Influence of dietary retrograded starch on the metabolism of neutral steroids and bile acids in rats

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Diets enriched in retrograded amylose (RS_3) have been shown to lower serum cholesterol concentrations in rats. The possibility was tested that this hypocholesterolaemic effect of RS_3 is caused by an increase in excretion of neutral steroids and/or bile acids. Six groups of ten rats were fed on purified diets containing either 12 or 140 g RS_3/kg solid ingredients with and without added cholesterol (5 g/kg). Low-RS_3 diets, with and without added cholesterol, to which the bile-acid-binding resin cholestyramine (20 g/kg) was added, were used as reference. The high-RS_3 diets *v.* the low-RS_3 diets tended to reduce the increase in the total serum cholesterol concentration during the course of the experiment ($P = 0{\cdot}067$), decreased serum triacylglycerol concentrations, raised total neutral steroids and total bile acids in caecal contents and faecal excretion of total bile acids, but lowered faecal excretion of neutral steroids. In addition, the serum concentration of total 3α-bile acids was markedly raised by the high-RS_3 diets. The high-RS_3 diets raised the faecal excretion of lithocholic and muricholic acids, but lowered that of hyodeoxycholic acid, and increased the caecal amounts of lithocholic, ursodeoxycholic, β-muricholic and ω-muricholic acids. Apart from the stimulation of faecal bile acids excretion, the effects of cholestyramine on bile acid metabolism differed at various points from those of RS_3. Cholesterol feeding had predictable effects on cholesterol metabolism and led to greater elevating effects of RS_3 on the faecal and caecal amounts of muricholic acids. The results suggest that the serum-cholesterol-lowering effect of high-RS_3 diets may be explained by an increased influx of neutral steroids and bile acids into the caecum, and increased faecal excretion of bile acids, and/or by an altered intestinal bile acid profile.

Resistant starch: Serum cholesterol: Bile acids

The expression dietary resistant starch (RS) refers to the fraction of starch that is not absorbed in the small intestine. Three types of RS can be distinguished: physically inaccessible starch (RS_1), resistant starch granules (RS_2) and retrograded amylose (RS_3) (Englyst *et al.* 1992). RS_3 is formed during the cooling of gelatinized, high-amylose starch and consists of small aggregates of hydrogen-bonded amylose. In the rat, both RS_2 and RS_3 were found to be resistant to digestion in the small intestine. RS_3 might be fermented by the caecal and colonic flora to a lesser extent than RS_2 (Schulz *et al.* 1993).

Previous studies in rats demonstrated that feeding of RS_3 instead of digestible starch lowered serum cholesterol concentrations (De Deckere *et al.* 1992, 1993). There is evidence that the hypocholesterolaemic effect of dietary RS_3 may also occur in humans (Behall *et al.* 1989). The metabolic basis for the cholesterol-lowering activity of RS_3 is unknown. Analogously to the way dietary soluble fibres affect cholesterol metabolism (Beynen &

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West, 1989), RS_3 may increase faecal excretion of neutral steroids and/or bile acids, thereby causing a drain on the whole-body cholesterol pool which in turn may lead to a fall in the serum cholesterol concentration. In addition, if RS_3 interacts with specific neutral steroids and/or bile acids, the composition of caecal and faecal steroids may be altered. In an attempt to unravel the mechanism by which dietary RS_3 influences cholesterol metabolism, we studied the effect of RS_3 ingestion *v.* that of digestible starch on serum and liver cholesterol concentrations, and on the amounts of neutral steroids and bile acids in the caecal contents and faeces of rats. Diets with and without added cholesterol were fed. Diets with added cholesterol were fed because effects of various dietary components on serum cholesterol concentrations in rats are generally amplified by simultaneous cholesterol loading (Beynen & West, 1989). Two additional groups of rats were given either a cholesterol-free or a cholesterol-rich diet containing cholestyramine and served as reference groups, because cholestyramine affects cholesterol metabolism by raising faecal bile acid excretion (Beynen *et al.* 1988).

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Experiments Committee of Unilever Research Laboratorium, Vlaardingen.

Animals, housing and diets

Sixty-five male, SPF Wistar rats (WU-strain) aged 9–10 weeks and weighing on average 275 g were purchased from Harlan/Cpb (Zeist, The Netherlands). They were housed individually, with free access to tap water, in cages with wire-mesh bottoms in an air-conditioned room (22–24°; relative humidity approximately 55%) with a fixed day–night rhythm (light on from 07.00 to 19.00 hours). After arrival the rats were acclimatized for 1 week in which they had free access to food. Next, they were accustomed to a meal-feeding regimen with free access to food from 07.00 to 07.30 hours and from 19.00 to 19.30 hours for 2 weeks. In these weeks the rats were fed on a commercial, pelleted natural-ingredient diet (R/M1(E)SQC, Special Diets Services, Witham, Essex). At the beginning of the third week body weights and serum total cholesterol and triacylglycerol concentrations were determined. At the start of the fourth week (day 0 in the experiment) the animals were allocated to six dietary groups of ten rats each (randomized design) with similar means of body weight, serum total cholesterol and triacylglycerol concentrations (see Tables 2 and 4). The experimental, purified diets (Table 1) were either cholesterol-poor or contained 5 g cholesterol/kg solid ingredients. Gelatinized starch was present in the form of starch preparations with either a low RS_3 (20 g/kg total starch) or high RS_3 content (230 g/kg total starch). Two diets, one with and the other without added cholesterol, contained 20 g cholestyramine/kg solid ingredients. Energy density of the experimental diets was 5·23 kJ/g on a wet basis and 15·11 kJ/g on the basis of solid ingredients.

Collection of samples

To determine cholesterol and triacylglycerol, tail blood samples were taken between 06.00 and 07.00 hours before the morning meal on both day −6 and day 21 of the experiment. Faeces of each rat were collected quantitatively during days 15–17. On days 21–24, between 09.00 and 10.00 hours, two or three rats of each dietary group were anaesthetized by exposure to diethyl ether in random order. Blood samples were obtained from the abdominal aorta for determination of serum 3α-bile acids. Caecums were removed and weighed. Caecal contents were collected, and weights and pH recorded. The livers were excised and stored at −20° until analysis. The rats were killed in small groups within 1 h in order to restrict the variation in the variables due to postprandial effects.

Table 1. *Composition of the experimental diets (g)*

Ingredients	High RS_3	High RS_3 + cholesterol	Low RS_3	Low RS_3 + cholesterol	Low RS_3 + cholestyramine	Low RS_3 + cholesterol + cholestyramine
Lard*	5·1	4·8	5·1	4·8	5·1	4·8
Starch, high RS_3†	40·4	40·4	—	—	—	—
Starch, low RS_3†	—	—	40·4	40·4	40·4	40·4
Cholesterol‡	—	0·33	—	0·33	—	0·33
Questran§	—	—	—	—	2·9	2·9
Sucrose	1·2	1·2	1·2	1·2	1·2§	1·2§
Constant components‖	144·46	144·46	144·46	144·46	144·46	144·46

RS_3, retrograded amylose.

* Smilde B.V., Heerenveen, The Netherlands; the preparation contained 600 mg cholesterol/kg.

† The starch with high RS_3 content was a modified maize starch (Ultra-set LT, National Starch and Chemical Company, Zutphen, The Netherlands) containing approximately 400 g amylose (of which 230 g RS_3)/kg starch. The starch with low RS_3 content was a common maize starch (Meritena A, N.V. Honig's Artikelen, Koog a/d Zaan, The Netherlands) containing 250 g amylose (of which 20 g RS_3)/kg starch. The starches were gelatinized with tap water (starch–water, 1:3, w/v) at 95° for 45 min (Ultra-Set) or 70° for 5 min (Meritena) under stirring, cooled down at room temperature and stored at −20° for 1–6 d.

‡ Merck, Darmstadt, Germany. Lard was exchanged for cholesterol.

§ Questran® (Bristol-Meyers, Woerden, The Netherlands) contained 444 g cholestyramine, 422 g sucrose and 134 g flavouring agents/kg. This amount of Questran gave 1·3 g cholestyramine and 1·2 g sucrose in the diet.

‖ Constant components consisted of (g): sunflower-seed oil (Union N.V., Merksem, Belgium), 0·2, casein (D.M.V., Veghel, The Netherlands) 14·6, mineral mixture 0·86, vitamin mixture 0·2, cellulose (Machery-Nagel GmbH, Dueren-Roelsdorg, Germany) 3·6, water 125. The compositions of the mineral and vitamin mixtures have been described earlier (De Deckere *et al.* 1993).

Chemical analyses

Resistant starch in the starch preparations used to prepare the diets was determined by the method of Berry (1986). Total cholesterol in serum was measured using a commercial test combination (CHOD-PAP, C-system, Boehringer, Mannheim, Germany). Total and free glycerol in serum were determined enzymically (GPO-PAP method, Roche Diagnostics, Basel, Switzerland and GPO-Trinder method, Sigma Chemical Co., St Louis, MO, USA). Serum triacylglycerol values were calculated as the difference between total and free glycerol concentrations. Total 3α-bile acids in serum were measured enzymically using a commercial test kit (Enzabile®, Nycomed Pharma AS, Oslo, Norway). The livers were homogenized in distilled water and samples were extracted and analysed for total cholesterol as described by Abell *et al.* (1952).

For analysis of neutral steroids and bile acids, faeces and caecal contents were freeze-dried and homogenized. Neutral steroids were determined in individual samples, but for bile acids analyses faeces and caecal contents were sampled proportionally to the total dry weight collected per rat and the samples pooled for each dietary group, which resulted in a value per dietary group equivalent to the mean of the ten individual rat values for the excretion of faecal total bile acids (μmol/d) and caecal total bile acids (μmol) only. In a glass tube 0·15 μmol 5α-cholestane (internal standard for neutral steroids), 0·6 μmol 7α,12α,-dihydroxy-5β-cholanic acid (internal standard for bile acids), 150 mg freeze-dried faeces or caecal contents, 2 ml methanol, and 0·65 ml 5 M-NaOH were mixed and saponified for 2 h at 80°. Neutral steroids were extracted three times with 3 ml petroleum ether (boiling range, 60–80°), the extracts were centrifuged for 5 min at 3000 ***g***, and the solvent was evaporated under a stream of N_2. The neutral steroids were converted into volatile trimethylsilyl ether derivatives by addition of 0·24 ml freshly prepared silylating agent (N,N-dimethylformamide–bis-silyl-trifluoracetamide, 2:1, by vol.) and heated at 80° for 1 h. After extraction of the neutral steroids the aqueous phase was acidified to pH 1 with HCl. Bile acids were extracted three times with 5 ml freshly distilled ether, and the extract was shaken and centrifuged for 5 min at 3000 ***g***. A portion of the extract was dried under N_2, and the bile acids were methylated by addition of a mixture of 2,2-dimethoxypropane–methanol–12 M-HCl (1:1:0·1, by vol.). After standing overnight at room temperature the solvent was evaporated under N_2, and trimethylsilyl ether derivatives were formed by incubating with a mixture of pyridine–bis-silyl-trifluoracetamide–trimethylchlorosilane (1:1:0·2, by vol.) for 1 h at 80°. After derivatization the solvent was evaporated under N_2, and the residue dissolved in hexane. Portions (1 μl) of the trimethylsilyl ether derivative solutions were injected into a gas chromatograph (Carlo Erba Model Mega 5160, Carlo Erba, Milan, Italy), which was equipped with a 25 m × 0·25 mm (inner diameter) fused silica capillary column (Chrompack, Middelburg, The Netherlands) and a flame-ionization detector. Neutral steroids were analysed on a CP Sil 5 CB column, and bile acids on a CP Sil 19 CB. H_2 was used as a carrier gas at a flow rate of 1·8 ml/min. Temperature programming was used under the following conditions: neutral steroids, 200° for 0·3 min, 200 to 240° in 4 min, isothermal for 20 min, 240 to 285° in 2·25 min, followed by 20 min at 285°; bile acids, 200° for 3 min, 200 to 275° in 7·5 min, followed by 15 min at 275°, 275 to 290° in 1·5 min, and 13 min isothermally at 290°. Steroids were calculated from the peak areas relative to the peak area of the internal standards. Identification of the major neutral steroids and bile acids was accomplished by matching their retention times with those of known standards of high purity. Differences in detector response among the various steroids were corrected on the basis of the response factors calculated from a mixture of pure steroids with known molar composition.

Table 2. *Body weight, feed intake and faeces production of rats fed on experimental diets containing resistant starch or cholestyramine**

(Mean values with their pooled standard error for ten rats per dietary group)

	Body weight (g)		Feed intake (g/d)	Faeces	
	Initial	Final		Production (g/d)	Dry matter content (g/kg)
Dietary treatment					
High RS_3	288	368	49·4	2·6	714
High RS_3 + cholesterol	287	367	48·9	2·9	668
Low RS_3	289	360	50·4	1·6	811
Low RS_3 + cholesterol	288	379	50·6	1·7	826
Low RS_3 + cholestyramine	284	365	52·4	3·1	677
Low RS_3 + cholestyramine + cholesterol	284	357	52·9	2·8	783
Analysis of variance					
Pooled SEM	6·0	9·2	1·6	0·2	24
Effects (*P* values)					
RS_3	—	0·866	0·339	< 0·001	< 0·001
Cholesterol	—	0·626	0·965	0·791	0·197
Cholestyramine	—	0·358	0·199	< 0·001	< 0·001
RS_3 × cholesterol	—	0·264	0·820	0·614	0·205
Cholesterol × cholestyramine	—	0·141	0·949	0·365	0·059

RS_3, retrograded amylose.

* For details of diets and procedures, see Table 1 and pp. 808–811.

Statistical analysis

Data, except those of bile acids in faeces and caecal contents, were examined by analysis of variance with diet as factor followed by evaluation using specific contrasts (Statistical Analysis System, SAS Institute Inc., 1987). The contrasts were high RS_3 *v.* low RS_3, cholesterol *v.* no added cholesterol, cholestyramine *v.* no cholestyramine in the diet, and the interactions between RS_3 and cholesterol, and between cholesterol and cholestyramine. Pooled SEM, being root mean square errors divided by $\sqrt{n}$, are presented, except for neutral steroids.

Data for individual and total neutral steroids were transformed to their natural logarithms. Geometric means are presented, and pooled variation coefficient of the mean (CVM) is given as $\sqrt{(s^2_{ln}/10)} \times 100\,\%$, where s^2_{ln} is the residual mean square in the logarithmic scale.

Analyses of total bile acids in faeces and caecal contents were performed only. Our experimental design, type of rats and analytical methods were very similar to those in experiments carried out previously (Beynen *et al.* 1984). Therefore, the number of degrees of freedom (10) and the variation coefficients for the excretion of bile acids were taken from these experiments. The mean variation coefficient was taken conservatively to be 0·30 for the faecal and caecal total bile acids. This procedure of estimating variation coefficients for pooled samples has been employed earlier by Lovati *et al.* (1990). For the groups without cholestyramine, approximate significance probability levels for the RS_3 and cholesterol effects were calculated by taking the square of the coefficient of variation (as a fraction) to be the residual mean square in the analysis of variance in the logarithmic scale and using the logarithms of the pooled sample values weighted by 10 (the number of rats contributing to the pool).

RESULTS

Body weight, feed intake and faeces production

Table 2 shows that body weight and feed intake were not affected by the dietary variables. Faeces production was significantly greater in the high-RS_3 groups and the cholestyramine groups than in the low-RS_3 groups. RS_3 and cholestyramine in the diet led to a reduced percentage of dry matter in the faeces.

Caecal characteristics

The weights of caecal tissue and contents were significantly higher in rats fed on the high-RS_3 diets than in rats fed on the low-RS_3 diets (Table 3). Cholestyramine in the diet also led to higher caecal weights. The high-RS_3 diets, in comparison with the low-RS_3 diets, reduced the percentage of dry matter in the caecal contents, whereas the cholestyramine-containing diets increased it. Caecal pH was lower in rats fed on the high-RS_3 diets than in rats fed on the low-RS_3 diets.

Serum cholesterol and triacylglycerols, and liver cholesterol

In the experimental period the serum total cholesterol and triacylgycerol concentrations increased with respect to the initial values (Table 4). Irrespective of the amount of cholesterol in the diet, high-RS_3 intake *v.* low-RS_3 intake tended to diminish the increase in the serum total cholesterol concentration. Cholesterol feeding did not significantly affect the serum total cholesterol concentration, and cholestyramine significantly raised the increase in the serum cholesterol concentration.

The high-RS_3 diets, in comparison with the low-RS_3 diets, led to significantly lower serum triacylglycerol concentrations (Table 4), whereas dietary cholesterol and cholestyramine did not significantly influence the concentrations.

Dietary RS_3 tended ($P = 0{\cdot}063$) to reduce the relative liver weight (Table 4). Cholesterol feeding raised the liver weight, and cholestyramine consumption antagonized this effect. The amount of RS_3 in the diet did not affect the liver cholesterol concentration. The high-cholesterol diets led to twofold higher hepatic cholesterol levels than did the cholesterol-free diet. However, this increase in liver cholesterol was prevented by cholestyramine in the diet.

Faecal and caecal total neutral steroids and serum 3α-bile acids

The high-RS_3 diet, in comparison with the low-RS_3 diet, reduced both the excretion (μmol/d) and the concentration of total neutral steroids (μmol/g dry faeces) in the faeces (Table 5). Dietary cholesterol and cholestyramine raised the faecal excretion of total neutral steroids, and a strong positive interaction between the effects of cholesterol and cholestyramine was found, but not between the effects of cholesterol and RS_3.

The high-RS_3 diet, in comparison with the low-RS_3 diet, increased the amount of total neutral steroids in the caecal contents, and decreased the concentration due to the increase in the contents (Table 5). Cholesterol and cholestyramine in the diet also raised the amount of total neutral steroids in the caecum, but only cholesterol raised the concentration.

The high-RS_3 diets *v.* the low-RS_3 diets raised the faecal excretion of the total bile acids, but did not affect the faecal concentration (Table 5). Both dietary cholesterol and cholestyramine increased the faecal excretion of bile acids. The high-RS_3 diet also led to an increase in the amount of the caecal total bile acids, but the concentration in the caecum was unchanged. Cholesterol and cholestyramine increased the total amounts and concentrations of bile acids in the caecum.

The serum total 3α-bile acids concentration was significantly raised by about 70% after feeding the high-RS_3 diets, in comparison with the low-RS_3 diets (Table 5). The addition

Table 3. *Caecal characteristics of rats fed on experimental diets containing resistant starch or cholestyramine**

(Mean values with their pooled standard error for ten rats per dietary group)

	Caecum			
	Tissue, wet wt (g)	Contents, wet wt (g)	Contents, dry matter (g/kg)	Contents, pH
Dietary treatment				
High RS_3	1·24	4·83	172·3	7·40
High RS_3 + cholesterol	1·27	5·18	169·2	6·91
Low RS_3	0·62	1·73	220·1	7·95
Low RS_3 + cholesterol	0·63	1·82	222·7	7·99
Low RS_3 + cholestyramine	0·72	2·39	242·9	7·70
Low RS_3 + cholestyramine + cholesterol	0·76	2·80	248·5	7·75
Analysis of variance				
Pooled SEM	0·05	0·28	6·3	0·15
Effects (*P* values)				
RS_3	< 0·001	< 0·001	< 0·001	< 0·001
Cholesterol	0·502	0·234	0·742	0·302
Cholestyramine	0·023	0·005	< 0·001	0·117
RS_3 × cholesterol	0·844	0·643	0·646	0·091
Cholesterol × cholestyramine	0·786	0·574	0·818	0·987

RS_3, retrograded amylose.

* For details of diets and procedures, see Table 1 and pp. 808–811.

of cholestyramine to the diets reduced the concentration of the serum 3α-bile acids by about 30%, and dietary cholesterol did not influence it.

Profiles of faecal and caecal neutral steroids

The high-RS_3 diets *v.* the low-RS_3 diets altered the profile of the faecal neutral steroids: the amount of coprostanol dropped and that of β-cholestanol rose (Table 6). However, the latter was a minor component. The high-RS_3 diets tended to raise the faecal excretion of cholesterol. Cholesterol and cholestyramine raised the faecal excretion of the three neutral steroids.

The high-RS_3 diets *v.* the low-RS_3 diets decreased coprostanol and increased cholesterol in the caecal contents, whereas cholesterol and cholestyramine increased the neutral steroids (Table 6).

Profiles of faecal and caecal bile acids

The high-RS_3 diets, compared with the low-RS_3 diets, raised the faecal excretion of lithocholic acid, and that of α-, β- and ω-muricholic acids, but lowered the excretion of hyodeoxycholic acid (Table 7). Dietary cholesterol generally raised the faecal excretion of all bile acids. Apart from α-muricholic acid, the faecal excretion of bile acids was markedly enhanced by dietary cholestyramine.

The amounts of chenodeoxycholic, lithocholic, ursodeoxycholic, β-muricholic and ω-muricholic acid in the caecum were elevated by the high-RS_3 diets *v.* the low-RS_3 diets (Table 7). Cholestyramine feeding lowered the caecal amounts of β- and ω-muricholic acids, but drastically raised those of the other bile acids. Depending on the composition of the diet, the feeding of cholesterol either raised or did not influence the caecal levels of the individual bile acids.

Table 4. *Serum and liver cholesterol and serum triacylglycerol concentrations of rats fed on the experimental diets containing resistant starch or cholestyramine**

(Mean values with their pooled standard errors for ten rats per dietary group)

	Serum total cholesterol			Serum triacylglycerols			Liver	
	Initial (mmol/l)	Final (mmol/l)	Change (mmol/l)	Initial (mmol/l)	Final (mmol/l)	Change (mmol/l)	Relative weight (g/kg body wt)	Cholesterol (μmol/g)
Dietary treatment								
High RS_3	2·40	2·52	+0·12	0·82	0·96	+0·14	31·5	5·14
High RS_3 + cholesterol	2·44	2·66	+0·22	0·83	1·25	+0·42	34·0	10·53
Low RS_3	2·47	2·71	+0·24	0·85	1·47	+0·62	32·6	4·91
Low RS_3 + cholesterol	2·36	2·81	+0·45	0·88	1·58	+0·70	35·2	10·24
Low RS_3 + cholestyramine	2·42	2·94	+0·52	0·79	1·27	+0·48	32·5	5·64
Low RS_3 + cholestyramine + cholesterol	2·39	2·97	+0·58	0·86	1·45	+0·59	3·0	5·42
Analysis of variance								
Pooled SEM	0·11	0·11	0·09	0·10	0·17	0·16	0·6	0·69
Effects (P values)								
RS_3	—	0·128	0·067	—	0·019	0·023	0·063	0·705
Cholesterol	—	0·319	0·109	—	0·181	0·242	0·001	0·001
Cholestyramine	—	0·089	0·037	—	0·345	0·443	0·053	0·005
RS_3 × cholesterol	—	0·837	0·527	—	0·601	0·540	0·921	0·971
Cholesterol × cholestyramine	—	0·778	0·430	—	0·848	0·928	0·089	0·001

RS_3, retrograded amylose.

* For details of diets and procedures, see Table 1 and pp. 808–811.

Table 5. *Faecal and caecal total steroids and serum 3α-bile acid concentration of rats fed on experimental diets containing resistant starch or cholestyramine**

(Values for total faecal and caecal neutral steroids (including coprostanone) are geometric means (*n* 10) and their pooled coefficients of variation (CVM). Values for bile acids, except those of serum 3α-bile acids which are means (*n* 10), are single values determined in pooled samples and were also expressed g/g dry weight of pooled samples for comparison with the data of the neutral steroids. (The within-group variation of the dry weight values of the faeces and the caecal contents was not more than a factor of 2.))

	Faecal total neutral steroids		Caecal total neutral steroids		Faecal total bile acids		Caecal total bile acids		Serum 3α-bile acids (μmol/l)
	(μmol/d)	(μmol/g dry faeces)	(μmol)	(μmol/g dry contents)	(μmol/d)	(μmol/g dry faeces)	(μmol)	(μmol/g dry contents)	
Dietary treatment									
High RS_3	11·07	6·42	4·91	6·21	23·85	13·29	17·99	21·31	10·77
High RS_3 + cholesterol	29·17	15·66	17·13	20·55	50·34	26·61	33·25	38·72	10·64
Low RS_3	14·26	11·51	3·26	8·96	17·01	13·55	8·33	21·99	6·27
Low RS_3 + cholesterol	38·43	28·13	15·56	39·50	37·20	27·06	18·46	45·78	6·16
Low RS_3 + cholestyramine	24·45	12·01	5·01	8·73	147·77	71·83	41·58	70·97	3·68
Low RS_3 + cholestyramine + cholesterol	156·30	71·10	27·25	40·05	178·22	80·69	46·76	67·47	4·65
Analysis of variance									
Pooled CVM (%)	9·7	8·3	11·3	8·6	—	—	—	—	—
Pooled SEM	—	—	—	—	—	—	—	—	1·08
Effects (*P* values)									
RS_3	0·009	< 0·001	0·030	< 0·001	0·007†	—	< 0·001†	—	< 0·001
Cholesterol	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001†	—	< 0·001†	—	0·783
Cholestyramine	< 0·001	< 0·001	< 0·001	0·944	—	—	—	—	0·063
RS_3 × cholesterol	0·907	0·993	0·173	0·103	0·859†	—	0·362†	—	0·989
Cholesterol × cholestyramine	< 0·001	< 0·001	0·559	0·818	—	—	—	—	0·619

RS_3, retrograded amylose.
* For details of diets and procedures, see Table 1 and pp. 808–811.
† Approximate *P* values.

Table 6. *Profiles of faecal and caecal neutral steroids of rats fed on experimental diets containing resistant starch or cholestyramine**†

(Geometric means with their pooled coefficients of variation (CVM) for ten rats per dietary group)

	Faeces (μmol/d)			Caecum (μmol)		
	Coprostanol	Cholesterol	β-Cholestanol	Coprostanol	Cholesterol	β-Cholestanol
Dietary treatment						
High RS_3	4·72	4·74	0·57	1·76	2·14	0·23
High RS_3 + cholesterol	3·07	20·02	0·57	1·42	11·84	0·41
Low RS_3	8·95	4·06	0·28	1·82	1·10	0·07
Low RS_3 + cholesterol	21·56	14·96	0·42	5·68	7·52	0·32
Low RS_3 + cholestyramine	16·58	6·73	0·78	2·75	1·80	0·14
Low RS_3 + cholestyramine + cholesterol	72·50	71·53	1·60	11·52	14·06	0·26
Analysis of variance						
Pooled CVM (%)	25·5	12·8	10·4	27·2	13·6	40·6
Effects (*P* values)						
RS_3	< 0·001	0·087	< 0·001	0·011	< 0·001	0·098
Cholesterol	0·003	< 0·001	< 0·001	< 0·001	< 0·001	0·009
Cholestyramine	< 0·001	< 0·001	< 0·001	0·044	< 0·001	0·601
RS_3 × cholesterol	0·013	0·598	0·055	0·016	0·427	0·262
Cholesterol × cholestyramine	0·248	< 0·001	0·142	0·588	0·621	0·305

RS_3, retrograded amylose.

* For details of diets and procedures, see Table 1 and pp. 808–811.

† Coprostanone amounts were low and in a number of samples not detectable. For this reason their geometric means were not given.

Table 7. *Profiles of faecal and caecal bile acids of rats fed on experimental diets containing resistant starch or cholestyramine**

(Single values determined in pooled samples of ten rats per dietary group)

Dietary treatment	DC	C	CDC	LC	HDC	UDC	α-MC	β-MC	ω-MC
Faecal bile acids (μmol/d)									
High RS_3	5·27	0·17	0·38	5·43	2·62	1·39	1·36	1·36	5·87
High RS_3 + cholesterol	9·51	0·71	0·27	9·06	6·57	2·19	5·37	6·89	9·76
Low RS_3	4·52	0·16	0·30	3·56	5·05	1·09	0·31	0·35	1·67
Low RS_3 + cholesterol	8·24	0·38	0·59	7·24	16·43	1·82	0·33	0·48	1·60
Low RS_3 + cholestyramine	71·23	1·82	1·93	51·30	14·40	2·09	n.d.	1·86	3·15
Low RS_3 + cholestyramine + cholesterol	81·06	1·45	2·80	65·06	20·81	2·27	n.d.	1·94	2·84
Caecal bile acids (μmol)									
High RS_3	6·74	0·38	0·16	2·58	2·85	0·96	0·78	0·56	2·98
High RS_3 + cholesterol	5·92	1·44	0·67	4·14	2·83	1·34	4·61	5·86	6·42
Low RS_3	2·66	0·38	0·04	1·10	2·39	0·52	n.d.	0·35	0·88
Low RS_3 + cholesterol	5·70	0·95	n.d.	2·20	6·15	0·74	n.d.	1·17	1·56
Low RS_3 + cholestyramine	21·58	0·51	1·02	13·92	2·86	0·76	n.d.	0·24	0·68
Low RS_3 + cholestyramine + cholesterol	23·05	n.d.	0·73	16·75	4·61	0·77	n.d.	0·31	0·54

DC, iso-, and deoxycholic acid; C, cholic acid; CDC, chenodeoxycholic acid; LC, iso-, and lithocholic acid, 12-keto-iso, and 12-keto-lithocholic acid; HDC, hyodeoxycholic acid; UDC, ursodeoxycholic acid; MC, muricholic acid; n.d., not detectable; RS_3, retrograded amylose.

* For details of diets and procedures, see Table 1 and pp. 808–811.

DISCUSSION

Feeding of diets containing a great amount of RS_3, in comparison with feeding of diets containing a low amount of RS_3, tended to reduce the mean serum cholesterol concentration (reduction by 6%). The effect of RS_3 was more pronounced for the increase in the serum cholesterol concentration due to the switch from the non-purified diet to the purified diet. In two earlier experiments with eight rats per group the hypocholesterolaemic effects of comparable high-RS_3 diets were 8 and 15% (De Deckere *et al.* 1992, 1993). Thus, high-RS_3 diets consistently have a small lowering effect on the serum cholesterol concentration which in the present study just failed to reach statistical significance.

The experimental diets also increased the serum triacylglycerol concentration in comparison with the non-purified diet. The effect was significantly lower for the high-RS_3 diets than for the low-RS_3 diets which may be explained either by a lower energy intake due to RS_3 (De Deckere *et al.* 1995), or by the lower amount of digestible starch in the high-RS_3 diet. Diets high in digestible carbohydrates generally raise the serum triacylglycerol concentration in rats (Herman *et al.* 1991; Zhang & Beynen, 1993).

The objective of the present study was to investigate whether the hypocholesterolaemic effect of dietary RS_3, as found in previous studies (De Deckere *et al.* 1992, 1993), is associated with changes in neutral steroid and bile acid metabolism. RS_3 lowered the faecal excretion of total neutral steroids, but raised that of bile acids. A similar effect of uncooked amylomaize starch on bile acid secretion was found by Sacquet *et al.* (1983). RS_3 raised the total amount of both neutral steroids and bile acids in the caecum, which suggests that the amounts of neutral steroids and bile acids in the digesta in the terminal ileum were increased or that intestinal transit time was decreased (Iwata *et al.* 1992). However, total gastrointestinal transit time has been found to be the same for a high-amylose diet *v.* a cooked potato-starch diet (Lajvardi *et al.* 1993). Binding of bile acids by RS_3 in the small intestine might have occurred because starch has been shown to bind bile acids (Bianchini *et al.* 1989). Although the nature of the interaction between RS_3 and bile acids in the small intestine is unknown, the hypocholesterolaemic effect of RS_3 as found in previous studies (De Deckere *et al.* 1992, 1993) might be explained by stimulation of bile acid excretion as found in the present study, leading to an increase in hepatic bile acid synthesis which in turn causes a drain on the body pool of cholesterol, which is the precursor of bile acids. The result is a decrease in the serum cholesterol concentration. The mechanism proposed here is identical to that underlying the hypocholesterolaemic effect in man of the bile acid-binding resin cholestyramine by direct ionic binding of bile acids (Grundy, 1986). In the rat, however, it has been reported that cholestyramine increases faecal excretion of bile acids without affecting serum cholesterol (Beynen & West, 1989). In the present study the rise in the serum total cholesterol concentration induced by the purified diets was greater for the diets with cholestyramine than for the diets without cholestyramine. This may be caused by the reported dramatic increase in hepatic cholesterol synthesis in rats fed with cholestyramine (Spady *et al.* 1985). However, cholestyramine did prevent the rise in liver cholesterol seen after cholesterol feeding. Cholestyramine also produced an increase in the faecal excretion of neutral steroids which might be explained by interference with fat absorption, including cholesterol absorption, due to reduced formation of mixed micelles.

The total amount of neutral sterols in the caecal contents of the rats fed on the high-RS_3 diet was greater than that of the rats fed on the low-RS_3 diet, whereas the faecal excretion of total neutral steroids was the opposite. Conversion of neutral steroids into non-detectable compounds or uptake of neutral steroids in the caecum (Molina *et al.* 1990) might have been greater in the rats fed on the high-RS_3 diets than in the rats fed on the low-RS_3 diets.

The high-RS_3 diets led to an increase in the concentration of serum total 3α-bile acids, whereas cholestyramine lowered this concentration. Cholestyramine effectively binds bile acids in the intestinal lumen and thus depresses their reabsorption, leading to a decrease in the concentration of serum total 3α-bile acids. RS_3 might also bind bile acids in the gut, but this effect was associated with an increase in the concentration of serum 3α-bile acids. The different effect of cholestyramine and RS_3 on the serum 3α-bile acids concentration may be due either to the difference in the extent of bile acid excretion or to the different caecal and faecal bile acid profiles induced by these compounds. Unlike cholestyramine, RS_3 specifically raised the amounts of lithocholic and muricholic acids. Perhaps the altered profile of intestinal bile acids in rats fed on a diet rich in RS_3 affects the enterohepatic circulation in such a way that the serum pool is enlarged.

Feeding of the high-RS_3 diet *v.* the low-RS_3 diet lowered the pH of the caecal contents, which may be associated with the differences in the amounts of cholesterol and bile acids. RS_3 in the diet raised cholesterol and decreased coprostanol in the caecum indicating that bacterial oxidation of cholesterol was depressed. This may be related to the lower pH value (Andrieux *et al.* 1989). The RS_3-induced alterations in the profiles of bile acids are more difficult to interpret because, apart from changes in bacterial transformation, there may also be differences in binding of the various bile acids. In addition, the increase in fermentation by RS_3 leading to a lower pH may reduce the solubility of bile acids. In any event, it can be concluded that in rats given high-RS_3 diets there was an enhanced hepatic formation of muricholic acids, a depressed bacterial formation of hyodeoxycholic acid, and an increased bacterial formation of ω-muricholic acid. The RS_3-induced change in the profile of intestinal bile acids could affect the enterohepatic circulation of total bile acids, depending on differences in the efficiency of reabsorption of individual bile acids.

Cholesterol feeding *per se* had predictable effects (Beynen *et al.* 1984): it raised the liver cholesterol concentration and the faecal excretion of total neutral steroids and bile acids. The results also suggest interactions between the effects of dietary cholesterol and RS_3 concerning the caecal and faecal amounts of muricholic acids. In the presence of cholesterol the feeding of RS_3 raised the amounts of muricholic acids to a greater extent. This may relate to cholesterol-induced stimulation of hepatic muricholic acid synthesis and competition between bile acids for binding to RS_3 in the small intestine.

In conclusion, the lower serum cholesterol concentration seen in rats after feeding of diets enriched in RS_3, in comparison with diets low in RS_3, may be explained, at least in part, by enhanced faecal excretion of bile acids as a result of an increase in influx of bile acids from the small intestine into the caecum (which was also found for the neutral steroids), and/or by altering the intestinal bile acid profile, so that the enterohepatic circulation of total bile acids is changed.

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