

## Mechanisms of cholesterol-lowering effects of dietary insoluble fibres: relationships with intestinal and hepatic cholesterol parameters

Ariëtte M. van Bennekum<sup>1</sup>, David V. Nguyen<sup>1</sup>, Georg Schulthess<sup>2</sup>, Helmut Hauser<sup>3</sup> and Michael C. Phillips<sup>1\*</sup>

<sup>1</sup>Department of GI/Nutrition, The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, 3615 Civic Center Blvd, Philadelphia, PA 19104-4318, USA

<sup>2</sup>Department of Internal Medicine, Medical Policlinic, University Hospital, CH-8091 Zurich, Switzerland

<sup>3</sup>Institute of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland

(Received 29 October 2004 – Revised 23 March 2005 – Accepted 5 April 2005)

Fibres with a range of abilities to perturb cholesterol homeostasis were used to investigate how the serum cholesterol-lowering effects of insoluble dietary fibres are related to parameters of intestinal cholesterol absorption and hepatic cholesterol homeostasis in mice. Cholestyramine, chitosan and cellulose were used as examples of fibres with high, intermediate and low bile acid-binding capacities, respectively. The serum cholesterol levels in a control group of mice fed a high fat/high cholesterol (HFHC) diet for 3 weeks increased about 2-fold to 4.3 mM and inclusion of any of these fibres at 7.5% of the diet prevented this increase from occurring. In addition, the amount of cholesterol accumulated in hepatic stores due to the HFHC diet was reduced by treatment with these fibres. The three kinds of fibres showed similar hypocholesterolaemic activity; however, cholesterol depletion of liver tissue was greatest with cholestyramine. The mechanisms underlying the cholesterol-lowering effect of cholestyramine were (1) decreased cholesterol (food) intake, (2) decreased cholesterol absorption efficiency, and (3) increased faecal bile acid and cholesterol excretion. The latter effects can be attributed to the high bile acid-binding capacity of cholestyramine. In contrast, incorporation of chitosan or cellulose in the diet reduced cholesterol (food) intake, but did not affect either intestinal cholesterol absorption or faecal sterol output. The present study provides strong evidence that above all satiation and satiety effects underlie the cholesterol-lowering properties of insoluble dietary fibres with moderate or low bile acid-binding capabilities.

### Dietary fibres: Cholesterol-lowering: Intestinal cholesterol absorption: Bile acid

Studies with animals and human subjects indicate that there is a considerable inter-individual variation in the rate of dietary cholesterol absorption in the small intestine (Grundy, 1983; Sehayek *et al.* 1998). Furthermore, intestinal cholesterol absorption efficiency is positively correlated with plasma cholesterol levels (Kesaniemi & Miettinen, 1987) and plasma cholesterol level is positively correlated with the incidence of CHD (Castelli, 1984). It follows that a reduction of cholesterol absorption in the small intestine should be beneficial in the prevention of CHD. In light of this, there has been great interest in the influence of food constituents, such as dietary fibres, on cholesterol absorption in the intestine.

Dietary fibres are non-digestible, water-soluble or water-insoluble carbohydrates that are present in the diet and are claimed to have beneficial effects in the lowering of serum cholesterol and the prevention of CHD (Story *et al.* 1997; Fernandez, 2001). The exact *in vivo* mechanisms for the lowering of serum and hepatic cholesterol levels by naturally occurring fibres are not well understood. On the other hand, the principal mechanism of action of the pharmacological agent, cholestyramine, is well known. This cationic polymer acts through the sequestration of bile acids in the intestine, resulting in an interruption of the enterohepatic circulation of bile acids and an up-regulation of bile acid synthesis (Casdorff, 1967). Moreover, the sequestration of bile acids is likely to perturb the process of intestinal cholesterol

absorption, and a decrease in cholesterol absorption efficiency by cholestyramine feeding has been established in various animal models, as well as in man (Gylling *et al.* 1989).

The aim of our studies was to use a mouse model to investigate the hypocholesterolaemic effects of insoluble dietary fibres. We examined three fibres that possess a range of abilities to perturb bile acid and sterol metabolism. The bile acid resin cholestyramine (a styrene–divinylbenzene copolymer containing quaternary ammonium groups) was included as a positive control, as it is a potent anion exchanger with a high bile acid-binding capacity. Chitosan was used because it has weak anion exchange properties; from *in vitro* studies, it was concluded that chitosan has about 50% of the bile acid-binding capacity of cholestyramine (Lee *et al.* 1999). Chitosan is the deacetylated form of chitin, which is *N*-acetylaminocellulose present in the exoskeleton of arthropods and is considered a fibre of animal origin (Furda, 1983). Cellulose (an unbranched polymer of  $\beta$ -1,4-linked glucose residues) is a common dietary fibre that is uncharged and has little or no bile acid-binding capacity (Vahouny *et al.* 1980*a,b*).

Our investigations focused on how the serum cholesterol-lowering effects of insoluble dietary fibres are related to parameters of intestinal cholesterol absorption, an aspect that has not received much attention yet, and hepatic cholesterol homeostasis.

**Abbreviation:** HFHC, high fat/high cholesterol.

\* **Corresponding author:** Dr Michael C. Phillips, fax +1 215 590 0583, email phillipsmi@email.chop.edu

## Experimental methods

### Animals and diets

Female C57BL/6 mice, aged 8–12 weeks, were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed three per cage in the Laboratory Animal Facility in a temperature- and humidity-controlled room with a 12 h light–dark cycle. They were fed a standard rodent chow (5P84 diet, North Penn Feeds, Hatfield, PA, USA) until the start of the dietary treatment. Subsequently, the mice were divided into four groups of six: the first group (control) was fed a high fat/high cholesterol (HFHC) diet containing no added fibre for 3 weeks while the other three groups were fed the same HFHC diet containing either 7.5% (w/w) cholestyramine, 7.5% chitosan (i.e. chitin which is minimally 85% deacetylated) or 7.5% cellulose. This dietary inclusion level of 7.5% (w/w) was chosen to ensure that chitosan would be effective in lowering serum cholesterol level without having any side-effects (Han *et al.* 1999). The HFHC diet (obtained from North Penn Feeds) consisted of Purina Mouse Chow, 5015; meal: 750 g/kg; casein, high protein: 75 g/kg; dextrose, monohydrate: 25 g/kg; sucrose: 16.25 g/kg; dextrin: 16.25 g/kg; cocoa butter: 75 g/kg; cholesterol: 12.5 g/kg; sodium cholate: 5 g/kg; cellulose (fibre): 12.5 g/kg; mineral mix, AIN-76: 8.75 g/kg; vitamin mix, AIN 76A(sub): 2.5 g/kg; choline chloride: 1.25 g/kg. The fibres were ground into the diet with a mortar and pestle and, after the addition of distilled water, new pellets were made and dried. Cholestyramine (cat. no. C-4650), chitosan (cat. no. C-3646) and cellulose (cat. no. C-6413) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Body weight (each mouse individually), food intake (averages of three mice) and the general appearance of the mice were recorded three times a week.

### Intestinal cholesterol absorption

At the end of the 3-week feeding period, animals were transferred to metabolic cages in the afternoon before the cholesterol uptake experiment. The following morning, the mice were fasted for 6 h, and 2 h before the start of the dark cycle they were gavaged 100  $\mu$ l corn oil containing 1  $\mu$ Ci [ $^{14}$ C]cholesterol ([4- $^{14}$ C]cholesterol, 45–60 mCi/mmol; NEN Life Science Products, Boston, MA, USA) and 0.4  $\mu$ Ci (8 pmol) [ $^3$ H] $\alpha$  $\beta$ -sitostanol ([5,6- $^3$ H] $\alpha$  $\beta$ -sitostanol 50 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO, USA). For this, aliquots of [ $^{14}$ C]cholesterol and [ $^3$ H- $\alpha$  $\beta$ ]sitostanol were dissolved in ethanol and mixed with corn oil, and the ethanol was subsequently removed by slowly bubbling N<sub>2</sub> through the solution for 3–4 h. Prior to use, the purity of the radiolabelled lipids was assessed by TLC and was at least 95% for [ $^3$ H] $\alpha$  $\beta$ -sitostanol and at least 98% for [ $^{14}$ C]cholesterol. After receiving the gavage, each mouse was returned to its metabolic cage where it immediately started to eat chow. Faeces were collected for 24 h periods, up to 72 h. Animals were anaesthetized using an intraperitoneal injection of a mixture of 150 mg ketamine plus 10 mg xylazine (Research Biomedical Instruments, Natick, MA, USA) per kg body weight. When killed, blood, gall bladder bile and liver were harvested for further analyses. Sera were obtained after clotting of the blood and centrifugation. All animal studies were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia.

### Analytical methods

Total collected faeces were weighed and homogenized in water (10%, w/v). Sterols were extracted from an aliquot of an appropriate dilution of the homogenate with an equal volume of chloroform–methanol (2:1, v/v), and [ $^{14}$ C]cholesterol and [ $^3$ H] $\alpha$  $\beta$ -sitostanol were determined by liquid scintillation counting. Appropriate standards were used to correct for spillover between the  $^3$ H- and  $^{14}$ C-counting channels. The cholesterol absorption efficiency was determined by the faecal dual-isotope ratio method (Grundy *et al.* 1968). Faecal cholesterol levels, and serum and biliary cholesterol levels were measured enzymatically using Sigma Diagnostics commercial kit no. 352, and biliary bile acid levels were determined using Sigma Diagnostics commercial kit no. 450-A, following the instructions of the manufacturer (Sigma Chemical Co.). For biliary bile acid analysis, a mixture of cholic acid and taurocholic acid (1:1 molar ratio) was used as a standard. Biliary phospholipids were determined using a phosphorus assay (Bartlett, 1959). Hepatic free cholesterol and total cholesterol were determined by GLC (Ishikawa *et al.* 1974) using cholesteryl methyl ether as an internal standard. Cholesteryl ester content was calculated as the difference between total and free cholesterol content. Bile acids were extracted from faeces essentially as described by Schiller *et al.* (1990). Briefly, 0.4 ml of an eight times dilution of a 10% faecal homogenate were added to a test tube containing 0.2 ml 10 M-NaOH and 2 ml 100% ethanol. An internal standard of 0.2  $\mu$ Ci (0.2 nmol) of [ $^3$ H(G)]taurocholic acid (2 Ci/mmol; NEN Life Science Products) was then added and the samples were refluxed on a heating block at 95–100°C for 1 h. Appropriate standards of a 1:1 molar mixture of cholic acid and taurocholic acid were treated similarly. After refluxing, samples were centrifuged for 15 min at 1500 g. Neutral sterols were extracted from two 0.5 ml aliquots of each sample supernatant using 0.75 ml petroleum ether three times. The remaining water–ethanol phase was evaporated and the residue redissolved in 0.5 ml water, after which the pH was adjusted to 7 using 1 M-HCl. Samples were dried and the residue was dissolved in methanol–Tris-HCl buffer (0.13 M-Tris-HCl, 0.67 mM-ethylene diamine tetraacetic acid, pH 9.5) (3.75:6.25, v/v). Aliquots were taken for liquid scintillation counting and bile acid analysis using the enzymatic assay of Turley & Dietschy (1978).

### Statistical analysis

Statistical evaluation was performed on a personal computer using Excel, Statistica version 6 (correlations; Statsoft Inc., Tulsa, OK, USA) and SPSS version 11.5 (SPSS, Chicago, IL, USA). Results are expressed as mean values and standard deviations. Variables were compared between the group of control animals and each group of fibre-treated animals by ANOVA followed by the two-sided Dunnett test.

## Results

### Effects of dietary fibres on cholesterol concentrations in the serum and liver

Table 1 shows the effects of the dietary fibres on serum cholesterol levels in female C57BL/6 mice after changing their diet from standard rodent chow to a HFHC diet containing 7.5% cocoa butter and 1.25% cholesterol. In control animals fed the HFHC diet without any of the three added fibres, the serum cholesterol level averaged 4.2 mM, consistent with published data (Carter *et al.* 1997). Upon feeding for 3 weeks with a

HFHC diet containing 7.5% cholestyramine, chitosan or cellulose, serum cholesterol levels were significantly lower with average values of 2.0, 2.2 and 1.6 mM, respectively. These values are similar to those obtained when the mice were fed a standard laboratory chow without added fat and cholesterol (data not shown). Thus, inclusion of any of the fibres in the diet prevented the increase in serum cholesterol level normally seen when the fat and cholesterol content of the diet is raised.

Hepatic steady-state sterol levels were determined as total cholesterol and as cholesteryl ester in all four groups of mice. When cholestyramine was included in the diet, the amount of total cholesterol did not increase to 72 mg cholesterol/g liver as observed with control animals fed a HFHC diet but remained low, i.e. 2.9 mg cholesterol/g liver (Table 1). Similar levels of liver cholesterol to the latter figure were observed when mice were fed a standard laboratory chow with no added fat and cholesterol (data not shown). Similar decreases in hepatic cholesterol stores have been reported in animal feeding studies using cholestyramine in the diet (Jennings *et al.* 1988; Turley *et al.* 1994, 1996). The inclusion of chitosan and cellulose in the diet also significantly reduced hepatic cholesterol concentrations, yielding values of 30 and 41 mg/g liver, respectively (Table 1). The chitosan effect is consistent with data in the literature (Jennings *et al.* 1988; Trautwein *et al.* 1997; Gallaher *et al.* 2000). Hepatic total cholesterol stores, calculated by multiplying the hepatic total cholesterol concentration (mg cholesterol/g liver) by the liver weight, decreased by 94, 57 and 40 mg cholesterol per mouse for the cholestyramine, chitosan and cellulose groups, respectively, relative to the total liver cholesterol of the control group. The effects of dietary fibres on liver total cholesterol levels were also reflected in their effects on hepatic cholesteryl ester stores (Table 1): the cholesteryl ester pools were significantly lower in animals fed cholestyramine-, chitosan- and cellulose-containing diets, as compared with the control group. The hepatic levels of cholesterol in the four animal groups correlated with the appearance of the livers: livers of the control group were light-coloured, those of mice fed the cholestyramine-containing diet were normal, dark red and those of the chitosan and cellulose groups were medium red-coloured.

#### Effects of dietary fibres on cholesterol and bile acid metabolism

In order to discriminate between decreases in cholesterol absorption and increases in cholesterol and bile acid excretion as possible mechanisms for the observed alterations in hepatic and serum cholesterol levels (Table 1), we investigated the effects of the dietary fibres on these parameters and on bile composition.

Cholestyramine caused a significant increase in faecal cholesterol output from about 0.5 mg/d per g body weight in control mice to about 1 mg/d per g body weight (Table 2). The other fibres had no significant effect on daily faecal cholesterol output. Faecal bile acid excretion levels averaged 3.3, 3.3 and 3.4  $\mu$ mol bile acid/d per body weight, respectively, in the control, chitosan and cellulose groups, and were significantly increased to 5.3  $\mu$ mol bile acid/d per g body weight in the cholestyramine group (Table 2). Cholestyramine also significantly affected the concentrations of cholesterol and bile acids in the bile of the mice. Thus, the biliary cholesterol level was in the range 16–19 mM for the control, chitosan and cellulose groups but only 3 mM in the cholestyramine-fed animals (Table 2). Inclusion of cholestyramine in the diet also reduced the biliary bile acid level significantly to 107 mM relative to the control (354 mM; Table 2). The biliary bile acid concentrations in the chitosan and cellulose groups were not significantly different from that of the control group. Biliary phospholipid concentrations averaged about 42 mM for all groups (data not shown).

Since cholesterol balance in the enterohepatic circulation is sensitive to the level of cholesterol acquired from food, we compared the effects of the dietary fibres on cholesterol absorption efficiency. The intestinal cholesterol absorption efficiency was 34% in the control group and 22% in the cholestyramine-fed group (Table 2). The cholesterol absorption efficiencies in the presence of chitosan and cellulose were identical within experimental error to that of the control. The absence of an effect of these fibres on cholesterol absorption efficiency was not due to a delayed appearance of the radiolabels in the stool because more than 95% of the  $^3$ H-labelled sitostanol was recovered in the faeces after 24 h (data not shown).

#### Effects of dietary fibres on body weight and food intake

Since the change from standard rodent chow to HFHC diet was expected to increase body weight, this parameter was monitored for the four groups of mice. At baseline, the mean body weights were similar: 19.3 g/animal (control), 19.7 g/animal (cholestyramine), 19.3 g/animal (chitosan) and 19.8 g/animal (cellulose). The mice on the control HFHC diet showed the greatest gain in weight over the 3-week diet period; the mean increase in body weight was +9.0%. The increase in body weight in this control group of mice was associated with the largest increase in serum and liver cholesterol concentrations (Table 1). In marked contrast, there was no increase in body weight of the cholestyramine-treated group (–0.2%) and there was no change in either serum cholesterol or hepatic cholesterol stores. Moderate

**Table 1.** Plasma and liver cholesterol concentrations\*†  
(Mean values, standard deviations and *P* values)

Parameter	Diet groups											
	Control ( <i>n</i> 6)			Cholestyramine ( <i>n</i> 6)			Chitosan ( <i>n</i> 6)			Cellulose ( <i>n</i> 6)		
	Mean	SD		Mean	SD	<i>P</i>	Mean	SD	<i>P</i>	Mean	SD	<i>P</i>
Serum cholesterol concentration (mM)	4.3	1.4		2.0	0.7	0.033	2.2	1.6	0.038	1.6	0.8	0.004
Hepatic total cholesterol concentration (mg/g liver)	72	19		2.9	0.3	<0.001	30	16	<0.001	41	17	0.006
Percentage of hepatic total cholesterol as cholesteryl ester	94	1		34	8	<0.001	82	5	<0.001	87	9	0.002

\* The variables were compared between the group of control mice and each group of fibre-treated animals; *P* values were calculated using ANOVA followed by the Dunnett test.

† For details of diets and procedures, see p. 332.

**Table 2.** Intestinal and biliary cholesterol and bile acid parameters\*†  
(Mean values, standard deviations and *P* values)

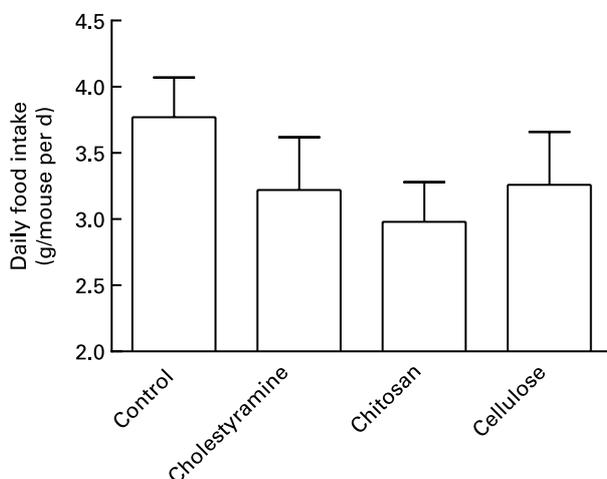
Parameter	Diet groups										
	Control ( <i>n</i> 6)		Cholestyramine ( <i>n</i> 5)			Chitosan ( <i>n</i> 6)			Cellulose ( <i>n</i> 6)		
	Mean	SD	Mean	SD	<i>P</i>	Mean	SD	<i>P</i>	Mean	SD	<i>P</i>
Faecal excretion of cholesterol (mg/d per g body wt)	0.55	0.18	1.06	0.31	0.002	0.59	0.07	0.67	0.56	0.10	0.90
Faecal excretion of bile acids ( $\mu\text{mol/d}$ per g body wt)	3.3	1.4	5.3	0.5	0.003	3.3	0.8	0.95	3.4	0.5	0.64
Biliary cholesterol concentration (mM)	19	6	3	1.5	<0.001	16	7	0.90	19	5	0.99
Biliary bile acid concentration (mM)	354	105	107	68	0.002	324	59	0.95	247	85	0.17
Cholesterol absorption efficiency (%)	34	8	22	12	0.05	36	6	0.99	33	8	0.99

\* The variables were compared between the group of control mice and each group of fibre-treated animals; *P*-values were calculated using ANOVA followed by the Dunnett test.

† For details of diets and procedures, see p. 332.

weight gains were observed in the chitosan-treated group (+3.1%) and in the cellulose-treated group (+2.1%). In these cases, the HFHC diet did not increase serum cholesterol although it did increase hepatic cholesterol stores to levels intermediate to those of the control and cholestyramine-treated groups (Table 1). All mice appeared healthy and were active throughout the feeding experiment.

As expected, the mean weight gain over the 3-week period of the diet for each group of mice was a function of the corresponding food intake. The dependence of weight increase (*W*, g/animal) on steady-state food intake (*F*, g/animal) was described by the equation  $W = 1.38F - 3.92$  ( $r^2$  0.8). There was an initial period of adjustment to the new diets and during this time the daily food intake fluctuated. However, for all groups of mice the food intakes became quite constant for days 10–21 of the diet regimen. Fig. 1 compares these steady-state daily food intakes for the four groups of mice. It is apparent that inclusion of the fibres in the diet reduced the food intake by about 15–20% relative to the control group in all cases. Since the intestinal absorption efficiency was similar for the control, chitosan and cellulose groups (Table 2), it follows that the amount of cholesterol absorbed in the chitosan and cellulose groups of mice also decreased by 15–20% relative to the value for the control



**Fig. 1.** Effects of the dietary insoluble fibres on steady-state daily food intake. Values are means (six mice per group) with standard deviations depicted by vertical bars. The food intakes for the three fibre groups were significantly lower than that of the control group ( $P < 0.05$ ).

group (about 16 mg cholesterol/mouse per d as calculated from the food intake data in Fig. 1 and the cholesterol intestinal absorption efficiencies in Table 2). Since cholestyramine treatment not only decreased food intake (Fig. 1) but also decreased cholesterol absorption efficiency (Table 2), the overall effect of this fibre on cholesterol uptake in the intestine was greater.

## Discussion

The results of the present study show that inclusion of 7.5% (w/w) cholestyramine, chitosan and cellulose in the diet had significant effects on the accumulation of dietary cholesterol in the mice. The change from basal to HFHC diet increased the daily intestinal absorption of dietary cholesterol for a control mouse from zero to about 16 mg/d. Feeding of cholestyramine completely prevented accumulation of this cholesterol in hepatic stores while feeding of chitosan and cellulose partially prevented this accumulation (Table 1). Several mechanistic theories for the hypocholesterolaemic effects of naturally occurring fibres have been put forward (Furda, 1990). One such mechanism involves binding of bile acid molecules in the intestinal lumen to the fibre, as occurs with cholestyramine. Such a mechanism would be expected to apply to a cationic polymer such as chitosan but not to a neutral fibre such as cellulose. Indeed, there is some question as to whether the insoluble cellulose fibre has any cholesterol-lowering effect (Chen & Anderson, 1979). A completely different mechanism of action of dietary fibres may relate to their ability to suppress energy intake. Dietary fibres can induce both satiation (i.e. limit the size of a meal) and satiety (i.e. inhibit the desire for another meal) (Burton-Freeman, 2000). Both effects can reduce the intake of any cholesterol present in the diet. Analysis of these cholesterol balance parameters for the four groups of mice has enabled us to compare the contributions to cholesterol reduction of the satiation and satiety-inducing properties and the bile acid-binding properties of the fibres.

### Mechanisms of cholesterol-lowering effects of cholestyramine

Cholestyramine is a well-known resin that has been widely used for the treatment of atherosclerosis and the prevention of CHD (Brown, 1990). The cholesterol-lowering effect of this compound (Table 1) has been clearly demonstrated in human and animal studies (Gylling *et al.* 1989; Turley *et al.* 1994). As a result of the extensive sequestration of bile acids in the intestines by cholestyramine, the amount of recycled bile acids, usually in excess of 95% (Cohen, 1999), was dramatically reduced and the demand

for hepatic cholesterol metabolism towards bile acid formation and secretion in cholestyramine-fed mice was consistent with the finding of lower biliary cholesterol and bile acid levels (Table 2). Feeding cholestyramine reduced the cholesterol absorption efficiency significantly from 34% in control mice to 22% (Table 2). This decrease was caused by the binding of bile acids to cholestyramine in the intestinal lumen which results in decreased cholesterol solubilization and reduced cholesterol uptake at the brush border membrane of enterocytes (cf. Homan & Krause, 1997). The binding of bile acids to cholestyramine led to increased excretion of bile acids and cholesterol in the faeces (Table 2) as has been found in human (Stanley *et al.* 1973) and other animal studies (Turley *et al.* 1996).

As a consequence of the changes, described earlier, in bile acid availability in the lumen of the intestines, the amount of dietary cholesterol absorbed was about 9 mg/mouse per d (calculated from the food intake data in Fig. 1 and the cholesterol intestinal absorption efficiencies in Table 2) in the cholestyramine group compared to about 16 mg/mouse per d in the control group. In the latter case, the 16 mg/mouse per d of absorbed cholesterol was associated with an increase of about 90 mg/mouse in liver cholesterol over the 3-week period (Table 1). This corresponds to a rate of accumulation of liver cholesterol of about 4 mg/d, with the excess being lost in the faeces as cholesterol or bile acid. In the cholestyramine group, the 9 mg/mouse per d of absorbed cholesterol did not lead to any accumulation of cholesterol in the liver (Table 1) over the feeding period. The 90 mg lower accumulation of liver cholesterol for the cholestyramine group compared to the control group was due, in part, to enhanced rates of loss of cholesterol and bile acid in the faeces (Table 2). It should be noted that the combined serum and biliary cholesterol pools amounted to about 1 mg/mouse and were relatively small compared to the hepatic cholesterol pool. The cholesterol present in adipose tissue was not assessed but this storage pool was probably not affected greatly during the feeding period because it turns over slowly (Farkas *et al.* 1973).

It is important to note that there were two contributions to the reduction in daily cholesterol absorption from 16 to 9 mg/mouse per d when cholestyramine was included in the diet. The decrease in daily cholesterol intake (calculated from the food intake data in Fig. 1) would have reduced the amount of absorbed cholesterol by about 2.4 mg/mouse per d if the absorption efficiency had remained at 34% (the value for control mice, see Table 2). The remainder of the 7 (16 - 9) mg/d per mouse reduction in absorbed cholesterol in cholestyramine-treated mice, i.e. about 4.6 mg/mouse per d, was a consequence of the decrease in cholesterol absorption efficiency to 22%. Thus, about one-third (2.4/7) of the reduction in daily cholesterol absorption arising from cholestyramine treatment was due to satiation and satiety effects (cf. Burton-Freeman, 2000) and two-thirds was due to bile acid sequestration effects.

#### *Mechanisms of cholesterol-lowering effects of chitosan and cellulose*

In contrast to the cholestyramine-fed mice, there was no change in cholesterol absorption efficiency or faecal sterol excretion with the groups of mice fed either chitosan or cellulose (Table 2). Hence, the hypocholesterolaemic effects (Table 1) of both chitosan and cellulose cannot be rationalized in terms of either a reduction in cholesterol absorption efficiency or a

significant increase in faecal sterol output. It follows that, in contrast to the situation with cholestyramine, the hypocholesterolaemic effects of chitosan and cellulose cannot be related to a major bile acid-binding capacity of these two fibres. The mechanism of action of these two dietary fibres seems to be related to a suppression of food intake by means of inducing satiation and satiety (Burton-Freeman, 2000). Thus, the average food intake of all three groups of mice receiving a dietary fibre decreased significantly with respect to the control groups of mice (Fig. 1). The daily amount of dietary cholesterol absorbed in the chitosan and cellulose groups of mice was about 13 mg/mouse in each case; this value was about 3 mg lower than the 16 mg/mouse per d seen with the control group. The daily absorption of 13 mg cholesterol per mouse was associated with accumulations of approximately 40 and 60 mg liver cholesterol over 3 weeks for the chitosan and cellulose groups, respectively (Table 1). It follows that the rates of accumulation of liver cholesterol were about 2 and 3 mg/mouse per d compared to the value of about 4 mg/mouse per d for the control group. The lower rates of cholesterol accumulation in the fibre-treated groups apparently were not due to differences in rates of faecal output of cholesterol and bile acid (Table 2). Overall, our results suggest that satiation and satiety effects (Burton-Freeman, 2000) contributed significantly to the cholesterol-lowering properties of dietary chitosan and cellulose seen in these mice. This finding is consistent with an earlier study (Trautwein *et al.* 1997) where it was shown that incorporation of chitosan into the diet of hamsters led to a reduction in food intake and a cholesterol-lowering effect.

#### *Clinical implications*

Mechanisms underlying the cholesterol-lowering effect of cholestyramine are (1) decreased cholesterol (food) intake, (2) decreased cholesterol absorption efficiency, and (3) increased faecal bile acid and cholesterol excretion. The latter effects can be attributed to the high bile acid-binding capacity of cholestyramine. In contrast, incorporation of chitosan or cellulose in the diet reduced cholesterol (food) intake, but did not significantly affect either intestinal cholesterol absorption or faecal sterol excretion in our study. We conclude that satiation and satiety effects contribute to the cholesterol-lowering properties of dietary fibres with moderate or low bile acid-binding capabilities. Knowledge about the mechanisms by which the different fibres prevent cholesterol accumulation will be helpful for investigating clinical effects of fibre therapy. A possible beneficial effect of fibres on cardiovascular risk might be related to the different mechanisms by which the fibres influence lipid metabolism. Thus, in future studies, the effect on cardiovascular morbidity and mortality as well as the occurrence of side-effects should be evaluated separately for each kind of fibre.

We have observed impressive effects of the fibres investigated in the present study, e.g. the satiation and satiety effects of chitosan and cellulose were sufficient to stabilize serum cholesterol levels against the increase induced by the HFHC diet, and reduce the degree to which liver cholesterol stores are increased by the HFHC diet. However, the dose of fibres used (7.5% of diet, w/w) was higher than doses usually used in human trials, e.g. 2% of chitosan incorporated into bread (Ausar *et al.* 2003) or 3–6 g of chitosan applied daily as capsules (Maizaki *et al.*

1993; Mhurchu *et al.* 2004). It should be noted that the cholesterol concentration used in the present study (1.25 % of diet, w/w) was much higher than the cholesterol content of diets usually consumed by man. Hence, a fibre content below 7.5 % might be sufficient to prevent cholesterol accumulation in man, as previously shown for cholestyramine (Brown, 1990) and chitosan (Maezaki *et al.* 1993; Pittler *et al.* 1999; Ylitalo *et al.* 2002; Bokura & Kobayashi, 2003). Investigation of cellulose in clinical studies is most intriguing, because a hypocholesterolaemic effect of cellulose has not been established as clearly (Chen & Anderson, 1979), although intake of high concentrations of cellulose as a food supplement in man was reported to lower serum cholesterol by about 25 % (Shurpalekar *et al.* 1971).

### Acknowledgements

This study was supported by NIH Grant HL22633 and a postdoctoral fellowship to Ariëtte van Bennekum from the American Heart Association (Mid-Atlantic Consortium).

### References

- Ausar SF, Morcillo M, Leon AE, *et al.* (2003) Improvement of HDL- and LDL-cholesterol levels in diabetic subjects by feeding bread containing chitosan. *J Med Food* **6**, 397–399.
- Bartlett GR (1959) Phosphorus assay in column chromatography. *J Biol Chem* **234**, 466–468.
- Bokura H & Kobayashi S (2003) Chitosan decreases total cholesterol in women: a randomized, double-blind, placebo-controlled trial. *Eur J Clin Nutr* **57**, 721–725.
- Brown WV (1990) Clinical trials including an update on the Helsinki Heart study. *Am J Cardiol* **66**, 11A–15A.
- Burton-Freeman B (2000) Dietary fiber and energy regulation. *J Nutr* **130**, 272S–275S.
- Carter CP, Howles PN & Hui DY (1997) Genetic variation in cholesterol absorption efficiency among inbred strains of mice. *J Nutr* **127**, 1344–1348.
- Casdorph HR (1967) Treatment of hypercholesterolemia with cholestyramine, a bile acid sequestering resin. *Vasc Dis* **4**, 305–308.
- Castelli WP (1984) Epidemiology of coronary heart disease: The Framingham study. *Am J Med* **76**, 4–12.
- Chen WJL & Anderson JW (1979) Effects of plant fiber in decreasing plasma total cholesterol and increasing high-density lipoprotein cholesterol. *Proc Soc Exp Biol Med* **162**, 310–313.
- Cohen DE (1999) Hepatocellular transport and secretion of biliary lipids. *Curr Opin Lipidol* **10**, 295–302.
- Farkas J, Angel A & Avigan MI (1973) Studies on the compartmentation of lipid in adipose cells. II. Cholesterol accumulation and distribution in adipose tissue components. *J Lipid Res* **14**, 344–356.
- Fernandez ML (2001) Soluble fiber and nondigestible carbohydrate effects on plasma lipids and cardiovascular risk. *Curr Opin Lipidol* **12**, 35–40.
- Furda I (1983) Aminopolysaccharides – their potential as dietary fiber. In *Unconventional Sources of Dietary Fiber*, pp. 105–122 [I Furda, editor]. ACS Symposium Series no. 214. Washington DC, USA: American Chemical Society.
- Furda I (1990) Interaction of dietary fiber with lipids – mechanistic theories and their limitations. *Adv Exp Med Biol* **270**, 67–82.
- Gallaher CM, Munion J, Hesslink RJ, Wise J & Gallaher DD (2000) Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats. *J Nutr* **130**, 2753–2759.
- Grundy SM (1983) Absorption and metabolism of dietary cholesterol. *Annu Rev Nutr* **3**, 71–96.
- Grundy SM, Ahrens EH & Salen G (1968) Dietary  $\beta$ -sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. *J Lipid Res* **9**, 374–387.
- Gylling H, Vanhanen H & Miettinen TA (1989) Effects of acipimox and cholestyramine on serum lipoproteins, non-cholesterol sterols and cholesterol absorption and elimination. *Eur J Clin Pharmacol* **37**, 111–115.
- Han LK, Kimura Y & Okuda H (1999) Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. *Int J Obesity* **23**, 174–179.
- Homan R & Krause BR (1997) Established and emerging strategies for inhibition of cholesterol absorption. *Curr Pharm Design* **3**, 29–44.
- Ishikawa TT, MacGee J, Morrison JA & Glueck CJ (1974) Quantitative analysis of cholesterol in 5 to 20  $\mu$ l of plasma. *J Lipid Res* **15**, 286–291.
- Jennings CD, Boleyn K, Bridges SR, Wood PJ & Anderson JW (1988) A comparison of the lipid-lowering and intestinal morphological effects of cholestyramine, chitosan, and oat gum in rats. *Proc Soc Exp Biol Med* **189**, 13–20.
- Kesaniemi YA & Miettinen TA (1987) Cholesterol absorption efficiency regulates plasma cholesterol level in the Finnish population. *Eur J Clin Invest* **17**, 391–395.
- Lee JK, Kim SU & Kim JH (1999) Modification of chitosan to improve its hypocholesterolemic capacity. *Biosci Biotechnol Biochem* **63**, 833–839.
- Maezaki Y, Tsuji K, Nakagawa Y, Kawai Y, Akimoto M, Tsugita T, Takekawa W, Terada A, Hara H & Mitsuoka T (1993) Hypocholesterolemic effect of chitosan in adult males. *Biosci Biotech Biochem* **57**, 1439–1444.
- Mhurchu CN, Poppitt SD, McGill AT, Leahy FE, Bennett DA, Lin R, Ormod D, Ward L, Strik C & Rodgers A (2004) The effect of the dietary supplement, chitosan, on body weight; a randomized controlled trial in 250 overweight and obese adults. *Int J Obes Relat Metab Disord* **28**, 1149–1156.
- Pittler MH, Abbot NC, Harkness EF & Ernst E (1999) Randomized, double-blind trial of chitosan for body weight reduction. *Eur J Clin Nutr* **53**, 379–381.
- Schiller LR, Bilhartz LE, Santa Ana CA & Fordtran JS (1990) Comparison of endogenous and radiolabeled bile acid excretion in patients with idiopathic chronic diarrhea. *Gastroenterology* **98**, 1036–1043.
- Sehayek E, Nath C, Heinemann T, McGee M, Seidman CE, Samuel P & Breslow JL (1998) U-shape relationship between change in dietary cholesterol absorption and plasma lipoprotein responsiveness and evidence for extreme interindividual variation in dietary cholesterol absorption in humans. *J Lipid Res* **39**, 2415–2422.
- Shurpalekar KS, Doraiswamy TR, Sundaravalli OE & Narayana Rao M (1971) Effect of inclusion of cellulose in an 'atherogenic' diet on the blood lipids of children. *Nature* **232**, 554–555.
- Stanley MM, Paul D, Gacke D & Murphy J (1973) Effects of cholestyramine, metacucil, and cellulose on fecal bile salt excretion in man. *Gastroenterology* **65**, 889–894.
- Story JA, Furumoto EJ & Buhman KK (1997) Dietary fiber and bile acid metabolism—an update. *Adv Exp Med Biol* **427**, 259–266.
- Trautwein EA, Jürgensen U & Erbersdobler HF (1997) Cholesterol-lowering and gallstone-preventing action of chitosans with different degrees of deacetylation in hamsters fed cholesterol-rich diets. *Nutr Res* **17**, 1053–1065.
- Turley SD, Daggy BP & Dietschy JM (1994) Psyllium augments the cholesterol-lowering action of cholestyramine in hamsters by enhancing sterol loss from the liver. *Gastroenterology* **107**, 444–452.
- Turley SD, Daggy BP & Dietschy JM (1996) Effect of feeding psyllium and cholestyramine in combination on low density lipoprotein metabolism and fecal bile acid excretion in hamsters with dietary-induced hypercholesterolemia. *J Cardiovasc Pharmacol* **27**, 71–79.

- Turley SD & Dietschy JM (1978) Re-evaluation of the 3 alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J Lipid Res* **19**, 924–928.
- Vahouny GV, Roy T, Gallo LL, Story JA, Kritchevsky D & Cassidy M (1980a) Dietary fibers III. Effects of chronic intake on cholesterol absorption and metabolism in the rat. *Am J Clin Nutr* **33**, 2182–2191.
- Vahouny GV, Tombes R, Cassidy MM, Kritchevsky D & Gallo LL (1980b) Dietary fibers: V. Binding of bile salts, phospholipids and cholesterol from mixed micelles by bile acid sequestrants and dietary fibers. *Lipids* **15**, 1012–1018.
- Ylitalo R, Lehtinen S, Wuolijoki E, Ylitalo P & Lehtimäki T (2002) Cholesterol-lowering properties and safety of chitosan. *Arzneimittelforschung* **52**, 1–7.