27.4 g resistant starch/100 g according to the procedure of Englyst *et al.* (1992).

[4] The constant components consisted of the following (g/kg diet): wheat gluten, 90; casein, 90; soybean oil, 20; cellulose, 50; $CaCO_3$, 12.5; $CaHPO_4$, 20; NaCl, 5; MgO, 2; $KHCO_3$, 15; $NaHCO_3$, 2.5; Cr_2O_3, 1.6; premix, 10; Cr_2O_3, 2.5. The premix consisted of the following (mg): MnO_2, 70; $FeSO_4.7H_2O$, 400; $ZnSO_4.H_2O$, 300; $Na_2SeO_3.5H_2O$, 0.2; KI, 0.5; $CuSO_4.5H_2O$, 100; $CoSO_4.7H_2O$, 2.5; thiamin, 2; riboflavin, 5; nicotinamide, 30; D,L-calcium pantothenic acid, 12; pyridoxine, 3; cyanocobalamin, 0.04; folic acid, 1; biotin, 0.1; ascorbic acid, 50; choline chloride, 1000; menadione, 3; D,L-alpha tocopheryl acetate, 40; retinyl acetate and retinyl palmitate, 18 (2700 retinol equivalents); cholecalciferol, 0.045; maize meal, 7962.615.

Prior to the experiment the piglets were fed a commercial diet. The piglets were divided into three groups of six animals each so that body weight distributions of the groups were similar. Each group of piglets was randomly assigned to either the glucose, RS_2 or RS_3

diet. In 4 d the ration gradually changed from commercial to each of the three experimental diets. After that, the experimental diets were given for another 10 d. It was considered important to standardize the intake of glucose equivalents and the nutritional status of the animals because ileal digesta were to be collected. Therefore, the piglets were fed on a restricted basis. The piglets were given an amount of feed that was equivalent to 2.6 times the maintenance requirement; this feeding regimen had already been installed prior to the start of the experiment. Maintenance level was assumed to be 420 kJ per kg metabolic weight. The feed was provided to the piglets in two meals of identical size, at 0800 and 1600 h during the adaptation period and at 0800 and 2000 h during the collection period, starting two days in advance. The piglets received tap water at a water:feed ratio of 2.35:1 (w/w). Body weights were measured at the beginning and at the end of the experiment.

Collection of faeces, urine and ileal digesta

On d 9-11 urine and faeces were collected quatitatively from each animal. Urine was collected in a bucket that was placed under the tray with a funnel that was present under the tenderfoot mesh floor of the cages. Faeces were removed from the cage floor and the tray. Urine and faeces collections were frozen at -20 °C until analysis.

On d 12-14 ileal digesta were collected quantitatively for 12-h periods, starting 15 min before the morning meal and ending 15 min before the evening meal. One hour prior to the collection period, the PVTC cannula was opened to adapt the animals and the digesta flow. During this hour the position of the valve changed and instead of protruding in the intestinal lumen it protruded into the lumen of the cannula. Digesta flowed through the cannula into a small plastic bag attached to the cannula with a selftightening nylon strap. Every hour the bags were replaced, weighed and frozen at -20 °C.

Chemical analyses

The faeces and ileal digesta were thawed, pooled per animal per 3 d, homogenized in demineralized water with a blender (Braun Multimix MX32; Braun, Frankfurt/Main, Germany) and then freeze-dried overnight. Dry matter content was determined as the weight difference before and after freeze-drying. Starch was measured in faecal and ileal samples as the difference between total glucose and free glucose, adapted from the method from Björck *et al.* (1987).

The urine was thawed and pooled per animal per 3 d. Creatinine was measured in lightly acidified urine with the use of a commercial test combination (Creatinine, MA-KIT 10 ROCHE; Roche Diagnostics, Basel, Switzerland) and a COBAS-BIO auto-analyser (Hoffmann-La Roche B.V., Mijdrecht, the Netherlands). Urea was measured in nonacidified urine by the urease method with the use of a commercial test combination (Urea UV, MA-KIT 10 ROCHE; Roche Diagnostics, Basel, Switzerland) and the auto-analyser.

N in feed, faecal, ileal and urine samples was measured by the Kjeldahl method. N balance was calculated as N intake minus N excretion via faeces and urine. Colonic N absorption was calculated as N in ileal digesta minus N in faeces.

Statistical analysis

For evaluation of the group comparisons that had been defined a priori, *i.e.* the RS_2 versus glucose group, the RS_3 versus glucose group, and the RS_2 versus RS_3 group, a two-tailed Student's t test was performed with a pre-set P value of 0.05. Because the contrasts were defined a priori, Bonferroni's adaptation was not applied. The statistical analysis package SAS, release 6.09 (SAS Institute Inc., Cary, NC) was used.

Results

Body weight and food intake

Both initial and final body weights did not differ between the dietary groups. Food intakes were also similar in the three groups (Table 6.2).

Ileal digesta and faeces

The piglets fed RS_3 had a markedly higher production of ileal digesta and faeces (Table 6.2) than the piglets fed glucose or RS_2 ($P < 0.05$). Feeding RS_2 also led to a significantly higher faecal output than feeding glucose ($P < 0.05$). The dry matter content of the ileal digesta was higher in the RS_2 than in the glucose group, and higher in the RS_3 than in the RS_2 and glucose groups ($P < 0.05$). The dry matter content of the faeces was approximately twice as high ($P < 0.05$) in the RS_2 and RS_3 groups compared with the glucose group (Table 6.2). A considerable amount of starch was recovered in

the ileal digesta from the piglets fed 114 g RS_2 or RS_3 per day: 50 and 81 g/d respectively, compared with only 1 g/d in the glucose group. The amounts of starch in ileal digesta were significantly different between all dietary groups ($P < 0.05$). In the faeces virtually no starch was recovered in either of the dietary groups (Table 6.2).

Table 6.2 *Body weight, food intake and production of faeces, urine and ileal digesta in piglets fed diets with either glucose, uncooked (RS_2) or retrograded resistant starch (RS_3)*[1]

	Diet		
	Glucose	RS_2	RS_3
Body weight (kg)			
Initial	16.6 ±0.4	16.4 ±0.6	16.7 ±0.4
Final	21.1 ±0.5	20.9 ±0.7	21.2 ±0.5
Food intake (g/d)	682 ±11	678 ±15	683 ±11
Ileal digesta			
Production (g/d)	577 ±92[a]	780 ±57[a]	1159 ±46[b]
Dry matter (g/d)	69 ±3[a]	149 ±8[b]	201 ±6[c]
Starch (g/d)	1 ±1[a]	50 ±2[b]	81 ±3[c]
Faeces			
Production (g/d)	55 ±8[a]	91 ±7[b]	142 ±15[c]
Dry matter (g/d)	27 ±4[a]	52 ±2[b]	52 ±4[b]
Starch (mg/d)	46 ±9	129 ±39	141 ±57
Urine			
Production (ml/d)	971 ±34	915 ±29	862 ±53
Creatinine (mmol/d)	7.0 ±0.4	7.2 ±0.3	6.9 ±0.4
Urea (mmol/d)	168 ±4[b]	160 ±6[b]	141 ±5[a]

[1] Values are means ±SEM for 6 piglets per dietary group. Values in a row with different superscripts are significantly different ($P < 0.05$). Note that starch is expressed in g/d for the ileal digesta and in mg/d for the faeces.

Urine

Urine production and urinary creatinine excretion were similar in the dietary groups (Table 6.2). Urinary urea excretion was lower in the RS_3 group compared with the glucose and RS_2 groups ($P < 0.05$).

N balance

The N intake was similar in the dietary groups (Table 6.3). The N content of the ileal digesta was significantly higher in the RS_2 and RS_3 groups compared with the glucose group ($P < 0.05$). Of the amount of N entering the colon (*i.e.* the N content of the ileal digesta since these were collected at the end of the ileum) about 1 g/d was absorbed by the colon in the glucose and RS_2 groups and only 0.6 g/d in the RS_3 group. The latter was significantly lower than in the glucose group ($P < 0.05$). In the RS_2 and RS_3 groups, N excretion via the faeces was respectively 100% and 150% ($P < 0.05$) higher than in the glucose group. N excretion via the urine (mainly as urea) was 6% lower in the RS_2 group ($P > 0.05$) and 14% lower in the RS_3 ($P < 0.05$) group compared with the glucose group. The N balance was similar in the dietary groups, approximately 11 g/d (Table 6.3).

Table 6.3 *N balance and apparent colonic N absorption in piglets fed diets with either glucose, uncooked (RS_2) or retrograded resistant starch (RS_3)*[1]

	Diet		
	Glucose	RS_2	RS_3
N intake (g/d)	16.4 ±0.3	16.7 ±0.4	17.1 ±0.3
N in ileal digesta (g/d)	1.7 ±0.1[a]	2.3 ±0.2[b]	2.1 ±0.1[b]
N in faeces (g/d)	0.6 ±0.1[a]	1.2 ±0.1[b]	1.5 ±0.2[b]
N in urine (g/d)	4.9 ±0.1[b]	4.6 ±0.2[a,b]	4.2 ±0.1[a]
N balance[2] (g/d)	10.9 ±0.1	10.9 ±0.2	11.4 ±0.3
N absorbed by colon[3] (g/d)	1.1 ±0.2[b]	1.0 ±0.2[a,b]	0.6 ±0.1[a]

[1] Values are means ±SEM for 6 piglets per dietary group. Values in a row with different superscripts are significantly different ($P < 0.05$).

[2] Calculated as N intake minus N in faeces and urine.

[3] Calculated as N in ileal digesta minus N in faeces.

Apparent N absorption

Apparent mouth-to-anus absorption of N was calculated as a percentage of intake. In piglets fed the glucose diet, mean (±SEM) N absorption was 96.3 ±0.4%. Feeding the

RS_2 and RS_3 diets resulted in percentages N absorption of 92.5 ±0.4% and 91.2 ±0.9%, respectively. The RS diets significantly reduced N absorption when compared with the glucose diet ($P < 0.05$).

Discussion

Dietary RS_2 and RS_3 versus glucose increased faecal N excretion resulting in a reduced apparent N absorption. The increase in faecal N after RS consumption was probably due to the combination of a decrease in ileal N absorption and an increase in bacterial N through stimulation of bacterial growth in the gut by fermentation of undigested RS. The latter is confirmed by the recovery of virtually no starch in the faeces of the piglets fed RS_2 and RS_3, indicating that overall both RS_2 and RS_3 were extensively fermented. Wünsche *et al.* (1987) also recovered no starch in the faeces of pigs that consumed RS_2 from raw potatoes. In man, 1% (van Munster *et al.* 1994*b*) to about 20% (Phillips *et al.* 1995, Heijnen *et al. submitted*) of the RS provided was recovered in the faeces. In rats, 9% of the RS_2 and 35% of the RS_3 consumed was recovered in faeces (Schulz *et al.* 1993). Thus, the pig seems a good RS fermenter in comparison with man and rat. The expected increase in faecal bacterial mass in the piglets fed RS_2 or RS_3 was confirmed by the increase in faecal mass. An increase in stool mass after RS cosumption also was found in man (van Munster *et al.* 1994, Phillips *et al.* 1995, Cummings *et al.* 1996, Heijnen *et al. submitted*) and rat (Schulz *et al.* 1993, Heijnen *et al.* 1996*b*). Fermentation of RS in the colon probably induced a lower colonic pH (not measured). A lower pH enhances the conversion of ammonia (NH_3) into ammonium (NH_4^+). Ammonium is less well absorbed by the colon than ammonia and will be excreted in the faeces. This process may also have contributed to the observed increase in faecal N excretion.

Dietary RS_3, but not RS_2, reduced urinary N excretion, mainly by reducing urinary urea excretion, which can be explained by the observed reduced colonic N absorption. A priori, we expected the effects of RS_2 and RS_3 to be just the other way around because we assumed that RS_2 is better fermentable than RS_3 (Schulz *et al.* 1993, Olesen *et al.* 1994, Cummings *et al.* 1995, Champ *et al. unpublished data*). The discrepancy between the results and our expectations can be explained by the finding that RS_2 was fermented in the small intestine to a greater extent than RS_3, as indicated by the lower amounts of

starch in the ileal digesta after RS_2 feeding. This means that less fermentable substrate entered the colon in the RS_2-fed piglets when compared with the RS_3-fed piglets. Thus, although the overall digestibility of RS_2 and RS_3 was equal, they differed in the site of fermentation: RS_2 was fermented for 56% in the ileum and for 44% in the colon, whereas RS_3 was fermented for 29% in the ileum and for 71% in the colon. Fermentation in the ileum of the pig is possible as especially the distal third of the ileum contains a significant amount of bacteria, *i.e.* about 10^8-10^9 viable counts/g digesta (Chesson *et al.* 1985, Liu *et al.* 1985, Bach Knudsen *et al.* 1993). Ileal fermentation of RS corresponds with the observed increase in the amounts of ileal N and digesta. However, the increase in ileal N after RS feeding may also reflect a decrease in protein digestion and absorption.

The effect of RS_3 on the routes of N excretion as found in the present study agrees with studies in which starch was infused into the terminal ileum of pigs (Gargallo & Zimmerman 1981, Misir & Sauer 1982, Mosenthin *et al.* 1992). In those studies fermentation also took place mainly in the colon and the increase in faecal starch excretion was balanced by a decrease in urinary N excretion, as in our study after RS_3 feeding. However, we have no explanation for the discrepancy between the effect of RS_2 on the routes of N excretion in the present study and in the study by Wünsche *et al.* (1987). In the latter, the increase in faecal N excretion was balanced by a decrease in urinary N excretion after pigs were fed RS_2, whereas in the present study this balancing did not occur after RS_2 feeding but only after RS_3 feeding.

In conclusion, replacement of glucose by either RS_2 or RS_3 in pig feed did not affect N retention but increased faecal N excretion due to ileal and colonic fermentation of RS. Ileal fermentation of RS_2 was greater than that of RS_3, resulting in a higher availability of fermentable substrate in the colon of the RS_3-fed piglets when compared with the RS_2-fed piglets. Thus, after RS_3 consumption more N could be trapped in bacteria in the colon than after RS_2 consumption, so that RS_3 but not RS_2 reduced colonic N absorption resulting in a reduced urinary N (urea) excretion. If these results can be extrapolated to man, consumption of RS_3 instead of digestible carbohydrate may lower the workload for the kidneys and the liver and may therefore be beneficial for patients with kidney or liver malfunction (Weber 1979, Rampton *et al.* 1984, Rivellese *et al.* 1985, Weber *et al.* 1985, Parillo *et al.* 1988).

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7

Effect of consumption of uncooked (RS_2) and retrograded (RS_3) resistant starch on apparent absorption of magnesium, calcium and phosphorus in pigs and man

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Abstract

The effect of uncooked (RS_2) and retrograded (RS_3) resistant starch on overall apparent absorption of magnesium, calcium and phosphorus was studied in man and the site of mineral absorption in swine. Twenty-three healthy men consumed in addition to their habitual diet daily supplements providing either 32 g glucose, RS_2 or RS_3. Each subject consumed each supplement for 1 wk in random order. Three groups of 6 piglets each consumed for 2 wk a diet containing either glucose, RS_2 or RS_3. The piglets were cannulated at the end of the ileum which allowed estimation of the ileal and colonic mineral absorption. Urine and faeces were collected, and in the piglets also ileal digesta, for measurement of magnesium, calcium and phosphorus. Supplementation of the diet with either RS_2 or RS_3, when compared with glucose, had no effect on mineral absorption in the healthy men. Dietary RS_2, but not RS_3, versus glucose reduced ($P < 0.05$) total absorption of magnesium and calcium and ileal absorption of phosphorus in the pig. Neither RS_2 nor RS_3 consumption stimulated mineral absorption in man as was found in rats. The amount of RS per MJ of energy intake that man is able to consume is much lower than the amounts used in experiments with rats. Differences between species or in RS and/or mineral intake may explain why RS_2 reduced the apparent absorption of magnesium and calcium in pigs in this study and raised it in rats in earlier studies.

Introduction

In rats, uncooked resistant starch (RS_2) compared with digestible starch raised apparent magnesium and calcium absorption (Rayssiguier & Rémésy 1977, Andrieux & Sacquet 1986, Schulz *et al.* 1993, Younes *et al.* 1993, 1996). Uncooked (RS_2) versus retrograded resistant starch (RS_3) also raised apparent magnesium and calcium absorption in rats (Schulz *et al.* 1993, Heijnen *et al.* 1996*b*). It has been proposed that RS_2 compared with either digestible starch or RS_3 raised apparent magnesium and calcium absorption by increasing ileal solubility of magnesium and calcium due to a reduction in pH (Heijnen *et al.* 1993, Schulz *et al.* 1993, Hara *et al.* 1996, Younes *et al.* 1996). In contrast to digestible starch, RS is not absorbed in the small intestine but may be fermented by the bacterial flora in the gut and some studies indicate that RS_2 is more fermentable than RS_3 (Schulz *et al.* 1993, Olesen *et al.* 1994, Cummings *et al.* 1995). However, since RS_2 compared with RS_3 increased only apparent but not true magnesium absorption by decreasing endogenous magnesium loss with faeces, the proposed mechanism seems to be incorrect (Heijnen *et al.* 1996*b*).

Magnesium (Hardwick *et al.* 1991) and calcium (Nellans & Goldsmith 1981, Ammann *et al.* 1986, Bronner *et al.* 1986, Trinidad *et al.* 1996) may not only be absorbed from the small but also from the large intestine, especially from the caecum in the rat (Hara *et al.* 1996). Distal magnesium and calcium absorption may be stimulated by fermentable RS_2 by increasing the soluble pool of the mineral (Schulz *et al.* 1993, Younes *et al.* 1993, 1996) through acidification of the caecal contents (Andrieux & Sacquet 1986, Demigné *et al.* 1989, Schulz *et al.* 1993, Younes *et al.* 1993, 1996) and/or by hypertrophy of the caecal wall, *i.e.* by increasing the surface area for absorption (Démigné *et al.* 1989, Levrat *et al.* 1991, Younes *et al.* 1993, 1996). Scharrer and Lutz (1990, 1992) have proposed that the short-chain fatty acids (SCFA) produced during carbohydrate fermentation in the gut may enhance magnesium absorption by a Mg^{2+}/H^+ exchanger located in the apical membrane of the epithelium in the distal colon. SCFA may also stimulate colonic cell proliferation (Lupton & Kurtz 1993) which could increase the mineral absorption capacity.

To our knowledge, the effect of RS consumption on mineral absorption in man has not been reported before. Therefore, we included measurements on magnesium, calcium and phosphorus absorption in a human experiment designed to measure the effect of RS_2 and

RS_3 on putative risk factors for colon cancer (Heijnen *et al. submitted*). To study the contribution of the small and the large intestine to the absorption of magnesium, calcium and phosphorus, we fed piglets that were cannulated at the end of the ileum glucose, RS_2 and RS_3. Of all domesticated animal species, the pig is in gastro-intestinal physiology, diet and size most similar to man (Graham & Åman 1986, Liu *et al.* 1985, Rowan *et al.* 1994). In the present studies with pigs and man, the same RS_2 and RS_3 preparations were used. We hypothesized that, compared with glucose, RS_2 but not RS_3 would raise magnesium and calcium absorption.

Methods

In a piglet experiment and a human experiment the effect of dietary RS_2 and RS_3 on the apparent absorption of magnesium, calcium and phosphorus was compared with that of dietary glucose. In each study the same RS preparations were used: uncooked high-amylose maize starch (Hylon VII; Cerestar, Vilvoorde, Belgium), containing 61.4%-63.3% RS_2 by wt as measured *in vitro* according to the procedure of Englyst *et al.* (1992); and retrograded high-amylose maize starch (extruded and retrograded Hylon VII; Cerestar, Vilvoorde, Belgium), containing 27.4%-29.9% RS_3 by wt as measured *in vitro* according to the procedure of Englyst *et al.* (1992). In the pig study, glucose, RS_2 or RS_3 was included in the feed. In the human study, subjects consumed dietary supplements containing glucose, RS_2 or RS_3 in addition to their habitual diet. The feeds and supplements used within a study only differed in the type of carbohydrate used. The amount of glucose equivalents was equal for the three diets/supplements within a study. Corrections were made for the different water contents of the carbohydrate preparations and for the water excluded during formation of glycosidic bonds. We did not try to equal the energy content of the diets and supplements within a study because there is no accurate estimate of the amount of energy that RS supplies. In diet, faeces, digesta and urine samples magnesium and calcium were analysed by atomic absorption spectrophotometry and phosphorus was measured colorimetrically.

Piglet study

The experimental procedure has been detailed elsewhere (Heijnen & Beynen *submitted*). The experimental protocol was approved by an Animal Ethical Committee. Crossbred castrates (FL*NL)*GY aged 10 wk and with an average body weight of 16 kg were used. At the age of 6 wk they had been fitted with a post-valve T-caecum cannula (PVTC) allowing quantitative collection of the ileal digesta, as described by van Leeuwen *et al.* (1991). No differences in growth performance, organ weights, nitrogen balance, mineral balances, and several blood variables were found between PVTC-pigs and intact pigs (Köhler *et al.* 1992*a,b*). The piglets were housed individually in stainless-steel metabolic crates.

Three groups of six animals each consumed either the glucose, RS_2 or RS_3 diet for 2 wk. It was considered important to standardize the intake of glucose equivalents and the nutritional status of the animals because ileal digesta were to be collected. Therefore, the piglets were fed on a restricted basis, *i.e.* an amount of feed that was equivalent to 2.6 times the maintenance requirement. Maintenance level was assumed to be 420 kJ per kg metabolic body weight. The feed was provided to the piglets in two meals of identical size, at 0800 and 2000 h. The piglets received tap water at a water:feed ratio of 2.35:1 (w/w). Food intake and initial and final body weights of the piglets did not differ significantly between the three diet groups. The piglets fed RS_2 or RS_3 consumed 114 g RS/d (Heijnen & Beynen *submitted*).

On d 9-11 faeces was collected quantitatively from each animal and frozen at -20 °C until analysis. On d 12-14 ileal digesta were collected quantitatively for 12-h periods, starting 15 min before the morning meal and ending 15 min before the evening meal. Digesta flowed through the cannula into a small plastic bag attached to the cannula with a self-tightening nylon strap. Every hour the bags were replaced, weighed and frozen at -20 °C. Faeces and ileal digesta were pooled per animal per 3 d.

Apparent total absorption of minerals was calculated as mineral intake minus faecal excretion and expressed as percentage of intake. Apparent ileal absorption was calculated as mineral intake minus ileal excretion and apparent colonic absorption was calculated as total absorption minus ileal absorption. Mineral intakes with tap water were less than 1% of the intakes with the diet and were ignored when calculating mineral absorptions.

Human study

The experimental procedure has been detailed elsewhere (Heijnen *et al. submitted*). Twenty-four apparently healthy men with a mean (±SD) age of 23 ±2 y and a mean BMI of 22.7 ±1.8 kg/m^2 participated in the study. One subject took antibiotics in wk 2: his data were excluded from statistical analysis. The experimental design of the study and possible discomforts were explained to the subjects before they gave their written informed consent. The study protocol was approved by the Medical-Ethical Committee of the Department of Human Nutrition of the Wageningen Agricultural University.

The subjects consumed for 4 wk a daily dietary supplement in addition to their habitual diet in a single-blind randomized multiple cross-over experiment. During the first wk (run-in period) each subject consumed the control supplement (glucose). Subsequently, every subject consumed the RS_2, RS_3 and the glucose supplement each during 1 wk. The 24 subjects were randomly divided into six groups before the start of the experiment. Each group consumed the supplements in one of the six possible sequences to eliminate variation due to residual effects of the previous diet or to drift of variables over time (Snedecor, 1980). The groups were not different with respect to age, height, body weight and BMI (*data not shown*).

The supplements consisted of a mixture of skim yogurt, skim milk, mashed canned fruit, and either glucose (4 g RS/d), RS_2 or RS_3 (32 g RS/d) and were consumed in three equal portions of approximately 170 g/d, essentially with breakfast, lunch and dinner.

To check compliance, 80 μmol lithium chloride was added to each supplement portion and lithium recovery in 24-h urine was measured by atomic absorption spectrophotometry as described before (Heijnen *et al.* 1996*a*). Mean lithium recovery was >90% for each supplement (Heijnen *et al. submitted*). Furthermore, the subjects were asked to report daily the times of consumption of the supplement portions in a diary. According to the diaries 99% of the supplements provided was consumed.

At d 6 and 7 of wk 2, 3 and 4 the subjects collected 24-h urine. During d 5-7 of wk 2, 3 and 4 the subjects produced two faecal samples at the Department of Human Nutrition. The faecal samples were weighed and frozen immediately at -20 °C. Radioopaque polyethylene rings were swallowed as a marker to determine which proportion of the daily faeces output had been collected (Branch & Cummings 1978). Every week a 24-h food consumption recall was obtained from each subject. No significant differences were found in energy and nutrient intake when the various

supplements were consumed and body weight remained constant throughout the study (Heijnen *et al. submitted*).

Assuming that the subjects were in mineral equilibrium and that minerals were excreted only via faeces and urine (thus ignoring losses via sweat, hair and nails) apparent absorption of magnesium, calcium and phosphorus was calculated as the amount of mineral in 24-h urine divided by the amount of mineral in 24-h faeces plus urine, corrected for marker recovery. This formula was used since the exact intake of magnesium, calcium and phosphorus was not determined.

Statistical analysis

Per study, differences between group means for each variable were evaluated by analysis of variance with the GLM (General Linear Model) procedure of SAS (release 6.09; Statistical Analysis Systems Institute Inc., Cary, NC, USA). The models contained 'diet' or 'supplement' as a fixed factor. In the human study, 'subject' was added as a random factor, thus taking the intrinsic individual levels into account. When the analysis of variance indicated a significant effect of diet or supplement ($P < 0.05$), Tukey's Studentized range test was used for pair-wise comparison of the group means. This method encompasses a downward adjustment of the significance limit for multiple testing.

Results

Magnesium

Apparent magnesium absorption was approximately 62% of intake in piglets (Table 7.1) and 35% of intake in men (Table 7.2). In the piglets, total magnesium absorption was lower ($P < 0.05$) in the RS_2 than in the glucose group (Table 7.1). Relatively less magnesium seemed to be absorbed from the colon and more from the ileum in the RS_2 and RS_3 groups compared with the glucose group. In men, magnesium absorption did not differ between glucose, RS_2 and RS_3 supplementation (Table 7.2).

Calcium

Calcium intake differed slightly between the three dietary groups in the piglet study (Table 7.1) due to small differences in calcium content measured in the diets. Apparent

calcium absorption was approximately 73% of intake in piglets (Table 7.1) and 14% of intake in men (Table 7.2). In the piglets, total absorption of calcium was lower ($P < 0.05$) in the RS_2 than in the glucose group (Table 7.1). No significant differences were found in ileal and colonic calcium absorption. In men, no differences were found in calcium absorption after supplementation with glucose, RS_2 or RS_3.

Table 7.1 *Apparent absorption of magnesium, calcium and phosphorus in cannulated piglets fed diets with either glucose, uncooked resistant starch (RS_2), or retrograded resistant starch (RS_3)*[1]

Mineral	Diet		
	Glucose	RS_2	RS_3
Magnesium			
Intake (mmol/d)	30 ±0.5	30 ±0.7	30 ±0.5
Ileal absorption (% of intake	33 ±6.0	33 ±5.2	37 ±2.8
Colonic absorption (% of intake)	34 ±3.9	21 ±3.8	26 ±4.2
Total absorption (% of intake)	67 ±3.7^{b}	54 ±3.6^{a}	63 ±3.3^{a,b}
Calcium			
Intake (mmol/d)	151 ±2^{b}	140 ±3^{a}	147 ±2^{a,b}
Ileal absorption (% of intake)	58 ±1.9	51 ±3.7	55 ±1.9
Colonic absorption (% of intake)	21 ±1.2	16 ±2.7	20 ±2.9
Total absorption (% of intake)	78 ±2.5^{b}	67 ±2.5^{a}	75 ±2.2^{a,b}
Phosphorus			
Intake (mmol/d)	107 ±2^{b}	100 ±2^{a}	101 ±2^{a,b}
Ileal absorption (% of intake)	69 ±2.0^{b}	56 ±3.8^{a}	63 ±1.5^{a,b}
Colonic absorption (% of intake)	7 ±5.3	13 ±2.6	12 ±2.1
Total absorption (% of intake)	75 ±6.8	69 ±2.4	75 ±2.3

[1] Values are means ±SEM for 6 piglets per dietary group. Values in the same row with different superscripts are significantly different.

Phosphorus

Phosphorus intake differed slightly between the three dietary groups in the piglet study (Table 7.1) due to small differences in phosphorus content measured in the diets. Apparent phosphorus absorption was approximately 73% of intake in piglets (Table 7.1)

and 50% of intake in men (Table 7.2). In both piglets and men, absorption of phosphorus was similar during glucose, RS_2 and RS_3 consumption (Table 7.1, 7.2). In the piglets, phosphorus was absorbed mainly from the ileum. Ileal absorption was lower ($P < 0.05$) in the RS_2 than in the glucose group (Table 7.1). No differences were found in colonic phosphorus absorption. Relatively more phosphorus seemed to be absorbed from the colon and less from the ileum in the RS_2 and RS_3 groups compared with the glucose group.

Table 7.2 *Apparent absorption of magnesium, calcium and phosphorus in men after daily supplementation of their habitual diet with either 32 g glucose, uncooked resistant starch (RS_2), or retrograded resistant starch (RS_3)*[1]

Mineral	Dietary supplement		
	Glucose $n=21$	RS_2 $n=20$	RS_3 $n=22$
Magnesium (% of intake)	36 ± 1.3	35 ± 1.5	35 ± 1.9
Calcium (% of intake)	14 ± 0.9	15 ± 1.7	14 ± 0.8
Phosphorus (% of intake)	51 ± 1.9	51 ± 1.8	50 ± 1.8

[1] Values are means ±SEM.

Discussion

In the present studies, dietary RS_2, but not RS_3, versus glucose reduced the apparent total absorption of magnesium and calcium and the apparent ileal absorption of phosphorus in the pig. Supplementation of the habitual diet with 32 g/d of either RS_2 or RS_3 for a week, when compared with glucose, had no effect on apparent magnesium, calcium and phosphorus absorption in healthy men.

In contrast to the present findings in the piglets, RS_2 enhanced the apparent absorption of magnesium and calcium in rats (Rayssiguier & Rémésy 1977, Andrieux & Sacquet 1986, Schulz *et al.* 1993, Younes *et al.* 1993, 1996, Heijnen *et al.* 1996*b*). This discrepancy may be due to species differences in *e.g.* hormonal control; bacterial flora,

anatomy or physiology of the digestive tract; or in eating pattern over the day. Moreover, the piglets had a much higher intake of RS and minerals per MJ of energy intake than the rats. The values for total, colonic and ileal absorption of magnesium, calcium and phosphorus are similar to those found by van der Heijden *et al.* (1995) but higher than those in the study of Larsen and Sandström (1993). However, in the latter study the pigs were older and the magnesium and phosphorus intakes were higher than in our study.

Magnesium absorption tended to be shifted from colon to ileum to some extent in the piglets fed RS_2 and RS_3 when compared with those fed glucose. This may be connected with the finding that RS is fermented already in the ileum, RS_2 to a greater extent than RS_3 (Heijnen & Beynen *submitted*). Fermentation in the ileum of the pig is possible as especially the distal third of the ileum containes a significant amount of bacteria (Chesson *et al.* 1985, Liu *et al.* 1985 Bach Knudsen *et al.* 1993), even though the pig is essentially a colon fermenter, like man (Graham & Åman 1986). The bacteria found in the ileum are part of the normal ileal flora, and are not airborne microorganisms that came into the gut when the piglet was operated (Chesson *et al.* 1985). In the study from van der Heijden *et al.* (1995) magnesium was absorbed mainly from the colon, in contrast to our findings. Both in our study and that from van der Heijden *et al.* (1995) calcium and phosphorus were absorbed mainly from the ileum. Larsen and Sandström (1993) found that minerals were absorbed from the small intestine and excreted in the large intestine. Because in the pig RS is fermented both in the ileum and the colon and in man probably almost exclusively in the colon, and mineral absorption may be affected by fermentation in the gut, the pig does not seem to be a good model for man to estimate the contribution of the various parts of the digestive tract to mineral absorption.

We found no effect of RS_2 or RS_3 supplementation on apparent absorption of magnesium, calcium and phosphorus absorption in man. Langkilde and Andersson found no effect of RS_2 (1995*b*) nor RS_3 (1992) compared with digestible starch on magnesium and calcium excretion in the ileal effluent from ileostomy patients. Possibly, our dose of RS was too small to influence mineral absorption. However, the daily dose of RS supplemented was six times larger than the current average estimated intake of RS in the Netherlands (Dysseler & Hoffem 1995*a*). Furthermore, man cannot consume more than about 30 g RS/d without unpleasant side-effects like flatulence and bloated feelings. Another explanation for the lack of effect of RS on mineral absorption in man may be the short duration of the supplementation. Each supplement was consumed for 1 wk only.

However, others (Balasubramanian *et al.* 1987, Brink *et al.* 1993, Siener & Hesse 1995) found differences in mineral absorption in man after a change in the diet within 5-10 d. Further, the present study was not a balance study designed to investigate effects on mineral absorption, so that minor changes cannot be excluded. It also cannot be excluded that RS affects mineral absorption when the mineral intake is marginal or inadequate.

The low percentage of calcium absorption in our human study may be explained by the high calcium intake in this group of young students (Allen 1982): about 2000 mg/d, of which 500 mg came from the supplements. The discrepancy between the present human study on one hand and the present pig study and rat studies reported by others on the other hand may be explained by *(i)* the much higher dose of RS per MJ of energy intake that the animals consumed (Table 7.3; RS intakes per MJ of energy intake in the rat studies were in between those from our human and pig study), *(ii)* differences in fermentation efficiency between the species or *(iii)* by other species differences like *e.g.* hormonal control or anatomy or physiology of the intestinal tract (Mathers 1991). Furthermore, the ileal starch digestibility in rats, piglets and man may be different (Roe *et al.* 1996). Therefore, the amount of starch that was truly resistant in each species is uncertain and may not have been the intended amount fed (based on *in vitro* RS analysis).

Table 7.3 *Comparison of the intakes of resistant starch, magnesium, calcium and phosphorus in the piglet and the human study*

	Study	
	Human[1]	Piglet
Resistant starch intake (g/MJ[2])	2	14
Magnesium intake (mg/MJ[2])	36	90
Calcium intake (mg/MJ[2])	133	727
Phosphorus intake (mg/MJ[2])	167	395

[1] For the human study the values of the mineral intakes are only indicative since they are calculated using a computerized food composition table. This HUVO-95 table, which is developed at the Department of Human Nutrition and is based on the NEVO-93 table (NEVO Foundation 1993), contains values for the magnesium, calcium and phosphorus content of 58%, 88% and 85% of the foods, respectively. Furthermore, the values are based on three 24-h recalls per subject only.

[2] The energy resistant starch supplies when it is fermented in the colon was not included.

In conclusion, neither RS_2 nor RS_3 consumption stimulated mineral absorption in man as was found in rats. The amount of RS per MJ of energy intake that man is able to consume is much lower than the amounts used in experiments with rats. RS_2, but not RS_3, reduced the apparent absorption of magnesium and calcium in pigs in the present study, in contrast to the increase found in rats in earlier studies. This discrepancy may be explained by differences between species and/or by differences in RS and/or mineral intake.

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8

Dietary raw versus retrograded resistant starch enhances apparent but not true magnesium absorption in rats

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Abstract

Dietary raw (RS_2) versus retrograded resistant starch (RS_3) raises apparent magnesium absorption in rats. The mechanism proposed is that RS_2 enhances magnesium availability for absorption; it does this by increasing ileal solubility of magnesium due to a reduction in pH as a consequence of RS_2 fermentation in the gut. The mechanism implies that dietary RS_2 versus RS_3 would raise true magnesium absorption and stimulate reabsorption of endogenous magnesium, leading to a lower faecal excretion of endogenous magnesium. Dietary lactulose versus glucose raises apparent magnesium absorption, and the mechanism proposed is similar to that for the stimulatory effect of RS_2 versus RS_3. Thus, we measured in rats fed RS_3, RS_2, glucose or lactulose true magnesium absorption on the basis of the retention of the orally and intraperitoneally administered radiotracer ^{28}Mg. Feeding rats with RS_2 instead of RS_3 significantly enhanced apparent but not true magnesium absorption, because RS_2 lowered faecal excretion of endogenous magnesium. When compared with dietary glucose, lactulose significantly raised both apparent and true magnesium absorption, but did not affect faecal excretion of endogenous magnesium. It is suggested that the proposed mechanism by which RS_2 and lactulose would enhance magnesium absorption is disproved by the present data.

Introduction

Magnesium absorption may be influenced by the type of resistant starch (RS) in the diet. Schulz *et al.* (1993) found that raw (RS_2) versus retrograded resistant starch (RS_3) raised apparent magnesium absorption in rats. RS is not absorbed in the small intestine but may be fermented by the bacterial flora in the gut, RS_2 being more fermentable than RS_3 (Olesen *et al.* 1992, Schulz *et al.* 1993, Cummings *et al.* 1995, Champ *et al. unpublished results*). The difference in fermentability between the two types of RS may explain their different effects on magnesium absorption. Fermentation in the gut lowers the pH which in turn raises magnesium concentration in the liquid phase of digesta because insoluble magnesium complexes in the solid phase will dissolve (Brink & Beynen 1992). Because only soluble magnesium may cross the intestinal epithelium (Brink *et al.* 1992*a*), an increase in magnesium solubility would stimulate absorption. Indeed, the stimulatory effect of RS_2 on apparent magnesium absorption, when compared with RS_3, was associated with a lower pH and higher magnesium solubility in the ileal lumen (Schulz *et al.* 1993). The mechanism by which RS_2 may stimulate magnesium absorption appears similar to that underlying the stimulatory effect of lactulose (β-1,4-galactosyl-fructose), another poorly digestible but well-fermentable carbohydrate. Dietary lactulose versus glucose lowered ileal pH, raised magnesium solubility in ileal contents, and improved apparent magnesium absorption in rats (Heijnen *et al.* 1993).

In light of the mechanism proposed earlier (Heijnen *et al.* 1993, Schulz *et al.* 1993) to explain the stimulatory effect of RS_2 on apparent magnesium absorption (magnesium intake minus faecal excretion), it would be expected that RS_2 enhances not only the absorption of dietary (exogenous) magnesium but also that of endogenous magnesium. It could be suggested that RS_2 versus RS_3 enhances true magnesium absorption (magnesium intake minus faecal excretion of exogenous magnesium) and lowers the faecal excretion of endogenous magnesium. To test the suggestion, we measured true magnesium absorption in rats fed either RS_2 or RS_3. On the basis of the retention of orally and intraperitoneally administered ^{28}Mg (van den Berg *et al.* 1995), true magnesium absorption was calculated using the method of Heth and Hoekstra (1965). Faecal excretion of endogenous magnesium was calculated as the difference between true and apparent absorption. The effect of RS_2 versus RS_3 was studied simultaneously with that of lactulose versus glucose. The effect of lactulose on apparent magnesium absorption

(Heijnen *et al.* 1993) is greater than that of an identical amount of RS_2 (Schulz *et al.* 1993). Therefore, the feeding of lactulose was expected to elicit greater, and thus more easily detectable effects on true magnesium absorption and faecal excretion of endogenous magnesium because RS_2 and lactulose are believed to share the same mechanism of action as mentioned above.

Materials and methods

The experimental protocol was approved by the Animal Experiments Committee of the Department of Medicine, Erasmus University, Rotterdam. The experiment was carried out at the Interfaculty Reactor Institute, Delft University of Technology.

Animals and housing

Outbred male Wistar rats (Hsd/Cpb:WU, Harlan, Zeist, the Netherlands) were used. On arrival, the rats were 5 wk old and had a mean body weight of 85 g. The rats were housed individually in metabolism cages (Tecniplast Gazzada, Buguggiata, Italy) in a room with controlled temperature (20-22 °C), relative humidity (50-60%) and light cycle (lights on 0600-1800 h).

Experimental diets

The composition of the four diets used is shown in Table 8.1. The diets differed with respect to the carbohydrate component and contained either RS_3, RS_2, glucose or lactulose. The amounts of monosaccharide equivalents were equal for the four diets. Corrections were made for the different water contents of the carbohydrate preparations and for the water excluded during formation of glycosidic bonds. When compared with the glucose diet, the lactulose diet contained lactulose at the expense of an identical amount of glucose equivalents. The RS_2 and RS_3 diets each contained 116 g RS/kg diet. The lactulose diet contained 90.5 g lactulose/ kg diet; this amount is based on earlier studies in rats (Heijnen *et al.* 1993). The powdered diets were stored at 4 °C until used for feeding.

Table 8.1 *Composition of the diets*

	Diet			
	RS_3	RS_2	Glucose	Lactulose
Ingredients				
Glucose[1] (g)	-	288.7	519.1	414.4
RS_2 preparation[2] (g)	-	208.8	-	-
RS_3 preparation[3] (g)	467.7	-	-	-
Lactulose[4] (g)	-	-	-	181.0
Constant components[5] (g)	397.1	397.1	397.1	397.1
Demineralized water (g)	135.2	105.4	83.8	7.5
Carbohydrates				
Monosaccharide equivalents (g/kg)	467.1	469.7	471.9	471.9
Glucose equivalents (g/kg)	467.1	469.7	471.9	376.7
Resistant starch (g/kg)	116.4	115.7	0	0
Chemical analysis				
Magnesium (mg/kg)	280.4	256.6	273.9	265.4

[1] Meritose, Cerestar, Vilvoorde, Belgium; dry weight 90.9%.

[2] Raw high-amylose maize starch, Cerestar, Vilvoorde, Belgium; dry weight 90.3%; 61.4 g resistant starch/100 g according to the procedure of Englyst *et al.* (1992).

[3] Retrograded high-amylose maize starch, Cerestar, Vilvoorde, Belgium; dry weight 90.8%; 27.4 g resistant starch/100 g according to the procedure of Englyst *et al.* (1992).

[4] Duphulac; Duphar BV, Amsterdam, the Netherlands; dry weight 50%.

[5] The constant components consisted of the following (g/kg diet): casein, 143.8; palm oil, 156.2; maize oil, 34.3; cellulose, 19.0; $CaHPO_4.2H_2O$, 4.0; $NaH_2PO_4.2H_2O$, 11.0; $MgCO_3$, 1.3; KCl, 6.6; mineral premix, 9.5; vitamin premix, 11.4. The mineral premix consisted of the following (mg/10 g): $FeSO_4.7H_2O$, 174; MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; KI, 0.2; $CrCl_3.6H_2O$, 1.5; $SnCl_2.H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9679.2. The vitamin premix consisted of the following (mg/12 g): thiamin, 4; riboflavin, 3; pyridoxine, 6; niacinamide, 20; DL-calcium pantothenate, 17.8; folic acid, 1; biotin, 2; choline chloride, 2000; cyanocobalamine, 50; menadione, 0.05; all-*rac*-α-tocopheryl acetate, 60; retinyl acetate and retinyl palmitate, 8 (1200 retinol equivalents); cholecalciferol, 0.025; maize meal, 9828.125.

For a run-in period of 9 d, all rats were given free access to the glucose diet and demineralized water. Then, the rats were divided into four groups of nine animals each so that body weight distributions of the groups were similar. One group of rats continued to receive the glucose diet and the other groups were switched to either the RS_2, RS_3 or

lactulose diet. The four diets were given for another 4 wk. The daily amount supplied was 80% of the mean of that consumed *ad libitum* during the last 3 d of the run-in period (mean ±SD: 13 ±2 g/d, n=36). Because the rats were growing, the amount of food given was increased by 1 g each week. We had decided to supply restricted amounts of feed so that all rats would consume identical amounts of glucose equivalents. Any food spilled was recorded. The rats had free access to demineralized water. Body weights were measured two times per week.

Radiotracer study

On d 14, after being deprived of food overnight, five rats of each group received $^{28}MgCl_2$ with an extrinsically labeled meal. The remaining four rats of each group were injected intraperitoneally with the radiotracer. To equalize handling and treatment of each rat, the rats receiving the radiotracer orally were injected intraperitoneally with distilled water, and the rats that were injected with ^{28}Mg were given a meal without the radiotracer. On d 21, the route of administration of radiotracer for each rat was reversed. On the days of radiotracer administration, the treatment order of the rats was randomized.

The $^{28}MgCl_2$ was prepared at the Interfaculty Reactor Institute according to a procedure described by Kolar *et al.* (1991). The radioactive meals were prepared by adding 100 μl of 150 mmol/l $^{28}MgCl_2$ (1.3 GBq/mol) in distilled water to 2 g of experimental diet. The added solution was dried and then mixed with the diet. For intraperitoneal administration, the 100 μl of radiotracer solution was injected. The meals with or without the radiotracer were presented to the rats after 16 h of food deprivation. In general, the meals were consumed within 10 min. Subsequently, the intraperitoneal injection was given. The rats were given the meal and injection sequentially with 10-min intervals. Throughout the experiment, measurements of radioactivity in the individual rats were carried out in the same order as ^{28}Mg was administered.

Radioactivity in individual rats was determined using a specially designed whole-animal gamma scintillation detector (van den Berg *et al.* 1995) within 10 min after administration of ^{28}Mg. Then, all rats received their habitual diets. For another 4 d, radioactivity in the rats was determined at regular intervals, *i.e.* 0, 3, 7, 20, 32, 43, 55, 68, 79 and 92 h after administration of ^{28}Mg. The efficiency of the whole-body counter for detection of ^{28}Mg was 65%, and its stability was monitored by counting a ^{65}Zn source.

Collection of faeces

From d 14 to 17 and d 21 to 24, faeces of each rat were collected quantitatively with the use of the metabolism cages. Faeces were frozen at -20 °C until analysis. Complete collection of urine was not possible due to whole-body counting.

Magnesium analyses

Diet samples and faeces (after drying for 48 h at 60 °C) were weighed, homogenized, and ashed at 500 °C for 17 h. The ash was dissolved in 5 ml of 6 mol/l HCl. After appropriate dilution of the samples, magnesium was analysed in the presence of 41 mmol/l $LaCl_3$ by atomic absorption spectrophotometry (Varian AA-475; Varian Techtron, Springvale, Australia), using an air-acetylene flame. Magnesium was measured at a wavelength of 285.3 nm.

Calculations

True magnesium absorption was calculated according to the method of Heth and Hoekstra (1965). Counting measurements were corrected for background and radioisotope decay and then expressed as percentage of the administered dose. Within dietary groups and per administration route, the data of the two measurement periods were pooled. Plots of the logarithm of the percentage of radioactivity retention after intraperitoneal and oral ^{28}Mg administration versus time were constructed. The equations for the radioactivity retention curves were fitted using the least-squares method, assuming parallelism for the oral and the intraperitoneal curve within each rat. The zero-time intercepts were determined by extrapolating the linear parts of the curve. The percentage of true absorption was calculated by dividing the intercept of the retention curve for oral ^{28}Mg by that of the retention curve of intraperitoneal ^{28}Mg and multiplying by 100. This calculation was performed for each rat. Absolute true magnesium absorption was calculated by multiplying magnesium intake and the percentage of true magnesium absorption. Apparent magnesium absorption was calculated as magnesium intake minus faecal magnesium excretion. Faecal excretion of endogenous magnesium was calculated as absolute true magnesium absorption minus absolute apparent magnesium absorption.

Statistics

Statistical analysis was restricted to group comparisons that had been defined *a priori*, *i.e.*, the RS_2 versus RS_3 group and the lactulose versus glucose group. For evaluation, a two-tailed Student's t test was performed. The statistical analysis package SAS, release 6.09 (SAS Institute Inc., Cary, NC, USA) was used.

Results

Body weight, food intake and faeces production

The initial and final body weights of the rats did not differ significantly among the four diet groups (Table 8.2). Group mean final body weight was somewhat lower in the rats fed lactulose (lactulose versus glucose, $P = 0.06$), which could relate to their greater food spillage. Faeces production was twofold higher in the RS_2 group and fourfold higher in the RS_3 group compared with the glucose and lactulose groups. Faeces production in the RS_3 group was higher ($P = 0.0001$) than in the RS_2 group.

Table 8.2 *Body weight, food intake and faeces production in rats fed the diets with either retrograded resistant starch (RS_3), raw resistant starch (RS_2), glucose or lactulose*[1]

	Diet			
	RS_3	RS_2	Glucose	Lactulose
Body weight (g)				
Initial[2]	120 ±3.4	120 ±3.1	123 ±3.0	124 ±3.2
Final	185 ±2.2	184 ±2.3	187 ±2.6	179 ±3.2
Food intake[3] (g/d)	14.0 ±0.1	14.1 ±0.2	13.6 ±0.2	12.1 ±0.4*
Faeces production[3] (g/d)	3.6 ±0.13	2.3 ±0.07*	0.7 ±0.04	0.8 ±0.10

[1] Values are means ±SEM, $n=9$.

[2] Body weight after the 9-d run-in period when the rats were 6 wk old.

[3] Average values for d 14-17 and d 21-24.

* Significant difference for RS_2 versus RS_3 or significant difference for lactulose versus glucose ($P < 0.05$).

Apparent magnesium absorption

Due to differences in food spillage and magnesium concentrations of the diets, magnesium intakes differed among the groups (Table 8.3); magnesium intake in the RS_3 group was higher than in the RS_2 group ($P = 0.0001$) and was higher in the glucose than in the lactulose group ($P = 0.0003$). Apparent magnesium absorption expressed as percentage of intake was higher ($P = 0.005$) in the RS_2 than in the RS_3 group (95% CI of the difference: 2-9%). Feeding of lactulose instead of glucose led to a 14 percentage units higher ($P = 0.0003$) apparent magnesium absorption (95% CI of the difference: 8-19%).

Table 8.3 *Apparent and true absorption of magnesium in rats fed the diets with either retrograded resistant starch (RS_3), raw resistant starch (RS_2), glucose or lactulose*[1]

	Diet			
	RS_3	RS_2	Glucose	Lactulose
Intake (μmol/d)	162 ±2	149 ±2*	153 ±2	132 ±4*
Apparent absorption (% of intake)	62 ±1	68 ±1*	69 ±2	83 ±2*
True absorption (% of intake)	83 ±3	82 ±1	82 ±4	96 ±4*
Endogenous loss with faeces (μmol/d)	34 ±4	20 ±2*	19 ±5	17 ±5

[1] Values are means ±SEM, $n=9$. Average values for d 14-17 and d 21-24.

* Significant difference for RS_2 versus RS_3 or significant difference for lactulose versus glucose ($P < 0.05$).

True magnesium absorption

For each dietary treatment, the semilogarithmic retention curves for orally and intraperitoneally administered ^{28}Mg were found to be linear between 30 and 100 h post-administration (Figure 8.1). True magnesium absorption was similar in the RS_2 and RS_3 groups, but was higher in the lactulose group compared with the glucose group ($P = 0.02$). Faecal excretion of endogenous magnesium was lower ($P = 0.01$) in the RS_2 than in the RS_3 group (95% CI of the difference: 5-24 μmol/d), but was similar in the lactulose and glucose groups (Table 8.3).

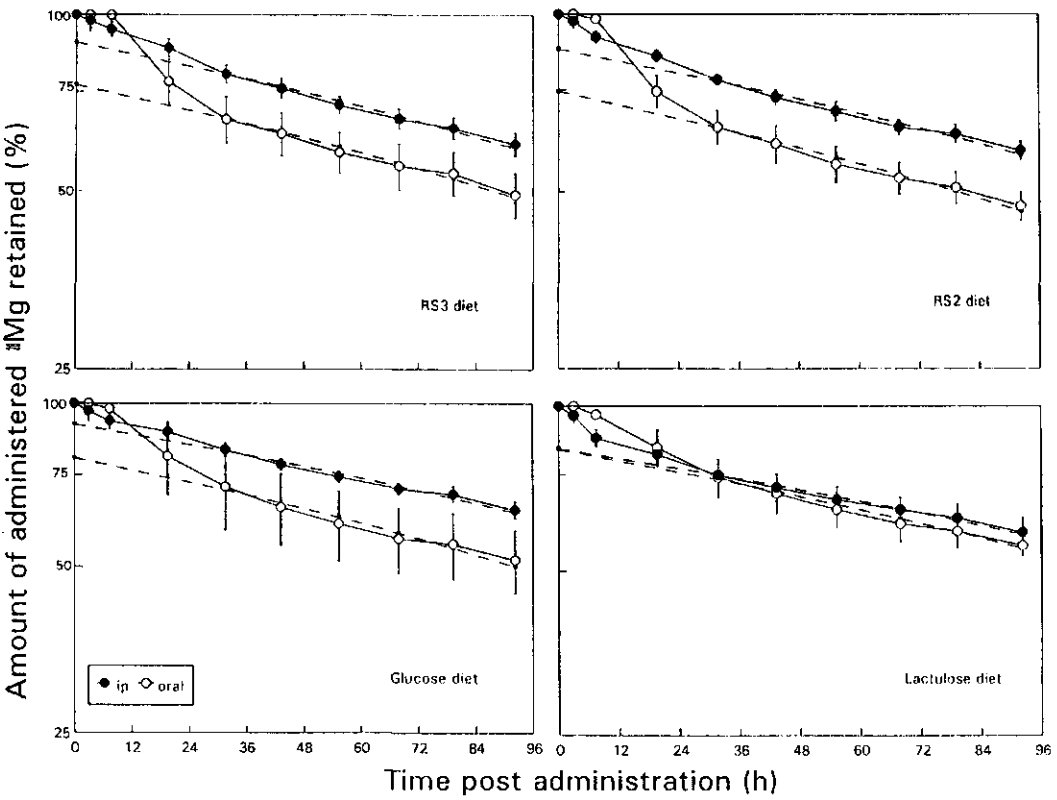

Figure 8.1 *Retention curves for radioactivity in rats fed on diets containing either retrograded resistant starch (RS_3), raw resistant starch (RS_2), glucose or lactulose after oral and intraperitoneal administration of ^{28}Mg. Values represent means ±SD, n=9; for the intraperitoneal curves, the SD sometimes were smaller than the symbols. Linear regression equations for each curve were established for the six timepoints beyond 30 h postadministration for each rat. Note that the y-axis has a logarithmic scale. RS_3 diet, intraperitoneal: log y = -0.0019 (SD 0.0002)x + 1.96 (SD 0.018); oral: log y = -0.0020 (SD 0.0001)x + 1.89 (SD 0.037). RS_2 diet, intraperitoneal: log y = -0.0019 (SD 0.0002)x + 1.95 (SD 0.012); oral: log y = -0.0022 (SD 0.0003)x + 1.87 (SD 0.035). Glucose diet, intraperitoneal: log y = -0.0017 (SD 0.0002)x + 1.97 (SD 0.012); oral: log y = -0.0021 (SD 0.0004)x + 1.90 (SD 0.085). Lactulose diet, intraperitoneal: log y = -0.0017 (SD 0.0002)x + 1.93 (SD 0.020); oral: log y = -0.0021 (SD 0.0002)x + 1.93 (SD 0.042).*

Discussion

In agreement with the study of Schulz *et al.* (1993) we found that the feeding of RS_2 instead of RS_3 significantly enhanced apparent magnesium absorption in rats. However, RS_2 did not raise true magnesium absorption when compared with RS_3. This implies that RS_2 feeding does not affect intestinal magnesium absorption, but depresses the faecal excretion of endogenous magnesium. Indeed, the rats fed RS_2 had a lower group mean loss of endogenous magnesium with faeces than the rats fed RS_3. We can only speculate as to the basis for a lower faecal loss of endogenous magnesium in rats fed RS_2. RS_2 might reduce intestinal fluid secretion or depress the turnover of epithelial cells, leading to less loss of endogenous magnesium. Another possibility is that RS_2 inhibits the magnesium efflux from the mucosa into the intestinal lumen, because dietary RS_2 raises the magnesium concentration in the liquid phase of ileal lumen (Schulz *et al.* 1993).

As in earlier work (Brink *et al.* 1992*b*, Verbeek *et al.* 1993, van der Heijden *et al.* 1994), true magnesium absorption was measured with the use of oral and intraperitoneal administration of tracer doses of ^{28}Mg. The initial loss of total body activity after oral administration of ^{28}Mg is caused by passage of the radiotracer through the intestine and its excretion in faeces. Dietary lactulose markedly reduced the initial loss of ^{28}Mg. This indicates, as was indeed found after calculation, that true magnesium absorption was enhanced by lactulose. Thus, the observed stimulatory effect of lactulose on apparent magnesium absorption, as was found previously (Heijnen *et al.* 1993), reflects true absorption rather than depressed loss of endogenous magnesium. In fact, faecal excretion of endogenous magnesium was not affected by lactulose in the diet.

We had proposed earlier (Heijnen *et al.* 1993, Schulz *et al.* 1993) that stimulation of apparent magnesium absorption by RS_2 versus RS_3 and by lactulose versus glucose is due to an increase in ileal solubility of magnesium as caused by a decrease in pH which in turn results from enhanced bacterial fermentation. Watkins *et al.* (1992) and Younes *et al.* (1996) have suggested a similar mechanism to explain their findings that apparent magnesium absorption in rats was enhanced by a diet containing wheat bran fibre compared with a fibre-free diet, or by a diet containing RS_2 compared with a diet containing digestible starch, respectively. In contrast, Scharrer and Lutz (1990, 1992) have proposed that the short-chain fatty acids (SCFA) produced during carbohydrate fermentation in the gut may enhance magnesium absorption by a Mg^{2+}/H^+ exchanger

located in the apical membrane of the epithelium in the distal colon. Both dietary lactulose (Demigné *et al.* 1989) and RS_2 (Demigné *et al.* 1989, Younes *et al.* 1996) have been shown to enlarge the SCFA pool in the caecum of rats. The two competing mechanisms proposed would imply simultaneous stimulation of the absorption of dietary and endogenous magnesium and thus would lead to a decrease in the loss of endogenous magnesium with faeces associated with an increase in true magnesium absorption. Clearly, the greater apparent magnesium absorption in rats fed RS_2 instead of RS_3 can be explained by a decrease in entry of endogenous magnesium into the intestinal tract rather than by the two mechanisms described. The stimulatory effect of dietary lactulose on apparent magnesium absorption can be explained by either mechanism, but then lactulose should simultaneously enhance the intestinal excretion and reabsorption of endogenous magnesium so that the amount of endogenous magnesium in faeces remains unaffected as was observed. We feel that the condition of an extra excretion and reabsorption of endogenous magnesium balancing each other is unlikely. It then becomes problematic to describe how dietary lactulose stimulates magnesium absorption.

In conclusion, feeding rats RS_2 instead of RS_3 enhanced apparent but not true magnesium absorption because RS_2 lowered the faecal excretion of endogenous magnesium. When compared with glucose feeding, RS_2 had no effect on magnesium absorption, whereas lactulose raised both apparent and true magnesium absorption. Schulz *et al.* (1993) and Heijnen *et al.* (1993) have proposed that both RS_2 and lactulose would enhance magnesium absorption by raising ileal solubility of magnesium due to a reduction in pH of ileal contents which in turn results from enhanced bacterial fermentation. The proposed meachanism now appears to be incorrect. According to the mechanism, RS_2 feeding should raise true magnesium absorption, and lactulose feeding should lower the faecal excretion of endogenous magnesium, but these effects were not observed.

Acknowledgements

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9

Neither raw nor retrograded resistant starch lowers fasting serum cholesterol concentrations in healthy normolipidaemic subjects

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Abstract

The question addressed was whether dietary resistant starch would lower serum cholesterol and triacylglycerol concentrations in healthy normolipidaemic subjects. In a randomized single-blind 3 x 3 Latin-square study with corrections for any carry-over effects, 27 males and 30 females consumed supplements containing glucose, or resistant starch (RS) from raw high-amylose maize starch (RS_2) or from retrograded high-amylose maize starch (RS_3). The RS_2 and RS_3 supplements provided 30 g RS/d. Each type of supplement was consumed in addition to the habitual diet for 3 wk. At the end of each 3-wk period, fasting blood samples and a 24-h food consumption recall were obtained from each subject. The subjects collected 24-h urine samples for lithium determination, which was added to the supplements to check compliance. Mean lithium recovery was 97% and did not differ between supplements. The mean composition of the background diet was similar when the three supplements were taken. Body weight remained constant throughout the study. There were no significant differences in the fasting concentrations of serum total, HDL, and LDL cholesterol; triacylglycerols, or 3α-hydroxy bile acids after consumption of glucose, RS_2, or RS_3. Evidence is presented that the lack of effect of RS_2 and RS_3 on serum lipid concentrations cannot be explained by insufficient statistical power, a low dose, or a short duration of treatment. The subjects reported softer stools and more gastrointestinal symptoms after supplementation with RS than after glucose. Neither the RS_2 nor the RS_3 supplements lowered serum lipid concentrations in healthy, normolipidaemic men and women.

The supplements were prepared in the kitchen of the Department of Human Nutrition three times a week and the subjects took them home for consumption. Supplements were stored at 4 °C until consumed. Supplements were consumed in three equal portions of approximately 115 g/d, essentially with breakfast, lunch and dinner. Supplements had to be consumed as provided and after stirring.

Table 9.2 *Composition of the supplements*[1]

	Dietary supplement		
	Glucose	RS_2	RS_3
RS[2] (in glucose equivalents, g/d)	0	30	30
Digestible glucose equivalents (g/d)	87	57	57
Protein (g/d)	10	10	10
Fat (g/d)	0.4	0.4	0.4
Cholesterol (mg/d)	10	10	10
Energy[3] (kJ/d)	1824	1314	1314

[1] Calculated by using a computerized food composition table (HUVO-95) that was developed at the Department of Human Nutrition and is based on the NEVO-93 table (NEVO Foundation 1993).
[2] As measured *in vitro* according to the procedure of Englyst *et al.* (1992).
[3] Ignoring the energy that RS provides when it is fermented in the colon.

Compliance

To check whether the subjects really consumed the supplements, 80 μmol lithium chloride was added per supplement portion (Sanchez-Castillo *et al.* 1987, van Houwelingen *et al.* 1987). This amount is 100 times the amount found in food and 100 times less than the dose used in antidepression drugs. About 95% of the ingested lithium is recovered in urine (Sanchez-Castillo *et al.* 1987, LeClercq *et al.* 1990). After a continuous intake of lithium, it takes 4-5 d for its excretion in urine to reach a steady concentration (LeClercq *et al.* 1990). Lithium was only added to the supplement portions that were to be consumed in the week before 24-h urine samples were collected (day 10 of each 3-wk period) to limit as much as possible the amount of lithium consumed. Subjects were told that a safe substance was added to the supplements to check their compliance. They were

given the impression that the substance was added to every supplement portion and that measurements in urine and blood could show their compliance over the last days before collection of urine or blood. When the urine was turned in, subjects were asked whether the collection had been successful. The urinary lithium concentration was measured by atomic-absorption spectrophotometry (model 2380; Perkin-Elmer, Norwalk, CT, USA), with a precision of 2.5%. Furthermore, the subjects were asked to report daily in a diary the times of consumption of the supplement portions.

Food consumption

The subjects were free to eat and drink what they wanted in addition to the supplements, but they were encouraged to maintain their habitual diet as much as possible. Subjects were instructed to minimize the consumption of products presumed to contribute significantly to their RS intake, such as muesli; unripe bananas; lentils; beans; fried or baked potatoes; cooked and cooled potatoes, rice and pasta; and potato chips. Subjects were asked to report the consumption of these foods and also deviations from their habitual diet or activity pattern in a diary. In the last week of every 3-wk period, a 24-h recall was obtained from each subject by one of the four dietitians involved. Each subject was interviewed by the same dietitian throughout the study. The way of interviewing and coding the foods was standardized. Energy and nutrient intakes were calculated using a computerized food-consumption table (HUVO-95) that was developed at the Department of Human Nutrition and based on the NEVO-93 table (NEVO Foundation 1993).

Because a change in coffee (Urgert *et al.* 1995) or alcohol consumption (Kris-Etherton *et al.* 1988) can influence serum cholesterol concentrations, subjects were told to maintain their usual coffee and alcohol consumption patterns. Subjects were asked to report daily in their diaries what type and the amount of coffee they drank and whether they had deviated from their habitual alcohol consumption.

Gastrointestinal complaints

In the same diary the subjects were asked to report daily whether they suffered from flatulence, a bloated feeling, belching, stomach ache, belly ache, nausea, vomiting, appetite disturbance, diarrhea, or constipation. The severity of the complaints was rated as 0 (absent), 1 (minor), 2 (moderate), or 3 (severe). For each subject a mean score for each type of complaint was calculated per supplement period. Furthermore, the subjects

were asked to record in their diaries illness, medicine use, start of menstruation, and time of defaecation. They also rated the consistency of their faeces from 1 (watery) to 5 (like pellets). A mean consistency score was calculated per subject for each supplement period.

Blood analysis

Blood was obtained by venipuncture and within 1 h serum was obtained by low-speed centrifugation for 10 min at 1500 x *g* and 4 °C (Sigma 4K10; Salm en Kipp B.V., Breukelen, the Netherlands) and analyzed enzymically for total cholesterol (Siedel *et al.* 1983), HDL cholesterol (Warnick *et al.* 1982), triacylglycerols (Fossati & Prencipe 1982), and 3α-hydroxy bile acids. Triacylglycerols and total and HDL cholesterol were analyzed with a Spectrum Analyzer (Abbott Laboratories, Chicago, IL, USA). 3α-Hydroxy bile acids were measured with a commercial test kit (Enzabile; Nycomed Pharma AS, Oslo, Norway) and a Cobas-Bio Analyzer (Roche Diagnostica, Basel, Switzerland). All samples from a particular subject were analyzed in one run. The CV within runs was 1.0% for total cholesterol, 0.8% for HDL cholesterol, 1.4% for triacylglycerols, and 5% for 3α-hydroxy bile acids. Mean bias with regard to the target values from serum pools provided by the Centers for Disease Control and Prevention, Atlanta, GA, USA, was 0.05 mmol/l for total cholesterol, 0.01 mmol/l for HDL cholesterol, and 0.05 mmol/l for triacylglycerols. LDL cholesterol was calculated by using the equation of Friedewald *et al.* (1972). The mean of the values of the two blood samples per period were used in the statistical analysis to exclude as much as possible the within-subject fluctuations in total and HDL cholesterol concentrations (Rotterdam *et al.* 1987).

Statistical analysis

Differences between the variables as induced by the three supplements were tested by analysis of variance with the GLM (general linear model) procedure of SAS (release 6.09; Statistical Analysis Systems Institute Inc., Cary, NC, USA). The model contained 'subject' as a random factor, thus taking the intrinsic individual concentrations into account, and 'supplement' as a fixed factor. When the analysis of variance indicated a significant ($P \leq 0.05$) effect of supplement, Tukey's Studentized range test was used for pair-wise comparison of the supplements and for calculation of the 95% CIs for the differences between two supplements. This method encompasses a downward adjustment

of the significance limit for multiple testing. With 60 subjects the *a priori* power was calculated to be $\geq 90\%$ for detecting a significant effect ($P \leq 0.05$) of RS compared with glucose on the serum total cholesterol concentration when testing one-sided, if the real population effect was ≥ 0.15 mmol/l and assuming the same within-subject variation as in previous studies at our department.

Results

Within 3 wk after the study began, three women dropped out for personal reasons. A fourth female participant had a traffic accident that leading to hospitalization; therefore, she could not finish the experiment. However, she had completed the RS_2 and RS_3 supplementation periods. One woman developed a bladder infection in the beginning of her RS_3 period; she took antibiotics for 1 wk, which might have affected her colonic flora. The results analyzed with and without the data from the subject that took antibiotics were similar unless stated otherwise.

Food consumption and body weight

No significant differences were found in energy and nutrient intakes when the various supplements were given (Table 9.3). Between the treatment periods, no changes in coffee and alcohol consumption were reported. Body weight remained constant throughout the study. The mean ($\pm$SD) change in body weight was -0.2 $\pm$0.8 kg (range: -2.0 to 1.7 kg) over the glucose periods, 0.2 $\pm$1.1 kg (range: -2.1 to 4.0 kg) over the RS_2 periods, and 0.0 $\pm$1.0 kg (range: -2.6 to 1.9 kg) over the RS_3 periods.

Compliance

According to the diaries, 99% of the supplements provided were consumed. It was reported that 1% of the glucose supplements, 1.1% of the RS_2 supplements, and 1.3% of the RS_3 supplements were not consumed. Mean lithium recovery was >95% and did not differ significantly among the three supplementation periods (Table 9.4). Mean lithium recovery increased by 1-2% when the data from urine collections that were reported to be incomplete were excluded. The three lowest lithium recoveries found in

individual subjects (36%, 39%, and 45%) corresponded with diaries reporting failure to consume all supplement portions.

Table 9.3 *Energy and nutrient intakes during daily supplementation of the habitual diet with either 30 g glucose, RS_2, or RS_3 for 3 wk*[1]

Nutrient	Dietary supplement		
	Glucose[2]	RS_2	RS_3
Energy[3] (MJ/d)	10.3 ± 0.5	10.9 ± 0.5	10.1 ± 0.4
Energy[4] (MJ/d)	12.1 ± 0.5	12.2 ± 0.5	11.4 ± 0.4
Protein (g/d)	88 ± 4	95 ± 4	89 ± 4
Fat (g/d)	89 ± 6	103 ± 6	92 ± 5
Carbohydrate (g/d)	309 ± 16	311 ± 15	293 ± 14
Alcohol (g/d)	10 ± 3	7 ± 2	7 ± 2
Dietary fibre (g/d)	20 ± 1	23 ± 2	20 ± 1
Cholesterol (mg/d)	258 ± 21	263 ± 26	218 ± 15

[1] Mean ±SEM. Supplements are not included in the calculations (n=57). Calculated by using a computerized food-composition table (HUVO-95) that was developed at the Department of Human Nutrition and is based on the NEVO-93 table (NEVO Foundation 1993). There were no significant differences by ANOVA.

[2] n=55; one subject had the flu on the day his 24-h recall was scheduled and one subject had had a traffic accident and was hospitalized.

[3] Supplements not included.

[4] Supplements included; the energy RS supplies when it is fermented in the colon was not included.

Serum concentrations of lipids and 3α-hydroxy bile acids

No treatment effects were found with regard to fasting concentrations of serum total cholesterol, HDL- and LDL-cholesterol, triacylglycerol, and 3α-hydroxy bile acids (Table 9.5). The mean difference (95% CI) in serum triacylglycerol concentration between glucose and RS_2 was 0.11 (-0.00;0.22) mmol/l ($P > 0.05$). When the GLM procedure as described above was run separately on the data from males and females, no significant treatment effects were found in either sex (*data not shown*). Within-subject variance was 0.42 mmol/l for total cholesterol, 0.12 mmol/l for HDL-cholesterol, and 0.34 mmol/l for triacylglycerol. When the data were analysed after exclusion of the data from the subjects

Table 9.4 *Lithium recovery in urine during daily supplementation of the habitual diet with either 30 g glucose, RS_2, or RS_3 for 3 wk*[1]

	All urine collections			Complete urine collections		
Dietary supplement:	Glucose	RS_2	RS_3	Glucose	RS_2	RS_3
n	53[2]	57	57	50[2]	51	55
Mean ±SD (%)	98 ±18	96 ±18	96 ±21	99 ±15	99 ±15	97 ±21
Range (%)	42-146	35-136	36-134	71-146	68-136	36-134

[1] Lithium chloride was added to the supplements for 1 wk before urine collection.

[2] Data from four subjects are missing: one was hospitalized after a traffic accident, the weight of the urine from one subject was not recorded, and urine bottles from two different subjects were accidentally pooled.

with a lithium recovery outside the range of the mean ±2 SD and from the subject that took antibiotics, only the treatment effect for triacylglycerol changed slightly so that it just reached the concentration of statistical significance. The mean difference (95% CI) in serum triacylglycerol concentration between glucose and RS_2 was 0.13 (0.03;0.22) mmol/l ($P < 0.05$), between glucose and RS_3 0.10 (0.00;0.19) mmol/l ($P < 0.05$), and between RS_2 and RS_3 -0.03 (-0.12;0.07) mmol/l ($P > 0.05$). Since the distribution of the serum triacylglycerol concentration was rather skewed, the statistical analysis was repeated on the logarithm of the triacylglycerol concentration. No significant differences were found then, neither when all data were analysed ($P = 0.09$) nor when the data of the above mentioned subjects were excluded ($P = 0.11$).

Gastrointestinal complaints

During supplementation with RS ($n=57$), more flatulence, bloated feelings, belching, and belly aches were reported than during glucose supplementation ($n=56$). The scores (mean ±SEM) for flatulence were 0.27 ±0.04 for glucose, 0.73 ±0.06 for RS_2, and 1.09 ±0.07 for RS_3 ($P < 0.0001$ for all comparisons). RS_3 ingestion caused more bloating (0.33 ±0.05) than did either the consumption of glucose (0.08 ±0.02; $P < 0.0001$) or RS_2 (0.19 ±0.05; $P < 0.05$). More belching ($P < 0.05$) was reported during supplementation with RS_3 (0.13 ±0.03) than during supplementation with either RS_2 (0.07

±0.02) or glucose (0.06 ±0.02). Belly ache was reported more during RS_2 (0.09 ±0.03) and RS_3 (0.10 ±0.02) supplementation than during glucose consumption (0.05 ±0.01, $P < 0.05$).

Table 9.5 *Fasting serum lipids and 3α-hydroxy bile acids after 3 wk of daily supplementation of the habitual diet with 30 g glucose, RS_2, or RS_3*[1]

	Dietary supplement		
	Glucose[2]	RS_2	RS_3
Total cholesterol (mmol/l)	4.69 ±0.14	4.61 ±0.13	4.61 ±0.13
HDL cholesterol (mmol/l)	1.47 ±0.04	1.45 ±0.05	1.45 ±0.04
LDL cholesterol[3] (mmol/l)	2.72 ±0.11	2.71 ±0.10	2.69 ±0.10
Triacylglycerol (mmol/l)	1.09 ±0.07	0.98 ±0.05	1.03 ±0.08
3α-Hydroxy bile acids (μmol/l)	3.25 ±0.28	3.08 ±0.28	3.08 ±0.28

[1] Mean ±SEM; $n=57$. For each subject, means from the two blood drawings on days 18 and 22 of each 3-wk period were used in the statistical analysis. One subject, during the glucose supplementation period, had blood drawn on day 18 only because he had the flu. There were no significant differences by ANOVA.

[2] $n=56$, one subject was hospitalized after a traffic accident.

[3] Calculated with the formula of Friedewald *et al.* (1972):
LDL-cholesterol = total cholesterol - HDL-cholesterol - (triacylglycerol/2.184).

Frequency and consistency of faeces

During supplementation with RS_3, a slightly higher number of bowel movements per day (mean ±SEM: 1.4 ±0.05; $n=57$) was reported than during supplementation with either RS_2 (1.3 ±0.05; $n=57$) or glucose (1.3 ±0.06, $P < 0.05$; $n=56$). During glucose supplementation, somewhat harder stools were reported (rated consistency/stool, mean ±SEM: 3.4 ±0.08; $n=56$) than during either the RS_2 (3.2 ±0.07, $P < 0.05$; $n=57$) or RS_3 (3.1 ±0.07, $P < 0.01$; $n=57$) supplementation periods.

Awareness of the nature of the supplements

The subjects were not told the sequence in which they would receive their supplements until they completed the experiment. At the end of the study the participants were asked

to guess their supplement sequence. The sequence was guessed right by 78% of the participants. Eighteen percent of the volunteers was able to discern the glucose from the RS supplements but could not discern between the RS_2 and RS_3 supplements. Two subjects (4%) guessed totally wrong.

Discussion

This study shows that in healthy normolipidaemic men and women, supplementation of their habitual diet for 3 wk with 30 g RS/d from either raw (RS_2) or retrograded (RS_3) starch did not lower fasting concentrations of serum lipids when compared with supplementation of the diet with glucose.

With 57 subjects and one-sided testing at $P \leq 0.05$, this study had a statistical power of 56% to detect a significant treatment effect on serum total cholesterol between RS and glucose ≥ 0.10 mmol/l. The power was 85% for a treatment-induced difference ≥ 0.15 mmol/l and 97% for a difference ≥ 0.20 mmol/l. A serum cholesterol lowering of ≥ 0.20 mmol/l, or $\geq 4\%$ for a baseline value of 5.0 mmol/l, is considered meaningful with regard to the risk of coronary heart disease.

Both reported compliance and compliance as assessed by lithium recovery in urine were high and did not differ between treatment periods. The variation in lithium recoveries was relatively large, but it was symmetrical around the mean and similar for all three supplement periods. The large variation most likely was due to day-to-day variation in urine production and composition, which was not accounted for by the single 24-h urine collection per treatment period. The sequences in which the supplements were consumed were guessed at least partly correctly by 96% of the subjects. However, it is unlikely that awareness of the nature of the supplements could have affected the study outcome with regard to serum lipid concentrations. The consumption of the different supplements was confirmed by the reported severity of gastrointestinal complaints and stool consistencies. As anticipated, consumption of RS_2 and RS_3 elicited more gastrointestinal complaints and softer stools than did consumption of glucose.

Serum lipid concentrations have been found to stabilize within 2 wk after a dietary change (Connor *et al.* 1961, Keys *et al.* 1965, Stasse-Wolthuis *et al.* 1980, Brussaard *et al.* 1982, Mensink & Katan 1987, Wolever *et al.* 1994), so that the 3-wk treatment period

used can be considered sufficiently long to detect any changes in serum lipid concentrations. The subjects consumed 30 g RS/d in addition to their habitual diet. This daily dose of RS is estimated to be about six times the average intake of RS in the Netherlands (Dysseler & Hoffem 1995*a*). Assuming that RS may be regarded as a kind of dietary fibre, the 30 g of RS applied in the supplements was a significant increase in dietary fibre intake compared with the habitual mean intake of 15 g/d in the Netherlands (Voorlichtingsbureau voor de Voeding 1993), or with the 20 g/d in our group of volunteers (Table 9.3). The glucose supplement provided at most 500 kJ (4% of total energy intake) more than did the RS supplements on the basis that RS supplies no energy at all. Probably, the subjects compensated for the difference in energy intake because no treatment effects on body weight were found.

It is often believed that women are less suitable subjects for studying dietary effects on serum lipids because of confounding effects of the menstrual cycle (Barclay *et al.* 1965, Kim & Kalkhoff 1979) or the use of oral contraceptives (Demacker *et al.* 1982). With a proper study design, however, such confounding effects are eliminated. In our randomized study the women entered the trial at different stages of their menstrual cycle so that the start of menstruation in the premenopausal women was equally spread over the three periods. The number of women starting menstruation was 17 during the glucose period, 22 during the RS_2 period, and 18 during the RS_3 period. Moreover, the supplements were given in random sequence, which provided that any effects of menstrual cycle would be averaged out and thus could not have systematically biased the comparisons of the supplements.

Our results do not agree with those of several studies in rats (Demigné & Rémésy 1982, de Deckere *et al.* 1992, 1993, Morand *et al.* 1994, Verbeek *et al.* 1995, Younes *et al.* 1995*c*) in which RS was found to lower blood cholesterol and triacylglycerol concentrations. This discrepancy might be due to either a species effect or to incomparable doses. The rats in the study of de Deckere *et al.* (1993) were fed daily 4.6 g RS/kg metabolic wt (body weight$^{0.75}$), in the rat study of Verbeek *et al.* (1995) 5.6 g RS/kg metabolic wt per day was given, and in the study of Younes *et al.* (1995*c*) rats were fed daily 12.3 g RS/kg metabolic wt. Other studies with rats reported insufficient information to calculate the intake of RS on the basis of metabolic weight. The subjects in our study consumed daily 1.2 g RS/kg metabolic wt. It is not feasible for humans to consume more RS per day as with 30 g/d, flatulence, bloating, and belching were

frequently reported in this study and also in other studies (van Munster *et al.* 1994*a*, Heijnen *et al.* 1995). Thus, it appears that the lack of effect of RS consumption on serum lipid concentrations in humans, as seen in this study, and the lowering effect found earlier in rats relates to the 4- to 10-fold higher RS doses administered to the rats.

To our knowledge a study on the intake of foods containing well-defined RS in relation to blood lipid concentrations in humans has not been reported before. In three cross-over studies the effect of high-amylose starch on blood lipid concentrations in humans was investigated (Behall *et al.* 1989, Reiser *et al.* 1989, Behall & Howe 1995). In these studies 10-12 healthy men consumed high-amylose foods that were incorporated into their diet for ≥ 5 wk. Reiser *et al.* (1989) and Behall *et al.* (1989) found a 7% decrease in the total cholesterol concentration and an 18% decrease in the triacylglycerol concentration. Behall & Howe (1995) reported that the total cholesterol concentration was elevated by 11% and the triacylglycerol concentration was lowered by 28%. Thus, a high- (compared with a low-amylose diet) did not consistently affect the blood cholesterol concentration, whereas it lowered the triacylglycerol concentration. It is difficult to compare results from the reported studies on high amylose intakes and those from the present study because the extent to which amylose intake was associated with either RS_2 or RS_3 is unknown. Furthermore, Reiser *et al.* (1989) used fructose administration as a control. Fructose compared with regular starches has been found to increase blood cholesterol and triacylglycerol concentrations in some studies (Hollenbeck 1993). Thus, when a high amylose intake is compared with a high fructose intake, as in the study of Reiser *et al.* (1989), the observed lipidaemic effects may be enhanced.

The major determinant of the serum bile acid concentration in healthy subjects is the rate of intestinal absorption of bile acids (LaRusso *et al.* 1978). In the present study neither RS_2 nor RS_3 affected the serum concentration of 3α-hydroxy bile acids. This finding is in line with the unaltered serum cholesterol concentrations and also points to an unaltered enterohepatic cycle and cholesterol absorption and synthesis. In contrast, Verbeek *et al.* (1995) found in rats that RS_3 (compared with digestible starch) significantly increased the serum 3α-hydroxy bile acid concentration by 72%. Again, the large RS dose used in the rat study could explain why an effect was found.

In conclusion, this study showed that daily supplementation of the habitual diet with 30 g RS from either raw or retrograded starch for 3 wk did not lower serum lipid concentrations in healthy normolipidaemic men and women. It is possible that RS

supplementation lowers serum cholesterol concentrations in hyperlipidaemic subjects. In any event, the lack of effect found in this study cannot be explained by insufficient statistical power, low RS doses, short duration of the trial, or inferior compliance by the subjects.

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10

Conclusions and recommendations

Conclusions

The purpose of the experiments described in this thesis was to study physiological effects of resistant starch (RS) consumption. The following conclusions can be drawn from these studies:

1. *Consumption of up to 32 g RS_2 or RS_3 per day is tolerated well by healthy individuals.*

After daily consumption of circa 30 g RS_2 from uncooked potato or high-amylose maize starch or of RS_3 from retrograded high-amylose maize starch, some subjects mentioned increased flatulence and bloated feelings, but severe discomforts were not reported (***Chapter 2-5, 9***). These findings agree with those of other studies (Tomlin & Read 1990, Muir *et al.* 1994, van Munster *et al.* 1994*a,b*, Phillips *et al.* 1995).

A supplementation dose of 30 g RS/d was used in the human studies described in this thesis because it was thought to be the maximum that would be tolerated well and because the amount was six times higher than the current estimated average RS intake of 5 g/d in the Netherlands (Dysseler & Hoffem 1995*a*, ***Chapter 1***). If RS is considered a type of dietary fibre, supplementation with 30 g RS/d represents also a considerable increase in fibre intake in the Netherlands, which is on average 15 g/d (Voorlichtingsbureau voor de Voeding 1993). The Dutch Health Council (1992) recommends to aim at an average intake of 28 g fibre/d.

Because the RS dose used was considerably higher than the estimated habitual RS intake, only well-defined RS supplements were given to the experimental subjects. Thus, the volunteers ate their habitual diet enriched with experimental, RS-containing supplements. This study design is justified by the observation that no differences in the background diet occurred during the various supplementation periods, as measured by 24-h food consumption recalls (***Chapter 3-5, 9***). Thus, any changes in the parameters studied were most likely caused by the RS supplements.

Each subject was provided with the same absolute amount of RS as was also done in other studies by *e.g.* van Munster *et al.* (1994*a,b*) and the group of Cummings (Silvester *et al.* 1995, Cummings *et al.* 1996). Alternatively, the amount of RS supplied could have been kept constant as expressed on the basis of kg (metabolic) body weight or unit of energy intake. The latter approach was used by *e.g.* the group of Muir (Muir *et al.* 1994,

Phillips *et al.* 1995). From a practical point of view, it is easier to supply each subject with the same amount of RS.

2. *RS_2 from uncooked potato or high-amylose maize starch and RS_3 from retrograded high-amylose maize starch are both fermented in the colon of healthy individuals.*

Consumption of 27 g RS_2 from uncooked potato starch in a single meal caused an increase in postprandial breath H_2 excretion when compared with consumption of an equivalent amount of pregelatinized potato starch (***Chapter 2***). Compared with glucose supplementation, 1 wk of daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch also increased breath H_2 excretion (***Chapter 4***). H_2 excretion in breath is a semi-quantitative index of colonic fermentation (Rumessen 1992). During consumption of the glucose supplements there also was a significant H_2 excretion in breath, probably because the background diet contained a significant amount of indigestible but fermentable material, *e.g.* dietary fibre. These findings are confirmed by studies from others (Muir *et al.* 1994, van Munster *et al.* 1994*a,b*).

The finding that RS_2 and RS_3 are fermented in the colon is confirmed by the small amount of starch found in the faeces, representing only 15-18% of the RS supplemented (***Chapter 4***). This agrees with the results from studies by others (van Munster *et al.* 1994*b*, Phillips *et al.* 1995, Cummings *et al.* 1996, Poppitt *et al.* 1996).

3. *RS_2 and RS_3 from high-amylose maize starch are equally fermentable in the colon of healthy individuals.*

One wk of daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch increased breath H_2 excretion to the same extent in a single-blind, randomized multiple cross-over study with 24 healthy men (***Chapter 4***). This is confirmed by the recovery of the same small amounts of starch in the faeces after consumption of the two types of RS (***Chapter 4***). It is important that the results from the H_2 measurements were confirmed by those from the amounts of starch in faeces, as Poppitt *et al.* (1996) showed recently that measurement of 24-h H_2 and CH_4 excretion in breath did not adequately predict the extent of fermentation of non-starch polysaccharides (NSP) and RS in either individuals or groups of healthy subjects.

The lack of a difference in fermentability between RS_2 and RS_3 seems to disagree with *in vitro* data (Cummings *et al.* 1995), a rat study (Schulz *et al.* 1993), and two human

studies (Olesen *et al.* 1994, Champ *et al. unpublished results*) which suggest that RS_2 is better and/or quicker fermentable than RS_3. However, the *in vitro* and human studies by others are difficult to interpret because not only the type of RS but also the amount of RS differed between treatments. Furthermore, *in vitro* studies may show inconsistent results depending on the inocula used. Some subjects fermented one kind of RS well and another type poorly as illustrated by the amount of starch in faeces, implying that different colonic flora ferment various RS sources differently (Cummings *et al.* 1996). In the rat study (Schulz *et al.* 1993), the amount of RS provided per kg metabolic body weight was circa 5 times larger than the presumed maximum amount that is tolerated well by man. Thus, the specific type and amount of RS as well as factors relating to the subject all seem to determine whether or not and to which extent the amount of H_2 in breath increases after consumption of RS.

It cannot be excluded on the basis of the findings in this thesis that RS_2 and RS_3 differ in the rate of fermentation and thus in the location of fermentation in the colon. As colonic fermentation occurs along the longitudinal transit of digesta, slow fermentation implies fermentation more distally in the intestines. The latter may be especially beneficial for colon cancer risk, since tumours in the sigmoid colon, rectosigmoid junction, and the rectum account for nearly 70% of all cases (Austoker 1994). The less fermentable, and thus more distally fermented dietary fibres were protective, whereas the more fermentable ones failed to protect or even enhanced the tumour growth in experimentally-induced colon cancer (Csordas 1996). At least RS_2 from uncooked potato starch seems to be fermented slowly as breath H_2 only started to rise 6 to 7 h after consumption (***Chapter 2***). In contrast, breath H_2 reached a peak value already at 3 h after consumption of lactulose, an indigestible disaccharide that is rapidly fermented in the colon (***Chapter 2***). These findings agree with those of Olesen *et al.* (1994). Nordgaard *et al.* (1995) demonstrated that pure RS of undefined type is fermented slowly *in vitro* as measured by SCFA production in faecal homogenates.

4. *Replacement of digestible starch by RS_2 from uncooked potato starch reduces postprandial glucose and insulin responses in healthy men.*

Replacement of 27 g digestible potato starch by 27 g RS_2 from uncooked potato starch reduced postprandial blood glucose and insulin concentrations in a single-blind, randomized cross-over study with 10 healthy men (***Chapter 2***). This agrees with findings

from others (Holm & Björck 1992, Olesen *et al.* 1994, Raben *et al.* 1994; Liljeberg & Björck 1994, Granfeldt *et al.* 1995).

However, the glucose and insulin responses after the meal containing RS_2 from uncooked potato starch were smaller than expected from the amount of rapidly digestible carbohydrate present in the meal, as was found by Raben *et al.* (1994) as well. Raben *et al.* (1994) found no change in gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) after a meal containing uncooked starch. GIP and GLP-1 are both potent stimulators of insulin secretion so that uncooked starch could indeed produce a lower insulin response than expected from the glucose-stimulated insulin secretion. In a study by Granfeldt *et al.* (1995) a meal with low-amylose bread, providing 45 g digestible starch, was taken as control meal. A meal with high-amylose bread, providing 45 g digestible starch plus 25 g RS, reduced postprandial glucose and insulin responses to the same extent as did a meal with high-amylose bread providing 29 g digestible starch plus 16 g RS. The reduction in blood glucose and insulin concentrations in the presence of RS was ascribed to reduced enzymic accessibility of the digestible starch in the presence of RS and not to a smaller amount of digestible carbohydrate in the meal (Granfeldt *et al.* 1995).

Supplementation of the habitual diet with 32 g RS_3/d, but not with RS_2 (both from high-amylose maize starch), lowered 24-h insulin secretion measured as urinary C-peptide in a single-blind, randomized multiple cross-over study with 24 healthy men (***Chapter 3***). It is as yet unclear why only RS_3 but not RS_2 reduced C-peptide excretion. Possibly, RS_3 reduced the gastric emptying rate more than RS_2 so that glucose entered the blood more gradually, resulting in less insulin secretion.

5. *Replacement of digestible starch by RS_2 from uncooked potato starch lowers diet-induced thermogenesis in healthy men.*

In a single-blind, randomized cross-over study with 10 healthy men, replacement of 27 g digestible potato starch by 27 g RS_2 from uncooked potato starch in a single meal lowered diet-induced thermogenesis (DIT) by on average 90 kJ during the first 5 h after the meal (***Chapter 2***). This reduction is also calculated when assuming that the indigestible carbohydrate in the meal does not contribute to the DIT. Others (Ranganathan *et al.* 1994, Tagliabue *et al.* 1995) also found that RS did not add to the total thermogenic effect of the meal during the first 5 to 6 h postprandially. Possibly, the RS_2-induced

decrease in insulin response (***Chapter 2***) explains the reduction in DIT because the facultative part of the DIT may be increased by insulin via stimulation of the sympathetic nervous system (Landsberg & Young 1983).

Consumption of 20 g lactulose (***Chapter 2***, Ritz *et al.* 1993) increased DIT, which was most probably caused by its fermentation products. Because the fermentation of RS_2 started only 6 to 7 h after consumption, as indicated by the H_2 concentration in end-expiratory air, and because DIT was measured during the first 5 h after the meal only, any possible contribution of fermentation products of RS_2 was not included in the DIT as presented in Chapter 2. As RS fermentation probably occurs slowly, the contribution of its fermentation to the DIT will most likely be too small to be measured by indirect calorimetry.

Replacement of digestible starch by RS may be used in weight-reducing diets because the metabolisable energy value of RS is estimated to be 8 kJ/g (Livesey 1995) whereas the energy value of digestible starch is 17 kJ/g. However, part of this difference seems to be counteracted by a RS-induced decrease of the DIT. The DIT of fermentation products of RS is postponed compared with the DIT of digestion products of digestible starch, so that the magnitude of the decrease in DIT measured in the first 5 h postprandially is an over-estimation of the true decrease in DIT that may occur.

6. *Daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch does not affect subjective feelings of hunger in healthy men.*

Daily supplementation of the habitual diet with 32 g RS_2 or RS_3, when compared with an equivalent amount of glucose, did not affect subjective ratings of hunger in a single-blind, randomized multiple cross-over study with 24 healthy men (***Chapter 3***). A few statistically significant differences were found that were, however, of no practical relevance. Each subject consumed each supplement for 1 wk and feelings of hunger were measured on d 6 of each week. Possibly, no effect on feelings of hunger was found because the subjects were allowed to eat and drink *ad libitum* in addition to the supplements. Thus, the participants could adjust their energy intake to the supplements so that their habitual pattern of hunger feelings would not change. However, energy intake computed from 24-h food consumption recalls remained constant during the three supplementation periods. Therefore, any possible compensation would have fallen within the variance in energy intake as assessed by 24-h food consumption recalls.

Raben *et al.* (1994) found that replacement of 27 g digestible starch by 27 g RS_2 from uncooked potato starch in a single meal induced significantly lower subjective ratings of satiety and fullness. This may be due to the lower metabolisable energy content of the RS meal compared with the digestible starch meal. In contrast, others (Holm & Björck 1992, Granfeldt *et al.* 1994, Holt & Brand Miller 1994) found increased subjective satiety scores after meals containing RS. Because of the single meal study design used, these experiments cannot be compared with the one described in Chapter 3. It appears that the satiating effect of RS might be studied better with a preload-test meal design as described by *e.g.* Delargy *et al.* (1993) and Hulshof *et al.* (1993).

7. *RS_2 and RS_3 from high-amylose maize starch increase stool weight in healthy men.*
Daily supplementation of the habitual diet with 32 g RS_2 or RS_3 from high-amylose maize starch, compared with an equivalent amount of glucose, increased stool weight by 1.4 g/g RS_2 and 2.2 g/g RS_3 (***Chapter 4***). Others reported similar results (Phillips *et al.* 1995, Cummings *et al.* 1996, van Munster *et al.* 1994*b*). However, consumption of 10 g RS_3/d from cornflakes was not sufficient to increase faecal bulk (Tomlin & Read 1990).

This RS-induced increase in faecal mass might be positive for human health because an inverse relationship has been reported between stool weight and colon cancer incidence (Cummings *et al.* 1992). Burkitt (1971) proposed a protective mechanism by "dilution" of the intestinal contents and reduction of intestinal transit time (due to an increase in digesta volume), thus reducing the contact of carcinogens with the colonic mucosa.

The increase in stool weight cannot be explained by extra starch or water in the stool, but is most likely due to an increase in bacterial mass. This is supported by the results from other studies (Scheppach *et al.* 1988*a*, Birkett *et al.* 1996, Cummings *et al.* 1996) and by the increase in colonic fermentation as illustrated by an increase in breath H_2 excretion (***Chapter 4***).

8. *One week of daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch has no effect on a number of putative risk factors for colon cancer in healthy men.*
When 24 healthy men consumed in addition to their habitual diet a daily supplement containing 32 g RS_2 or RS_3 or glucose in a single-blind, randomized multiple cross-over study, no differences were found in faecal pH and SCFA concentrations, nor in the pH,

bile acid concentrations, and cytotoxicity of faecal water (***Chapter 4***). Effects might have been found when the supplementation period would have been increased to several weeks as fermentative activity (indicated by an increase in breath H_2 excretion) and stool weight were increased by RS supplementation. Further, the variance in faecal parameters would be reduced if all faeces produced during 3-5 d would have been collected (Setchell *et al.* 1987) instead of two stools produced within 3 d, as in the study described in Chapter 4.

Daily supplementation with 28 g RS_2 from high-amylose maize starch during 2 wk positively affected putative risk factors for colon cancer in 13 healthy subjects (van Munster *et al.* 1994*b*). However, that study lacked a control group. In a subsequent parallel study with patients with one or more recently removed adenomas, daily supplementation with 28 g RS_2 from high-amylose maize starch for 4 wk increased the concentration of primary bile acids in faecal water and decreased the concentration of secondary bile acids when compared with glucose supplementation (Grubben *et al.* 1996). However, faecal wet and dry weight, pH, SCFA excretion and rectal mucosal proliferation were not affected by RS_2. The authors suggest that the discrepancy between their study and that by van Munster *et al.* (1994*b*) may be due to a relatively higher fibre and lower fat intake in the patients. In a randomized cross-over trial 11 healthy subjects consumed two diets, one providing 5 g RS/d and the other 39 g RS/d (Phillips *et al.* 1995). Each diet was consumed for 3 wk by each subject. The high-RS diet induced a lower faecal pH and increased the faecal concentration and absolute excretion of butyrate and acetate. However, as the diets contained a mixture of RS_1, RS_2 and RS_3 it is unclear to what extent the effects observed are caused by the various types of RS.

Recently, three studies have been published that investigated the effect of dietary RS on chemically-induced colon cancer in rats (Caderni *et al.* 1994, Young *et al.* 1996, Sakamoto *et al.* 1996). However, it is difficult to draw conclusions from these experiments for the human situation because the amount and/or the type of RS used in those rat studies was not defined. Mazière *et al.* (1996) found that dietary RS_3 reduced the amount of aberrant crypt foci (ACF) in the colon of 1,2-dimethylhydrazine (DMH)-injected rats. Further, RS_3 induced a lower caecal pH, and increased faecal and caecal mass and butyrate production. Rats fed RS_3 or lactulose exhibited decreased levels of carcinogen-induced DNA damage in the colonic mucosa when compared with rats fed sucrose, digestible starch or soy fibre (Rowland & Rumney 1996).

Thus, some studies, particularly those in the rat, suggest a possible favourable effect of dietary RS on putative risk factors for colon cancer, while others do not. It should be noted that there is no conclusive evidence that the biomarkers used are indeed causally related to the development of colon cancer.

9. *One week of daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch has no effect on faecal ammonia excretion in healthy men, but RS_3 decreases faecal ammonia concentration.*

One wk of daily supplementation of the habitual diet with 32 g RS_2 or RS_3 or glucose induced the same amounts of ammonia excretion with faeces in a randomized, single-blind multiple cross-over study with 24 healthy men (***Chapter 5***). Because RS_3 supplementation increased faecal weight significantly, when compared with glucose, RS_3 also decreased the faecal ammonia concentration. This may be advantageous in the protection against colon cancer (Lin & Visek 1991). Birkett *et al.* (1996) also reported that in healthy subjects consumption of RS lowered faecal concentration of ammonia. They compared a diet providing 39 g RS/d with a diet containing 5 g RS/d in a randomized cross-over study of 2 x 3 wk. The RS in the diets was a mixture of RS_1, RS_2 and RS_3. Thus, their finding may have been specifically caused by the RS_3 component.

As both Birkett *et al.* (1996) and we (***Chapter 5***) found no effect of RS on absolute faecal ammonia excretion nor on urinary urea excretion, ammonia was not used for RS-induced bacterial growth because there was no shift of nitrogen excretion from urine to faeces. In some studies (Flourié *et al.* 1986) RS had no effect on absolute ammonia and nitrogen excretion in faeces whereas in others (Birkett *et al.* 1996; Cummings *et al.* 1996) RS increased absolute faecal nitrogen excretion. The latter may point at increased bacterial growth as 60% of faecal nitrogen is bacterial nitrogen (Stephen & Cummings 1980). Starch malabsorption as induced by the glucosidase inhibitor acarbose increased faecal nitrogen excretion, faecal bacterial mass and faecal bacterial nitrogen in healthy subjects (Scheppach *et al.* 1988*a*). Thus, it is not clear yet how RS consumption affects nitrogen metabolism in man.

10. *Consumption of RS_3 but not of RS_2 from high-amylose maize starch shifts nitrogen excretion from urine to faeces in piglets.*

Replacement of glucose by RS_2 or RS_3 in the feed of piglets cannulated at the end of the ileum did not affect nitrogen retention but increased faecal nitrogen excretion (***Chapter 6***). The latter was probably due to the combination of a decrease in ileal nitrogen absorption and an increase in nitrogen trapping by bacteria. Bacterial growth probably was enhanced as a result of fermentation of RS_2 and RS_3 because virtually no starch was recovered in faeces and faecal mass had increased (***Chapter 6***, *see conclusion 11*). Fermentation of RS in the colon probably induced a lower colonic pH (not measured). A lower pH enhances the conversion of ammonia (NH_3) into ammonium (NH_4^+). Ammonium is less well absorbed by the colon than ammonia and will be excreted in the faeces. This process may also have contributed to the observed increase in faecal nitrogen excretion.

Only in the RS_3-fed piglets, and not in the RS_2-fed piglets, the increase in faecal nitrogen excretion was balanced by a decrease in urinary nitrogen excretion, mainly in the form of urea, which can be explained by the observed reduced colonic nitrogen absorption. *A priori*, we expected the effects of RS_2 and RS_3 to be just the other way around because we assumed that RS_2 is better fermentable than RS_3 (Schulz *et al.* 1993, Olesen *et al.* 1994, Cummings *et al.* 1995, Champ *et al. unpublished data*). The discrepancy between the results and our expectations can be explained by the greater ileal fermentation of RS_2 compared with RS_3, as indicated by the lower amounts of starch in the ileal digesta after RS_2 feeding (***Chapter 6***, *see conclusion 11*). Thus, more fermentable substrate was available in the colon of the RS_3-fed piglets so that more nitrogen could be trapped in bacteria in the colon after RS_3 instead of RS_2 feeding. If these results can be extrapolated to man, consumption of RS_3 rather than digestible carbohydrate may lower the workload for the kidneys and the liver and may therefore be beneficial for patients with kidney or liver malfunction.

The effect of RS_3 on the routes of nitrogen excretion as found in the present study agrees with studies in which starch was infused into the terminal ileum of pigs (Gargallo & Zimmerman 1981, Misir & Sauer 1982, Mosenthin *et al.* 1992). In those studies fermentation also took place mainly in the colon and the increase in faecal starch excretion was balanced by a decrease in urinary nitrogen excretion, as in our study after RS_3 feeding. However, we have no explanation for the discrepancy between the effect of

RS_2 in the present study and in the study by Wünsche *et al.* (1987). In the latter, the increase in feacal nitrogen excretion was balanced by a decrease in urinary nitrogen excretion after pigs were fed RS_2 from raw potato products, whereas in the present study this balancing did not occur after RS_2 feeding but after RS_3 feeding.

11. *RS_2 and RS_3 from high-amylose maize starch are fully fermented in the digestive tract of the pig, and a considerable part is fermented in the small intestine.*

In the faeces of piglets cannulated at the end of the ileum virtually none of the 114 g RS_2 or RS_3 fed per day was recovered (***Chapter 6***), indicating that RS was fully digested in the gastrointestinal tract. At the end of the ileum 56% of the ingested RS_2 and 29% of the RS_3 had disappeared. This means that either RS was fermented in the ileum and/or that RS as measured *in vitro* by the procedure of Englyst *et al.* (1992) does not correspond to the amount of the physiologically defined RS *in vivo* in the pig. The ileum of the pig, especially the distal third, contains a significant amount of bacteria (Chesson *et al.* 1985, Liu *et al.* 1985, Bach Knudsen *et al.* 1993) so that ileal fermentation of RS is possible. Ileal and colonic fermentation is indicated further by the increase in nitrogen content and weight of the ileal digesta and faeces (***Chapter 6***) which could, at least partly, be caused by an increase in the number of bacteria. Another indication of increased fermentative activity in the ileum is the fact that the increase in dry matter content of the ileal digesta was only partly accounted for by undigested starch. Thus, although the overall digestibility of RS_2 and RS_3 in the pig was equal, they differed in the location of fermentation: RS_2 was fermented for 56% in the ileum and for 44% in the colon, whereas RS_3 was fermented for 29% in the ileum and for 71% in the colon.

12. *Dietary RS_2 but not RS_3 from high-amylose maize starch reduces apparent absorption of magnesium and calcium in piglets.*

When compared with glucose, dietary RS_2, but not RS_3, reduced total apparent absorption of magnesium and calcium in piglets cannulated at the end of the ileum (***Chapter 7***). No other studies on the effect of RS consumption on mineral absorption in pigs have been published so far. These results do not agree with those of human and rat studies which may be due to differences in RS and/or mineral intake or to species differences (*see conclusion 13*).

Magnesium absorption tended to be shifted from the colon to the ileum to some extent in the piglets fed RS_2 and RS_3 when compared with those fed glucose. This may be connected with the finding that RS is fermented already in the ileum (*see conclusion 11*), inducing a lower ileal pH, increasing magnesium solubility and thereby magnesium absorption (Heijnen *et al.* 1993, Schulz *et al.* 1993, Younes *et al.* 1996). In a pig study comparing dietary fructose with glucose (van der Heijden *et al.* 1995) magnesium was absorbed mainly from the colon, which is in contrast to our findings. Both in our study (***Chapter 7***) and that from van der Heijden *et al.* (1995) calcium and phosphorus were absorbed mainly from the ileum. RS_2, but not RS_3, significantly reduced the ileal absorption of phosphorus while the reduction in total phosphorus absorption did not reach statistical significance (***Chapter 7***).

13. *One week of daily supplementation with 32 g RS_2 or RS_3 from high-amylose maize starch has no effect on the apparent absorption of magnesium, calcium and phosphorus in healthy men.*

In a single-blind, randomized multiple cross-over study with 24 healthy men, 1 wk of daily supplementation of the habitual diet with 32 g RS_2 or RS_3 or glucose induced the same apparent absorption of magnesium, calcium and phosphorus expressed as percentage of intake (***Chapter 7***). Langkilde and Andersson also found no effect of RS_2 (1995*b*) or RS_3 (1992) versus digestible starch on ileal absorption of magnesium and calcium in ileostomy patients. No other human experiments studying the effect of RS consumption on mineral absorption have been published so far.

In rats, RS_2 compared with digestible starch raised apparent magnesium and calcium absorption (Rayssiguier & Rémésy 1977, Andrieux & Sacquet 1986, Schulz *et al.* 1993, Younes *et al.* 1993, 1996). Schulz *et al.* (1993) and we (***Chapter 8***) found that RS_2 versus RS_3 also raised apparent magnesium and calcium absorption in rats. The discrepancy between the results from our human study (***Chapter 7***) and from the above mentioned rat studies and our pig experiment (***Chapter 7***) may be explained by the 5-10 times higher dose of RS per kg metabolic body weight that the rats and piglets consumed, respectively. Other factors involved could be species differences in mineral intake per kg metabolic body weight, in fermentation efficiency, or in hormonal control, anatomy or physiology of the intestinal tract (Mathers 1991). Furthermore, the ileal starch digestibility in rats,

pigs and man may be different (Roe *et al.* 1996) so that the amount of starch that was truly resistant in each species is uncertain.

It cannot be excluded on the basis of the study described in Chapter 7 that RS could affect mineral absorption if the mineral intake was marginal or inadequate, or if RS supplementation was continued over a longer period. However, differences in mineral absorption were found within 5-10 d after a dietary change (Balasubramanian *et al.* 1987, Brink *et al.* 1993, Siener & Hesse 1995).

14. *RS_2 from high-amylose maize starch does not enhance true magnesium absorption in rats.*

Schulz *et al.* (1993) and Heijnen *et al.* (1993) have proposed a mechanism to explain the observed enhancing effect of RS_2 on apparent magnesium absorption in rats (Rayssiguier & Rémésy 1977, Andrieux & Sacquet 1986, Schulz *et al.* 1993, Younes *et al.* 1996, ***Chapter 8***). RS_2 would raise the ileal solubility of magnesium due to a reduction in ileal pH resulting from enhanced bacterial fermentation of RS. However, RS_2 did not enhance true magnesium absorption because it lowered the faecal excretion of endogenous magnesium (***Chapter 8***). If the earlier proposed mechanism would be true then the absorption of both dietary exogenous and endogenous magnesium should be enhanced, resulting not only in an increased apparent absorption but also in an increased true absorption.

15. *Neither RS_2 nor RS_3 from high-amylose maize starch lowers fasting serum lipid concentrations in healthy normolipidaemic subjects.*

Daily supplementation of the habitual diet with 30 g RS_2 or RS_3, when compared with an equivalent amount of glucose, did not affect serum concentrations of triacylglycerols, and total, LDL and HDL cholesterol in a single-blind, randomized multiple cross-over study with 57 healthy men and women (***Chapter 9***). So far, no other human trials that studied the effect of well-defined (experimental) foods rich in RS have been published. In three cross-over studies (Reiser *et al.* 1989, Behall *et al.* 1989, Behall & Howe 1995) the effect of high-amylose maize starch on serum lipid concentrations in healthy men was studied. However, these studies showed conflicting results with respect to serum cholesterol concentration, and it is unclear whether the effects found are due to RS, to amylose, or to a combination of RS and amylose.

In several rat studies (Demigné & Rémésy 1982, Sacquet *et al.* 1983, de Deckere *et al.* 1992, 1993, 1995, Mathé *et al.* 1993, Morand *et al.* 1994, Verbeek *et al.* 1995, Younes *et al.* 1995*c*) high-amylose maize starch, RS_2 or RS_3 lowered serum triacylglycerol and cholesterol concentrations considerably. However, the dose of RS per kg metabolic body weight that the rats consumed was five times higher than the presumed maximum dose humans can consume without negative side effects. Furthermore, the cholesterol metabolism in the rat is different from that in man.

Normolipidaemic subjects were enroled in the study described in ***Chapter 9*** because these people were easily accessible to us. Various changes in the diet, such as an increase in the soluble fibre content, have been shown to induce both statistically significant and physiologically meaningful decreases in serum cholesterol concentration both in normolipidaemic and in hyperlipidaemic individuals (Stasse-Wolthuis *et al.* 1980, Topping 1991, Truswell 1995). The power calculation in the study described in ***Chapter 9*** was based on intra-individual variation in normolipidaemic subjects. The power was 97% for a treatment-induced difference in total cholesterol of ≥ 0.20 mmol/L. A serum cholesterol lowering of ≥ 0.20 mmol/L, or $\geq 4\%$ for a baseline value of 5.0 mmol/L, is considered meaningful with regard to the risk of coronary heart disease. Moreover, the lack of effect in the study cannot be explained by *(i)* a low dose of RS as the daily RS dose provided was six times the current estimated RS intake in the Netherlands (Dysseler & Hoffem 1995*a*), *(ii)* insufficient compliance as compliance measured as urinary lithium recovery was $>95\%$, *(iii)* other dietary changes as the amount and composition of the background diet did not differ during the various supplementation periods, and *(iv)* a too short intervention period as serum lipid concentrations have been found to stabilize within 2 wk after a dietary change (Keys *et al.* 1965, Stasse-Wolthuis *et al.* 1980, Brussaard *et al.* 1982, Wolever *et al.* 1994) so that the 3-wk treatment period can be considered sufficiently long to detect any changes in serum lipid concentrations.

Recommendations

1. More methodological studies should be performed to obtain a widely accepted standardized, precise and reproducible *in vitro* method that is validated by *in vivo* measurements to determine RS in foods as eaten.

2. RS should be included in food composition tables, so that epidemiological studies on the putative health effects of RS consumption become feasible.
3. In food consumption studies the preparation method of starchy foods should be inquired about in detail, so that RS intake can be estimated better.
4. If animal experiments are conducted to study mechanisms of action, the dose of RS provided should be equivalent, when expressed on the basis of kg metabolic body weight, to the presumed maximum dose that humans can consume without negative side effects, *i.e.* circa 30 g/d.
5. More evidence should be obtained to confirm that the parameters considered risk factors for developing colon cancer are indeed true biomarkers predicting colon cancer risk.
6. Studies on the possible role of RS in the prevention of colon cancer should be continued and extended.
7. The significance for human physiology, metabolism and health of increased activity and site of fermentation in the colon should be studied further.

In conclusion, daily consumption of up to 32 g RS_2 or RS_3 is not unfavourable for healthy individuals, but it also does not have great beneficial effects on human physiology, at least for the parameters and time span studied in this thesis. Especially the significance for human health of increased activity and site of fermentation in the colon, and the possible role of different types of RS in the prevention of colon cancer should be studied further.

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Samenvatting

Hiermee wil ik aan mijn familie en vrienden buiten de 'voedingswereld' uitleggen waar ik me de afgelopen vijf jaar op m'n werk mee bezig heb gehouden.

Zetmeel

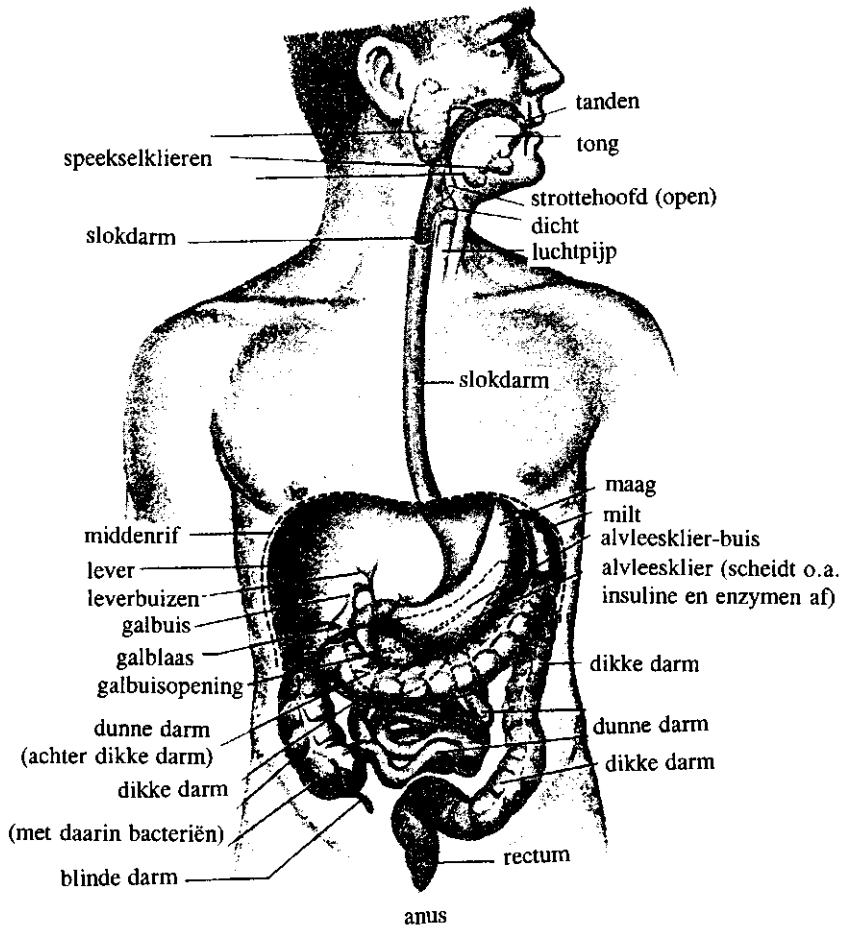
Zetmeel is een belangrijk bestanddeel van onze voeding, naast vet en eiwit. Nederlanders eten gemiddeld 126 gram zetmeel per dag, grotendeels afkomstig uit brood, aardappelen en pasta. Zetmeel is als het ware een lange ketting van 'suiker-kralen' (glucose-eenheden). Na het eten van bijvoorbeeld een boterham wordt het zetmeel daaruit in de dunne darm (***zie figuur***) in stukjes geknipt door stoffen die we enzymen noemen. Zo komen de afzonderlijke glucose-eenheden ('suiker-kralen') vrij, die dan uit de dunne darm opgenomen worden in het bloed. We zeggen dan dat het zetmeel is verteerd. Glucose (suiker) is de brandstof voor lichaamscellen. Het bloed met daarin glucose stroomt langs de lichaamscellen en die nemen daaruit het glucose op met behulp van het hormoon insuline.

Onverteerbaar zetmeel (RS)

Vijftien jaar geleden is ontdekt dat niet al het zetmeel dat we eten op deze manier in stukjes geknipt wordt in de dunne darm om als glucose opgenomen te worden in het bloed. Een deel van het zetmeel in onze voeding kan dus niet verteerd worden. Dit onverteerbare zetmeel wordt 'resistant starch' (RS) genoemd in het Engels. Deze Engelse term wordt ook in Nederland gebruikt. Er worden drie soorten resistant starch onderscheiden: RS_1, RS_2 en RS_3. Deze drie soorten verschillen in de reden waarom het zetmeel niet door enzymen in glucose-eenheden geknipt kan worden. Met andere woorden: RS_1, RS_2 en RS_3 verschillen in de oorzaak van hun onverteerbaarheid.

RS in de dikke darm

Omdat RS niet in de vorm van glucose-eenheden in het bloed opgenomen wordt uit de dunne darm, komt het in de dikke darm terecht (***zie figuur***). In de dikke darm zitten bacteriën die RS (gedeeltelijk) kunnen vergisten (fermenteren). Daarbij wordt RS omgezet in onder andere waterstofgas dat gedeeltelijk opgenomen wordt in het bloed. Als het bloed langs de longen stroomt, gaat het waterstofgas grotendeels uit het bloed de longen



Het spijsverteringskanaal van de mens. *Voedsel gaat na het kauwen in de mond via de slokdarm naar de maag. Van daaruit gaat het via de dunne darm, de dikke darm en het rectum naar de anus. Onderweg wordt het voedsel in hele kleine stukjes 'geknipt' door maagzuur en stoffen die we enzymen noemen. Enzymen worden door onder andere de speekselklieren en de alvleesklier afgegeven. Gal komt uit de galblaas in de dunne darm en helpt bij de afbraak van vet. De hele kleine voedselstukjes worden uit de darm opgenomen in het bloed en zo afgeleverd bij de lichaamscellen. Die worden zo voorzien van energie en bouwstoffen waardoor ons lichaam kan functioneren.*

in. Het wordt dan vervolgens uitgeademd. Je kunt de hoeveelheid waterstofgas in uitademingslucht meten om te weten of er vergisting plaats vindt in de dikke darm.

Consumptie van RS

Er wordt geschat dat Nederlanders nu gemiddeld vijf gram RS per dag eten. Er zit een klein beetje RS in bijvoorbeeld oud geworden brood, gekookte en weer afgekoelde pasta of aardappelen, peulvruchten en onrijpe (groene) bananen. Vijf gram RS per dag is maar een klein deel van de 126 gram zetmeel die we in totaal eten. Maar het is mogelijk om de hoeveelheid RS die je eet te beïnvloeden door de keuze van je voedingsmiddelen en de manier waarop je ze bereidt. Als je bijvoorbeeld gekookte aardappelen koud eet als salade, eet je meer RS dan wanneer je vers gekookte aardappelen eet. Ook de levensmiddelenindustrie kan de hoeveelheid RS in een produkt beïnvloeden, nl. door de keuze van de grondstoffen (b.v. zetmeel uit maïs, aardappelen of tarwe) en van de industriële bereidingswijze.

Is RS goed voor de gezondheid?

Het kan interessant zijn, zowel voor de levensmiddelindustrie als voor de consument, om de hoeveelheid RS in bepaalde voedingsmiddelen te verhogen, als RS goed zou zijn voor de gezondheid. Uit eerder onderzoek zijn bepaalde ideeën voortgekomen waarom en hoe het eten van RS gunstig zou kunnen zijn voor de gezondheid van de mens. Ik heb, met hulp van anderen, experimenten uitgevoerd om een aantal van deze mogelijk gunstige effecten van het eten van RS te onderzoeken. Deze experimenten staan in dit proefschrift beschreven.

Experimenten in dit proefschrift

Omdat de verschillende soorten RS verschillende effecten zouden kunnen hebben, heb ik in de meeste experimenten RS_2 en RS_3 onderzocht. RS_1 heb ik niet bestudeerd omdat het moeilijk is om voedingsmiddelen te maken met RS_1 erin die iedere keer precies hetzelfde zijn. Dat gaat wel goed met RS_2 en RS_3. Bovendien waren er aanwijzingen uit eerder onderzoek dat RS_2 beter vergist zou worden dan RS_3. Het effect van het eten van RS_2 en RS_3 is steeds vergeleken met dat van verteerbaar zetmeel of glucose (suiker) omdat de laatste twee, in tegenstelling tot RS, volledig verteerbaar zijn en opgenomen worden in het bloed. Aan de meeste experimenten deden gezonde vrijwilligers mee. Zij aten

gedurende een aantal weken ongeveer 30 gram RS per dag naast hun gebruikelijke voeding. Deze hoeveelheid RS is zes keer zoveel als hun gebruikelijke RS consumptie. De vrijwilligers aten RS in de vorm van meel dat was vermengd met melk en yoghurt en waaraan voor de smaak limonadesiroop of fruit uit blik was toegevoegd. Verder heb ik twee experimenten met ratten gedaan (waarvan er één in dit proefschrift staat beschreven) en één met biggen.

Geen bijwerkingen

Het eten van 30 gram RS extra per dag werd goed verdragen door gezonde mensen. Sommige vrijwilligers rapporteerden toegenomen winderigheid en een opgeblazen gevoel, maar er werden geen ernstige negatieve bijwerkingen gemeld (hoofdstuk 2-5, 9).

Vergisting van RS

RS_2 en RS_3 werden inderdaad vergist door de bacteriën in de dikke darm want de hoeveelheid waterstofgas in de uitademingslucht van gezonde vrijwilligers die RS_2 of RS_3 hadden gegeten, nam toe (hoofdstuk 2, 4, 9). Ik vond ook nauwelijks zetmeel terug in de ontlasting na het eten van RS_2 of RS_3. Het eten van RS_2 en RS_3 leidde tot evenveel waterstofgas in de uitademingslucht en even weinig zetmeel in de ontlasting (hoofdstuk 4, 9). RS_2 en RS_3 lijken dus even goed vergist te kunnen worden.

Glucose en insuline in het bloed

Omdat RS niet door enzymen in glucose-stukjes geknipt kan worden om zo opgenomen te worden in het bloed, verwachtte ik dat het glucose-gehalte in het bloed na het eten van RS minder zou stijgen dan na het eten van verteerbaar zetmeel. Dat heb ik inderdaad gemeten bij gezonde vrijwilligers die RS_2 hadden gegeten (hoofdstuk 2). Als er minder glucose in het bloed wordt opgenomen, is er ook minder van het hormoon insuline nodig voor de opname van glucose in de lichaamscellen. Ik heb ook een lager insuline-gehalte in het bloed gemeten na het eten van RS_2 in plaats van verteerbaar zetmeel (hoofdstuk 2). Minder stijging van de glucose- en insuline-gehaltes in het bloed is gunstig voor suikerpatiënten, maar ook voor gezonde mensen.

Energie

RS levert minder energie dan verteerbaar zetmeel omdat het eten van RS niet leidt tot opname van glucose (brandstof) in de lichaamscellen. Dit kan nuttig zijn in vermageringsdiëten. Het voordeel is echter minder groot dan verwacht omdat het afbreken van verteerbaar zetmeel tot glucose, het opnemen daarvan in het bloed en de lichaamscellen, en het verwerken van glucose in de lichaamscellen ook energie kost. Dat gold niet voor RS_2, althans niet in de eerste vijf uur na de maaltijd (hoofdstuk 2). RS eten in plaats van verteerbaar zetmeel betekent dus minder energie opnemen, maar ook minder energie verbruiken.

Hongergevoelens

Het eten van RS zou tot minder hongergevoelens kunnen leiden als het werkt als maagvulling (een 'vol' gevoel geeft). Dan zou het eten van RS in plaats van verteerbaar zetmeel ook op deze manier kunnen helpen in vermageringsdiëten. Maar RS zou juist ook tot meer hongergevoelens kunnen leiden omdat het minder energie levert dan verteerbaar zetmeel. Gezonde vrijwilligers rapporteerden evenveel hongergevoelens als ze naast hun gebruikelijke voeding 32 gram RS_2 of RS_3 per dag aten als wanneer ze 32 gram glucose per dag extra aten (hoofdstuk 3). RS lijkt dus geen verzadigend effect te hebben.

Hoeveelheid ontlasting

Het eten van 32 gram RS_2 of RS_3 extra per dag gedurende een week vergrootte de hoeveelheid ontlasting (hoofdstuk 4). Dit kan gunstig zijn voor de stoelgang. Het kan mogelijk ook helpen om dikke-darm-kanker te voorkomen doordat schadelijke stoffen die in de dikke darm aanwezig kunnen zijn als het ware verdund worden als er meer 'brei' in de dikke darm zit. Verder zorgt meer brei in de darmen ervoor dat alles sneller door de darmen heen stroomt. Eventueel aanwezige schadelijke stoffen hebben dan minder tijd om de dikke darm te beschadigen. De toename van de hoeveelheid ontlasting werd waarschijnlijk veroorzaakt door een toename van het aantal bacteriën. Als RS in de dikke darm komt, betekent dat meer 'voedsel' voor de daar aanwezige bacteriën, zodat ze zich kunnen vermeerderen.

Dikke-darm-kanker

Er zijn aanwijzingen dat vergisting van RS in de dikke darm de kans op kanker aan de dikke darm kan verkleinen. Er is gesuggereerd dat je aanwijzingen daarvoor kunt vinden door bepaalde stoffen in de ontlasting te meten. Ik heb die gemeten in de ontlasting van gezonde vrijwilligers die gedurende een week iedere dag 32 gram extra RS_2, RS_3 of glucose hebben gegeten (hoofdstuk 4-5). Ik vond geen verschillen tussen glucose, RS_2 en RS_3. Ik vond dus geen aanwijzingen dat RS zou kunnen helpen om dikke-darm-kanker te voorkomen, met uitzondering van een toename van de hoeveelheid ontlasting. Het is mogelijk dat het gedurende langere tijd eten van RS wel een effect heeft op de gehaltes van bepaalde stoffen in de ontlasting en op het risico voor dikke-darm-kanker.

Cholesterol-gehalte in het bloed

Er waren aanwijzingen dat het eten van RS het cholesterol-gehalte in het bloed zou kunnen verlagen. Een te hoog cholesterol-gehalte in het bloed kan hart- en vaatziekten veroorzaken. Het eten van 30 gram RS_2 of RS_3 extra per dag gedurende drie weken had geen effect op het cholesterol-gehalte in het bloed van gezonde vrijwilligers (hoofdstuk 9). Het eten van RS kan dus niet helpen om het cholesterol-gehalte in het bloed te verlagen.

Stikstof in biggen

In biggen leidde het eten van RS_3, maar niet van RS_2, er toe dat de hoeveelheid stikstof in de ontlasting toenam, terwijl de hoeveelheid stikstof in de urine juist afnam (hoofdstuk 6). Dat is gunstig voor het milieu omdat het minder vervuiling met ammoniak betekent. RS_2 en RS_3 werden in de big volledig vergist. De vergisting begon al in de dunne darm (hoofdstuk 6). Dat kan omdat daar bij de big, in tegenstelling tot bij de mens, ook al bacteriën zitten.

Opname van calcium en magnesium

Calcium (kalk) en magnesium zijn noodzakelijk voor allerlei lichaamsprocessen. Zo is calcium bijvoorbeeld een belangrijke bouwsteen van de botten en is magnesium nodig voor bijvoorbeeld de werking van spieren. In vergelijking met glucose en RS_3 leidde het eten van RS_2 in de big tot een verminderde opname van calcium en magnesium uit het voer (hoofdstuk 7). Ik weet nog niet waarom. Het eten van 32 gram RS_2 of RS_3 extra per

dag gedurende een week had geen effect op de opname van magnesium en calcium in gezonde vrijwilligers (hoofdstuk 7). Het is mogelijk dat RS wel een effect heeft op de opname van calcium en magnesium als deze stoffen in zeer kleine hoeveelheden in de voeding voorkomen, of als RS gedurende langere tijd gegeten zou worden. In ratten verhoogde RS_2 ten opzichte van RS_3 de opname van magnesium uit het voer (hoofdstuk 8). Dit werd echter niet door vergisting van RS veroorzaakt (hoofdstuk 8), zoals eerder onderzoek suggereerde. RS_2 en RS_3 hebben dus verschillende effecten op de opname van calcium en magnesium in rat, big en mens. Dit kan mogelijk verklaard worden doordat de rat, big en mens per kilogram lichaamsgewicht verschillende hoeveelheden RS, calcium en magnesium aten. Een andere mogelijke verklaring zijn de verschillen tussen de rat, big en mens in bijvoorbeeld het spijsverteringskanaal en de bacteriën in de dikke darm.

Conclusie

Uit de experimenten in dit proefschrift blijkt dat het eten van RS niet ongunstig is voor de mens, maar ook niet leidt tot grote positieve effecten op de gezondheid. Ik adviseer om nader onderzoek te doen naar het effect van verschillende soorten RS op het risico voor dikke-darm-kanker en naar de betekenis van meer vergisting in de dikke darm voor de gezondheid.

Curriculum vitae

Marie-Louise Heijnen was born on 8 December 1966 in Roosendaal, the Netherlands. In 1985 she completed secondary school (Gymnasium β) at the Norbertus College in Roosendaal and started the study Human Nutrition at the Wageningen Agricultural University (WAU). As part of this study, she conducted research projects in human physiology (Department of Physiology of Man and Animal, WAU), clinical nutrition (University Hospital Gent, Belgium), and human nutrition (Department of Human Nutrition, WAU; Department of Human Nutrition, University of Helsinki, Finland; Unilever Research Laboratory, Vlaardingen). In June 1991 she received the MSc degree *with distinction*.

From January 1992 till August 1996 she was appointed as PhD fellow at the Department of Human Nutrition, WAU. During this appointment she conducted the research described in this thesis. She participated in EURESTA, an European-Community-funded collaboration of forty European research groups, investigating the physiological implications of consumption of resistant starch in man. In July 1994 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. She was co-organiser of the symposium 'The editors speak out' on the occasion of the 25th anniversary of the Department of Human Nutrition in September 1994. Further, she was a board member of the Wageningen PhD organisation, of the PhD-council of the research school VLAG and of the council of the Department of Human Nutrition.

She was one of the organisers of a workshop on dietary fibre for members of the Dutch Society for Nutrition and Food Technology in October 1996. In December 1996 she participated in the international course 'Ecophysiology of the gastrointestinal tract', organised by the research school VLAG. She was elected to participate in the fourth European Nutrition Leadership Programme in Luxembourg in March 1997.

Curriculum vitae

Marie-Louise Heijnen werd op 8 december 1966 geboren in Roosendaal. In 1985 behaalde ze haar gymnasium-β diploma aan het Norbertuscollege te Roosendaal en begon ze met de studie Voeding van de Mens aan de Landbouwuniversiteit in Wageningen (LUW). Als onderdeel van deze studie voerde ze onderzoeksprojecten uit op het gebied van de humane fysiologie (vakgroep Fysiologie van Mens en Dier, LUW), klinische voeding (Universiteits Ziekenhuis Gent, België) en humane voeding (vakgroep Humane Voeding, LUW; vakgroep Humane Voeding, Universiteit van Helsinki, Finland; Unilever Research Laboratorium, Vlaardingen). In juni 1991 ontving ze haar bul met lof.

Van januari 1992 tot en met augustus 1996 was ze aangesteld als assistent-in-opleiding (AIO) op de vakgroep Humane Voeding, LUW. Tijdens deze aanstelling voerde ze het in dit proefschrift beschreven onderzoek uit. Ze nam deel aan EURESTA, een door de Europese Commissie financieel gesteund samenwerkingsverband van veertig Europese onderzoeksgroepen, die de fysiologische effecten van consumptie van resistant starch voor de mens bestudeerden. In juli 1994 volgde ze gedurende twee weken internationale cursussen op het gebied van statistiek en epidemiologie aan de Tufts universiteit in Boston, USA. Ze was mede-organisator van het symposium 'The editors speak out' ter gelegenheid van het 25-jarige jubileum van de vakgroep Humane Voeding. Verder was ze bestuurslid van het WAIOO, een belangenorganisatie voor Wageningse AIO's en OIO's, en lid van de AIO-raad van de onderzoekschool VLAG en het vakgroepsbestuur.

Ze was één van de organisatoren van een workshop over voedingsvezel voor leden van de Nederlandse Vereniging voor Voeding en Levensmiddelentechnologie (NVVL) in oktober 1996. In december 1996 nam ze deel aan de vijfdaagse internationale cursus 'Ecofysiologie van het maagdarmkanaal', georganiseerd door de onderzoekschool VLAG. Ze werd uitgekozen om deel te nemen aan het vierde European Nutrition Leadership Programme in Luxemburg in maart 1997.

Het is eindelijk af: zjoepie de poekie!