Cholesterol crystallization in gall-bladder bile of pigs given cholesterol–β-cyclodextrin-enriched diets with either casein or soyabean concentrate as protein sources

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(Received 3 February 1999 – Revised 20 September 1999 – Accepted 21 October 1999)

Cholesterol precipitation from supersaturated bile is the earliest and determinant step in the formation of cholesterol gallstones, which is thought to be diet-dependent. Bile composition, appearance and growth of cholesterol crystals were studied in fresh gall-bladder biles from pigs adapted to four different protein-containing diets over 3 weeks: 160 g dietary protein/kg as casein (C16; *n* 6), or as soyabean-protein concentrate (S16; *n* 6), or a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w) (CS16; *n* 6), or 320 g of the mixed protein/kg (CS32; *n* 6). Moreover, all four diets contained 3 g cholesterol/kg and 50 g β-cyclodextrin/kg as modifiers of bile composition towards cholesterol pro-crystallization. Cholesterol precipitation was most active after the high-protein diet, CS32, and the casein diet, C16, and lowest after the soyabean-protein diet, S16. It was intermediate after the mixed diet, CS16, but still much lower than in the former two groups. These diet-induced variations were suggested to be mediated through modifications in the biliary profile of bile acids, whereas all other biliary constituents studied were essentially unchanged. The fasting level of plasma cholesterol was lowest in both 160 g protein/kg diets containing soyabean protein (S16 and CS16), highest for the high-protein diet CS32, and intermediate for the C16 diet. These results should encourage clinical studies on the effect of soyabean protein, or other vegetable proteins, for primary or recurrence prevention of cholelithiasis at its earliest stage.

Plasma lipids: Bile composition: Soyabean protein: Cholesterol crystals

Cholesterol gallstones are predominantly reported in Western countries and are believed to be related to diet (Heaton, 1984; Pixley *et al.* 1985; Hayes *et al.* 1992; Kern, 1994; Attili *et al.* 1998; Carolibosc *et al.* 1998). Although this pathogenesis is complex and involves several recognized steps (Hay & Carey, 1990), precipitation of cholesterol crystals from supersaturated gall-bladder bile is the earliest and determinant step in the genesis of cholesterol-rich gallstones. Delaying cholesterol precipitation through nutritional intervention protocols would thus be of great interest for preventing cholelithiasis at its earliest stage in human subjects. This, however, is an unexplored field, due until recently to the lack of appropriate animal models. In this context, we proposed growing pigs given dietary cholesterol–β-cyclodextrin as new donors of sufficiently large quantities of nucleating gall-bladder bile (Juste *et al.* 1997*b*). We effectively demonstrated that including 3 g cholesterol/kg and 50 g β-cyclodextrin/kg into otherwise well-balanced pig diets led to an increase in chenodeoxycholic acid in bile up to levels observed in human subjects, and thus favoured cholesterol nucleation. This model allows reliable measurements of the cholesterol crystallization rate under various treatments and concomitant screening of biliary candidates for the modulation of cholesterol precipitation. In the present study we investigated whether the protein intake interfered with precipitation of cholesterol crystals in gall-bladder bile of our animal model. In an effort to identify the biliary compounds supporting the diet-induced differences in cholesterol precipitation rate, bile acids, phospholipids, cholesterol and biliary proteins were measured quantitatively.

Abbreviations: CHc, cholesterol crystal concentrations; C16, diet containing 160 g casein/kg; CS16, diet containing 160 g casein–soyabean protein (70 : 30, w/w)/kg; CS32, diet containing 320 g casein–soyabean protein/kg; S16, diet containing 160 g soyabean protein/kg.

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Different species of bile acids and phosphatidylcholines, which are known to interfere with cholesterol precipitation (Halpern *et al.* 1993; Konikoff *et al.* 1994; Tazuma *et al.* 1994), were further analysed. Biliary proteins, which are proposed as crystallization effectors in pig bile (Catala *et al.* 1998), were also purified and assayed for their cholesterol crystallization-influencing activity. Lastly, blood lipids, as well as sterol excretion in the faeces, were studied concomitantly, in order to provide a complete description of the current model and ascertain whether a beneficial shift in bile composition towards anti-crystallization would or would not result in a harmful modification of blood variables.

Materials and methods

Animals

We used twenty-four castrated male pigs of the Large White strain (six litters of four pigs), weighing 34·7 (SEM 0·5) kg at the beginning of the experiment. They were housed individually, maintained at 25° under normal light (from 07.00 hours to 19.00 hours) and dark cycles, and fed daily at 08.00 hours and 16.00 hours with free access to water. All pigs received humane care in compliance with the 'Principles for Biomedical Research Involving Animals' developed by the Council for the International Organisations of Medical Sciences (1986). The experimental protocol was approved by our Institute in agreement with the Department of Health and Human Services (USA, identification number: #A5105-01), and conditions of housing were approved by the French Direction des Services Vétérinaires (Ministry of Agriculture).

Experimental design

Over a 3 d period, the standard growing diet was gradually replaced by a semi-purified cholesterol–β-cyclodextrin-enriched diet, containing 160 g protein/kg as casein (C16 diet). Pigs were stabilized on this diet for 1 week. They were then weighed, and divided into four groups, in such a way that four siblings were assigned to different experimental diets formulated to measure both the effects of protein origin and protein level in the diet: 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w) (CS16), or 320 g of the mixed protein/kg diet (CS32) (Table 1). Casein hydrochloride (Union Coopérative des Caséineries, Surgères, France) was used as the source of casein and was found to be 90 % pure. Soyabean proteins were introduced into the diet as a soyabean-protein concentrate (DANPRO H, 669 g protein/kg; Central Soya, Aarhus, Denmark). All four diets contained sucrose, 3 g cholesterol/kg and 50 g β-cyclodextrin/kg, as inductors of cholesterol crystallization in pig bile (Juste *et al.* 1997*b*). They were isoenergetic, and provided the same quantities of total lipids and fibre, as well as a constant starch : sucrose ratio (Table 1).

All four dietary groups were provided with their respective experimental diets (two meals of 800 g each per d) in powdered form mixed with water for 3 weeks, before blood and bile collection. Growth was measured over the last 2 weeks, and stools were collected, weighed and frozen under vacuum within the last 4 d. On the final day of the experiment the animals underwent general anaesthesia to obtain a gall-bladder bile aspirate, according to the protocol detailed previously (Juste *et al.* 1997*b*), as well as a peripheral blood sample (20 ml taken into Na_2EDTA as an anticoagulant). Plasma was immediately separated by centrifugation at 4°, then divided for lipid and lipoprotein analyses, and stored at −20° after addition of monoiodoacetamide (1 mg/ml

Table 1. Ingredients (g/kg diet) and analysis (/kg diet) of the experimental semi-purified diets containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32)

Diet...	C16	S16	CS16	CS32
Ingredients				
Casein*	178·0	0·0	124·4	248·9
Soyabean-protein concentrate†	0·0	239·0	71·7	143·5
Soyabean oil	150·0	150·0	150·0	150·0
Maize starch	383·0	368·3	378·7	245·2
β-Cyclodextrin	50·0	50·0	50·0	50·0
Sucrose	145·0	143·9	144·8	95·5
Cholesterol	3·0	3·0	3·0	3·0
Cellulose‡	50·0	4·8	36·4	22·9
Mineral mix	30·0	30·0	30·0	30·0
Vitamin mix	10·0	10·0	10·0	10·0
Antioxidant	1·0	1·0	1·0	1·0
Analysis				
Energy (MJ)	19·6	19·2	19.6	20.4
Protein (g; N × 6.25)	160·1	158·3	159·5	318·4
Lipids (g)	152·6	150·0	150·7	150·8
Cholesterol (g)	3·0	3·0	3·0	3·0
Fibre‡ (g)	41·3	40·0	40·8	39·1

* Casein hydrochloride, consisting of (g/kg): protein 900, fat 14, ash 5 and moisture 81.
† Soyabean-protein concentrate consisting of (g/kg): protein 669, carbohydrates 49, fat 5, ash 38, total fibre 189 and moisture 50.
‡ Total fibre measured according to the method of Prosky *et al.* (1988).

plasma) as an inhibitor of cholesterol esterification (Férézou *et al.* 1997). Gall-bladder aspirates (50–60 ml) were mixed thoroughly and immediately fractionated under N_2 and sterile conditions. Cholesterol crystallization was monitored from fresh samples. Biochemical analyses and protein purification were carried out from samples frozen rapidly in liquid N_2 and then stored at −80° until use.

Analytical methods

Plasma lipids. Plasma levels of total cholesterol, phospholipids and triacylglycerols were measured using commercially available kits (CHPOP-method, Boehringer, Meylan, France for cholesterol, and Wako kits, Osaka, Japan for phospholipids and triacylglycerols). Lipoproteins were fractionated by ultracentrifugation in a density gradient (Férézou *et al.* 1997), and each fraction was analysed for lipids as described earlier.

Biliary lipids. Cholesterol, phospholipids, and bile acids in bile were measured enzymically on decolorized isopropanolic dilutions (1 : 10, v/v). For the analysis of molecular species of phosphatidylcholines, total biliary lipids were extracted from 500 μl gall-bladder bile (Folch *et al.* 1957). Phosphatidylcholines were then separated (Singleton *et al.* 1965), and their different molecular species were resolved by HPLC (Juste *et al.* 1997*b*). Further GLC was performed to identify methyl esters of fatty acids derived from each molecular species of phosphatidylcholine (Leonardi *et al.* 1987). Individual molecular species of conjugated bile acids were determined by HPLC (Legrand-Defretin *et al.* 1991). The hydrophobicity index of each gall-bladder bile was calculated from its bile acid composition, using the hydrophobicity indices known for common conjugated bile acids (Heuman, 1989) and assigning to the hydrophobicity index of the less common highly hydrophilic glycine conjugate of 3α-hydroxy-6-oxo-5β-cholanoic acid a value of −0·35. Concentrations of cholesterol, phospholipids, and bile acids were expressed as mmol/l, as well as mol/100 mol. Individual molecular species of bile acids or phospholipids were expressed as mol/100 mol. Total lipid concentration (g/l) was determined from the sum of the concentrations of bile acids, phospholipids and cholesterol using the appropriate molecular masses of the different molecular species for bile acids and phosphatidylcholines previously quantified.

Faecal steroids. For each pig, faeces collected over 4 d were thawed under vacuum, pooled, and homogenized. After adding predetermined amounts of radioactive markers ([1,2-^{3}H]cholesterol and [^{14}C]taurocholate sodium salt), neutral sterols and bile acids were extracted from 2 mg faeces (Riottot *et al.* 1993). They were then separated through liquid–liquid extraction, and bile acids were deconjugated. Finally, trimethylsilyl derivatives of neutral sterols and bile acids were assayed by GC in the presence of cholestane as an internal standard (Juste *et al.* 1997*b*). Daily faecal outputs of cholesterol and bile acids were calculated after correction for faecal flow, on the basis of a theoretical 90 % recovery of dietary β-sitosterol taking into account that this phytosterol is a reliable marker in pigs (Marsh *et al.* 1972).

Cholesterol crystallization in native bile. This was monitored from 2 ml freshly obtained gall-bladder aspirate, as previously detailed (Juste *et al.* 1997*b*). Cholesterol crystal concentrations (CHc), as enzymically determined, were plotted *v.* time (t), as previously described (Juste *et al.* 1995) and expressed as a sigmoidal function of t, according to the equation:

$$CHc = a + ([b - a]/1 + e^{[-4c/b][t-m]}),$$

where *a* is CHc at the initiation of crystallization (*a* was set to zero, as no cholesterol crystal could be initially observed in microscopy or measured enzymically), *b* is maximum CHc at equilibrium (in μg/ml), *c* is maximum crystal growth rate (in μg/ml per d) and *m* is the time required for maximum crystal growth rate (in d). Sets of data (one set of data represents one experiment on one gall-bladder bile) for the same diet were averaged, and a single curve was fitted for each treatment (*n* 6).

Cholesterol crystallization-influencing activity of biliary proteins. Biliary proteins from 2 ml of each gall-bladder aspirate were purified, according to the method previously detailed (Catala *et al.* 1998). Half the purified protein fraction was lyophilized and its cholesterol crystallization-influencing activity was assayed in 1 ml reconstituted supersaturated model bile (sodium taurocholate–egg yolk lecithin–cholesterol 72·9 : 17·5 : 9·6 molar ratio; final lipid concentration, 95 g/l) (Catala *et al.* 1998). Monitoring and modelling of cholesterol crystallization in artificial protein-enriched biles was the same as that described earlier for native bile. The protein concentration in a sample was derived from its amino acid composition (Catala *et al.* 1998). The remainding protein fraction was frozen at −80° for further protein identification (results not reported).

Statistical analysis

Data were analysed by means of the S-plus software system (version 3.4) running under the UNIX operating system (AT&T Software Sales, Greensboro, NC, USA) on a Sun 670 MP computer. Data are presented as mean values with their standard errors. ANOVA followed by the Newman-Keuls test for multiple comparisons was used when comparing the four diets. Differences were considered significant at $P < 0{\cdot}05$. Parameter values of crystallization curves were obtained by maximum likelihood estimation and compared between treatments using a maximum likelihood ratio test (Bouvier & Huet, 1994; Huet *et al.* 1996).

Results

Food consumption was complete in all animals, and they remained in good health throughout the experiment, without any significant difference in body-weight gain (864 (SEM 38), 791 (SEM 26), 843 (SEM 54) and 903 (SEM 34) g/d for diets C16, S16, CS16 and CS32 respectively).

Plasma lipids

Triacylglycerol and phospholipid levels in fasting plasma were not affected by the nature or the level of protein intake (triacylglycerols 236 (SEM 20), 173 (SEM 36), 223 (SEM 57) and 195 (SEM 34) mg/l for diets C16, S16, CS16 and CS32 respectively; phospholipids 1164 (SEM 74), 1077 (SEM 55),

1087 (SEM 103) and 1150 (SEM 78) mg/l for the same diets). Fasting cholesterol concentrations were significantly highest for the high-protein diet CS32 (1354 (SEM 146) mg/l), and lowest in both 160 g protein/kg diets containing soyabean-protein concentrate (938 (SEM 64) and 936 (SEM 80) mg/l for diets S16 and CS16 respectively). The concentration was intermediate for the 160 g protein/kg diet C16 (1101 (SEM 90) mg/l), but not significantly different from the other three groups. These differences in plasma cholesterol level were essentially located in the LDL fraction, which was significantly highest in pigs receiving the CS32 diet (902 (SEM 94) mg/l) when compared with the three diets containing 160 g protein/kg (584 (SEM 37), 508 (SEM 34) and 502 (SEM 42) mg/l for diets C16, S16 and CS16 respectively, all three not significantly different).

Lipid and protein composition of bile

Table 2 indicates that neither absolute concentrations nor molar percentages of bile acids or cholesterol, neither total lipid nor protein concentrations, differed significantly between groups. In contrast, the absolute concentration of phospholipids was significantly lowest with the high-protein diet CS32 when compared with the two diets containing 160 g protein/kg as casein (C16) or soyabean concentrate (S16). However, it was not significantly different when compared with the CS16 diet. The molar percentages of phospholipids followed the same variation between groups but the difference was never statistically significant.

The respective proportions of the individual conjugated bile acids presented in Table 3 differed consistently between groups. Conversion of the primary hyocholic acid to its bacterial metabolite hyodeoxycholic acid was strikingly increased with the two diets containing high levels of soyabean-protein concentrate (S16 and CS32). The less-represented secondary bile acids 3α-hydroxy-6oxo-5β-cholanoic acid and deoxycholic acid followed the same trend. As a result, the overall proportion of secondary bile acids in pigs fed on S16 or CS32 diets was twice that measured for pigs fed on C16 or CS16 diets. The chenodeoxycholic level was highest for pigs fed on C16 and CS32 diets, intermediate for those fed on the CS16 diet and lowest for those fed on the S16 diet. Another result concerns the hydrophobicity index that increased regularly as dietary casein supply increased (S16 < CS16 < C16 < CS32). The difference, however, was only significant between the extreme values S16 and CS32.

Table 4 displays the distribution of the molecular species of biliary lecithins which were, for all four groups, in decreasing order (and in *sn*-1–*sn*-2 order): 16:0–18:2 > 18:0–18:2 > 16:0–18:1(18:0–20:4) > 18:2–18:2≅ 18:1–20:4. The two *sn*-1-palmitoyl species, 16:0–18:2 and 16:0–18:1, followed reciprocal changes and did not differ significantly between C16 and S16 diets on the one hand, and CS16 and CS32 diets on the other. The S16 group was characterized by the highest proportion of the 18:0–18:2 lecithin species, whereas the CS32 diet was characterized by the lowest proportion of the 18:2–18:2 species.

Faecal steroid excretion

As shown in Table 5, faecal neutral steroid excretion (g/d) was lowest with the C16 diet, highest with the S16 diet and intermediate with the two mixed diets. The proportion of soyabean concentrate in the diet was therefore related to the faecal neutral steroid excretion. Total excretion of bile acids in faeces was less influenced by dietary protein, but their distribution was considerably changed through a striking decrease in the percentage of hyocholic acid, as well as increased proportions of chenodeoxycholic, lithocholic, and oxo bile acids for high soyabean concentrate consumption (diets S16 and CS32). In contrast, the proportion of hyodeoxycholic acid in faeces was not affected by the type of protein in the diet.

Cholesterol crystallization in native bile

Fig. 1(a) displays increases in CHc, as a function of time, in

Table 2. Absolute and relative concentrations of bile acids, phospholipids, and cholesterol in gall-bladder bile of pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32)*

(Mean values with their standard errors for six pigs per diet)

Diet . . .	C16		S16		CS16		CS32	
Bile component	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Absolute concentration (mmol/l)								
Bile acids	152·90[a]	8·86	192·18[a]	30·40	156·60[a]	23·53	135·88[a]	17·28
Phospholipids	22·38[a]	2·26	22·16[a]	4·23	19·88[ab]	2·14	13·29[b]	1·88
Cholesterol	6·47[a]	0·28	6·19[a]	1·21	5·60[a]	0·50	4·24[a]	0·76
Relative concentration (mol/100 mol)								
Bile acids	84·12[a]	1.06	86·86[a]	2·10	85·49[a]	1·13	88·49[a]	0·98
Phospholipids	12·29[a]	1·06	10·41[a]	1·85	11·22[a]	0·78	8·75[a]	0·87
Cholesterol	3·59[a]	0·17	2·73[a]	0·32	3·29[a]	0·43	2·76[a]	0·43
Total lipids (g/l)	92·6[a]	16·8	111·0[a]	5·1	92·1[a]	12·7	76·6[a]	9·3
Total proteins (μg/ml)	479[a]	42	514[a]	14	585[a]	38	430[a]	38

[a,b] Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (ANOVA followed by Newman-Keuls test for multiple comparisons).

* For details of diets and procedures, see Table 1 and p. 413.

Table 3. Distribution (mol/100 mol) and mean hydrophobicity* of bile acids in gall-bladder bile of pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32)†

(Mean values with their standard errors for six pigs per diet)

Diet...	C16		S16		CS16		CS32	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Bile acids								
Glycodeoxycholic	0·06^{a}	0·06	0·48^{a}	0·37	0·04^{a}	0·04	0·17^{a}	0·14
Chenodeoxycholic	37·71^{a}	1·97	28·53^{b}	1·92	33·56^{ab}	2·52	37·18^{a}	3·46
Glyco	35·25^{a}	1·93	26·47^{b}	1·83	32·56^{ab}	2·50	31·64^{ab}	1·00
Tauro	2·46^{a}	0·27	2·06^{a}	0·36	1·00^{a}	0·45	5·54^{b}	0·72
Cholic	1·59^{a}	0·67	0·63^{a}	0·22	2·94^{a}	1·27	1·60^{a}	0·81
Glyco	0·89^{a}	0·56	0·22^{a}	0·20	2·49^{a}	1·35	0·97^{a}	0·68
Tauro	0·71^{a}	0·21	0·42^{a}	0·20	0·45^{a}	0·16	0·62^{a}	0·22
Hyodeoxycholic	21·71^{a}	4·41	50·58^{b}	1·50	26·59^{a}	8.55	45·36^{b}	4·52
Glyco	20·69^{a}	4·23	48·35^{b}	1·62	25·94^{ac}	8·50	39·91^{bc}	4·07
Tauro	1·02^{a}	0·38	2·23^{b}	0·48	0·64^{a}	0·15	5·45^{c}	0·50
Hyocholic	35·71^{a}	5·03	9·35^{b}	1·93	32·64^{a}	7·27	8·58^{b}	1·07
Glyco	34·78^{a}	4·69	9·14^{b}	1·78	32·36^{a}	7·28	8·03^{b}	1·10
Tauro	0·92^{a}	0·43	0·20^{a}	0·17	0·28^{a}	0·14	0·55^{a}	0·25
Glyco-3αOH-6oxo‡	3·21^{a}	0·40	10·42^{b}	1·46	4·23^{ac}	1·63	7·11^{bc}	0·85
Sum of secondary	24·99^{a}	4·65	61·48^{b}	2·06	30·86^{a}	9·80	52·64^{b}	4·67
Sum of glyco-conjugates	94·89^{a}	0·67	95·09^{a}	0·67	97·63^{b}	0·66	87·83^{c}	0·76
Hydrophobicity index	–0·028^{ab}	0·018	–0·079^{a}	0·018	–0·053^{ab}	0·019	0·010^{b}	0·029

^{a,b,c} Mean values within a row not sharing a common superscript letter were significantly different, $P < 0·05$ (ANOVA followed by Newman-Keuls test for multiple comparisons).

* Hydrophobicity indices calculated according to Heuman (1989).

† For details of diets and procedures, see Table 1 and p. 413.

‡ Glyco-3α-hydroxy-6-oxo-5β-cholanoic acid.

the incubated gall-bladder biles. Crystallization curves essentially differed between dietary groups. Biles from pigs fed on the CS32 diet started crystallizing first, followed by biles from the C16 dietary group, then those from the CS16 dietary group. Crystallization from group S16 bile was the most delayed. The maximum crystal growth rate (*c*, expressed as μg/ml per d, Table 6), measured over the equilibration period, was significantly lower in native biles from the pigs fed on the S16 or CS16 diets compared with the other two groups that consumed the C16 or the CS32 diet. In the latter two groups, crystal formation reached equilibrium within 3 d, as inferred from the plateau portion of the crystallization curves (Fig. 1(a)). In contrast, crystals continued forming at a slow rate for an additional 2 d in biles from the CS16 group and for an additional 5 d in the S16 group. As a result, the time required for maximum crystal growth rate (*m*, expressed as d, Table 6), where CHc reached half its value at equilibrium, was maximum for the S16 group, minimum for the two groups C16 and CS32 and intermediate for the CS16 group. Cholesterol crystallization time, classically defined as the day of first appearance of crystals under polarized light microscopy, was in the same order (Table 6). Finally, values for CHc at equilibrium (*b*, expressed as μg/ml, Table 6) were, in decreasing order: CS32 > C16 > CS16 = S16.

Cholesterol crystallization in artificial model bile enriched with biliary proteins

Fig. 1(b) displays increases in CHc as a function of time in

Table 4. Distribution (mol/100 mol) of lecithin species in gall-bladder biles of pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32)*

(Mean values with their standard errors for six pigs per diet)

Diet...	C16		S16		CS16		CS32	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Lecithin species								
18:2–18:2	5·03^{ab}	0·73	5·66^{a}	0·75	5·75^{a}	0·83	3·51^{b}	0·29
18:1–20:4	6·86^{a}	0·43	5·30^{a}	0·97	5·28^{a}	0·77	6·46^{a}	0·81
16:0–18:2	51·57^{ab}	1·39	49·66^{a}	1·74	54·28^{bc}	1·59	56·57^{c}	1·31
16:0–18:1 (18:0-20:4)	13·31^{a}	0·54	12·98^{ab}	0·26	11·42^{bc}	0·68	11·37^{c}	0·59
18:0–18:2	23·23^{a}	1·07	26·39^{b}	1·19	23·12^{a}	1·06	22·09^{a}	0·94

^{a,b,c} Mean values within a row not sharing a common superscript letter were significantly different, $P < 0·05$ (ANOVA followed by Newman-Keuls test for multiple comparisons).

* For details of diets and procedures, see Table 1 and p. 413.

Table 5. Daily faecal excretion of steroids (g/d) and distribution of faecal bile acids (mol/100 mol) in pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32)*

(Mean values with their standard errors for six pigs per diet)

Diet . . .	C16		S16		CS16		CS32	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Faecal excretion (g/d)								
Neutral steroids	3·34[a]	0·43	9·91[b]	1·24	5·76[ac]	1·38	6·55[c]	0·71
Bile acids	2·92[a]	0·52	3·47[ab]	0·38	4·66[b]	0·43	3·17[a]	0·22
Total steroids	6·26[a]	0·93	13·38[b]	1·47	10·42[b]	1·72	9·72[ab]	0·61
Distribution of faecal bile acids (mol/100 mol)								
Chenodeoxycholic	1·27[a]	0·24	4·63[bc]	0·79	2·78[ab]	0·64	4·97[c]	0·98
Hyodeoxycholic	65·73[a]	10·19	70·53[a]	3·58	52·83[a]	10·19	63·75[a]	4·66
Ursodeoxycholic	1·19[a]	0·47	0·78[a]	0·10	3·26[b]	1·06	1·56[ab]	0·58
Hyocholic	25·09[a]	9·98	3·71[b]	1·07	29·93[a]	10·13	3·22[b]	1·15
Lithocholic	2·92[a]	0·69	10·03[b]	1·68	5·01[a]	1·46	19·04[c]	2·12
Oxo acids	3·79[a]	0·47	10·32[b]	1·85	6·20[ab]	1·62	7·46[ab]	2·47

[a,b,c] Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (ANOVA followed by Newman-Keuls test for multiple comparisons).

* For details of diets and procedures, see Table 1 and p. 413.

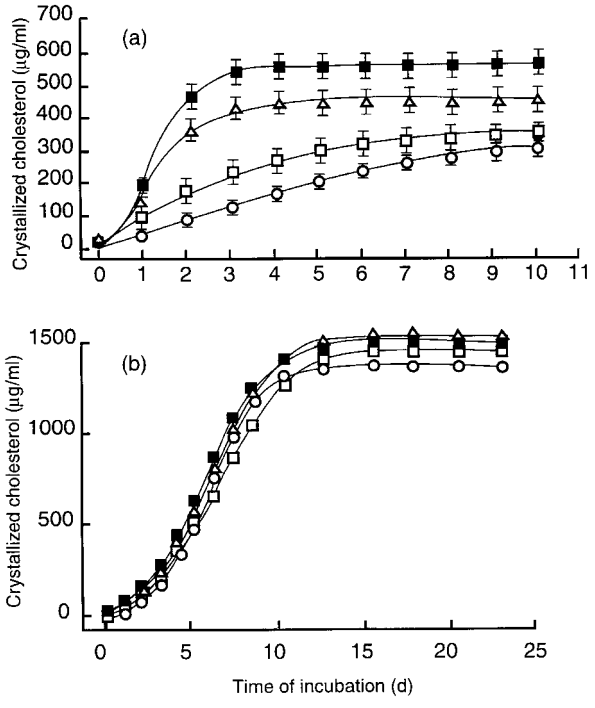

Fig. 1. Development of cholesterol crystals in (a) fresh gall-bladder biles and (b) supersaturated model biles enriched with biliary proteins purified from the same fresh gall-bladder biles. Gall-bladder biles were aspirated from pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (△), or as a soyabean-protein concentrate (○), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; □), or 320 g of the mixed protein/kg diet (■). Fresh gall-bladder biles and model biles were incubated *in vitro*, as described on p. 413. Values are means for six pigs per dietary group, with standard deviations represented by vertical bars (small standard deviations (Fig 1(b)) were obscured by the symbols). Data were fitted using a non-linear package on a Sun 670 MP Computer.

supersaturated model biles enriched with biliary proteins purified from gall-bladder bile of pigs adapted to the various protein diets. All model biles began crystallizing simultaneously, and the crystal growth rates did not differ significantly over the equilibration period. Likewise, microscopic observations did not allow discrimination between samples, which invariably formed first crystals on day 1 of incubation. Only the maximum CHc at equilibrium (*b*, expressed as μg/ml, Table 6) was affected by dietary protein. It was slightly, but significantly, lower in model biles enriched with biliary proteins from pigs fed on a soyabean-protein concentrate as the only protein supply, compared with the other three dietary groups (all three not significantly different). This occurred without any significant change in the total protein content of bile (Table 2).

Discussion

Soyabean-protein concentrate is high in protein but also contains many other constituents (Anderson & Wolf, 1995; Potter, 1995), which may have been responsible, at least partially, for the effects reported in the present work. However, in another study (Juste *et al*. 1997*a*), we demonstrated that soyabean isolate containing as much as 855 g protein/kg (with a concomitant decrease in other constituents) was still more effective than soyabean concentrate in decreasing cholesterol crystallization and LDL-cholesterol in pigs, thus suggesting a determining role of the protein fraction, rather than other constituents.

In the present study, both time-sequential enzymic determination of cholesterol crystals and concomitant microscopic observations demonstrated that replacing casein with soyabean concentrate as the only protein source (S16) considerably lowered cholesterol precipitation from pig bile. When this substitution was restricted to 30 % of the dietary protein supply (diet CS16), cholesterol crystallization was still efficiently decreased. These results are consistent with the demonstration of a decreased cholesterol gallstone risk in rabbits (Ozben, 1989) and hamsters

Table 6. Crystallization time as microscopically observed, and parameter estimates* for the cholesterol crystallization kinetics in gall-bladder biles of pigs fed on a semi-purified diet containing 160 g protein/kg diet as casein (C16), or as soyabean-protein concentrate (S16), or as a mixture of both protein sources (casein–soyabean protein, 70 : 30, w/w; CS16), or 320 g of the mixed protein/kg diet (CS32), and in model bile enriched with purified biliary proteins from the same pigs†

(Mean values and standard deviations from six observations)

Diet...	C16		S16		CS16		CS32	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Gall-bladder bile								
Crystallization time (d)	1·2[a]	0·5	6·0[b]	1·2	4·5[ab]	1·8	1·5[a]	0·9
Kinetic parameters‡								
b (μg/ml)	420[a]	18	296[b]	50	315[b]	25	530[c]	18
c (μg/ml per d)	227[a]	74	37[b]	11	60[b]	20	295[a]	80
m (d)	1·38[a]	0·16	3·75[b]	1·14	1·92[a]	0·54	1·34[a]	0·12
Model bile								
Crystallization time (d)	1·0[a]	0·0	1·0[a]	0·0	1·0[a]	0·0	1·0[a]	0·0
Kinetic parameters‡								
b (μg/ml)	1529[a]	15	1389[b]	15	1482[a]	16	1512[a]	15
c (μg/ml per d)	248[a]	30	223[a]	24	196[a]	20	233[a]	24
m (d)	6·17[a]	0·17	5·52[a]	0·17	6·43[a]	0·22	5·60[a]	0·17

[a,b,c] Mean values within a row not sharing a common superscript letter were significantly different, $P < 0·05$ (maximum likelihood ratio test).

* Values and SD were derived by mathematical modelling of crystallized cholesterol concentrations as sigmoidal functions of time.

† For details of diets and procedures, see Table 1 and p. 413.

‡ Parameters *b*, *c*, and *m* are interpreted as (*b*) maximum cholesterol crystal concentration at equilibrium, (*c*) maximum crystal growth rate and (*m*) time required for maximum crystal growth rate. For details of cholesterol crystallization monitoring in native and model bile, see p. 413.

(Kritchevsky & Klurfeld, 1983; Sullivan-Gorman *et al.* 1985; Trautwein *et al.* 1993) fed with soyabean protein in total or partial replacement of casein. It is of particular interest that, in our study, these diet-induced changes in the propensity of bile to crystallize cholesterol among groups supplied with 160 g protein/kg diet occurred without any significant change in total biliary lipid and protein content or in the relative proportions of bile acids, phospholipids, and cholesterol. Thus it can be argued that the S16 and CS16 diets acted as potent promoters of antinucleating qualitative effectors in pig bile, while the C16 diet induced potent pronucleating activity. Cholesterol crystallization, however, was still more substantial when a high-protein diet, even one containing soyabean concentrate (CS32), was administered to the animals. In this case, the observed low concentration of biliary phospholipids could contribute to promoting cholesterol precipitation.

We then wanted to identify the qualitative biliary effectors that could have participated in inducing those differences in cholesterol crystallization between groups. Today, biliary proteins are suggested to be cholesterol-crystallization modifiers and their 'balance' or 'imbalance' is thought to be essential for defining cholesterol precipitation from native bile (Catala *et al.* 1998). In the present study, however, we could not demonstrate any obvious diet-dependent differences in the crystallization-influencing activity of the protein bulk purified from native bile and assayed into reconstituted supersaturated model bile.

Molecular species of bile acids present in bile are another qualitative effector of cholesterol crystallization (Juste *et al.* 1995). In the present study, the respective proportions of the different molecular species of bile acids were consistently modified by the dietary protein source. As a result, the overall hydrophobicity index of the bile acid pool shifted towards decreasing hydrophobicity, according to the order CS32 > C16 > CS16 > S16, which coincided exactly with decreasing cholesterol crystal precipitation from the corresponding gall-bladder biles. Those variations in the hydrophobicity index, despite integrating various changes in the pattern of the bile acid pool, were essentially related to diet-induced fluctuations of chenodeoxycholic acid, the main hydrophobic bile acid in pig bile. The proportion of chenodeoxycholic acid was lower in gall-bladder biles from both groups S16 and CS16 exhibiting the lowest crystal growth rate. This corroborates other demonstrations in native (Dusserre *et al.* 1988; Trautwein *et al.* 1993; Juste *et al.* 1997*b*) and artificial (Salvioli *et al.* 1983; Dusserre *et al.* 1988; Donovan *et al.* 1989; Stolk *et al.* 1994; Juste *et al.* 1995; van de Heijning *et al.* 1995) biles where, for a given bile acid : phospholipid : cholesterol ratio and an unchanged total lipid concentration, as observed between the different dietary groups in our present study, the cholesterol-holding capacity of bile was greater in the presence of more hydrophilic bile acids. Another remark concerns the mechanism underlying these diet-induced fluctuations of biliary chenodeoxycholic acid. Between the three groups C16, CS16 and S16, decreasing levels of chenodeoxycholic acid in bile coincided with increasing excretion of this acid and its main bacterial product, lithocholic acid, in the faeces. It can thus be argued that chenodeoxycholic acid would be less efficiently recycled in pigs fed on soyabean-protein concentrate, as a result of (1) accelerated passage of digesta through the small intestine (van der Meer & Beynen, 1987; Beynen *et al.* 1990), where chenodeoxycholic acid would be less efficiently absorbed through passive diffusion (Aldini *et al.* 1996), and (2) increased bacterial conversion of chenodeoxycholic acid to poorly absorbable lithocholic acid in the hindgut. However, in the CS32 group, chenodeoxycholic acid increased both in bile and faeces. To explain this apparent paradox, it could be argued that the CS32 diet containing 224 g casein and 96 g soyabean protein/kg (1) had a C16-like effect in the small intestine,

as long as casein was not completely digested, and (2) had an S16-like effect in the hindgut. The latter assumption was reinforced by the analogy of the bile acid distribution in faeces from the CS32 and S16 groups. The tremendous increase in bacterial transformation of primary to secondary bile acids in response to a high consumption of soyabean-protein concentrate was also shown by the respective proportions of hyocholic acid and its bacterial products, hyodeoxycholic and 3α-hydroxy-6-oxo-5β-cholanoic acids, in bile from the CS32 and S16 groups. A tendency for secondary bile acid content to increase in the small intestine of pigs given soyabean isolate instead of casein, has already been reported (Scholz *et al.* 1985).

In our present study, faecal loss of total bile acids was essentially unchanged, regardless of the type of protein in the diet, whereas that of total neutral steroids increased with increasing intake of soyabean-protein concentrate (C16 < CS16 < CS32 < S16). In other studies, excretion of both neutral and acidic steroids was shown to increase with soyabean protein consumption (Kim *et al.* 1980; Fumagalli *et al.* 1982; Tanaka *et al.* 1984; van der Meer & Beynen, 1987; Beynen, 1990*a*,*b*). We previously demonstrated that β-cyclodextrin, when incorporated at a level of 50 g/kg in the diet, represented a major driving force for bile acid loss in the faeces, but interfered minimally with neutral steroid elimination (Férézou *et al.* 1997; Juste *et al.* 1997*b*). We therefore suggest that, in the present study, β-cyclodextrin may have masked the effect of the dietary protein supply on the faecal efflux of total acidic but not neutral steroids. In another study (C Juste, M Riottot and I Catala, unpublished results), we confirmed that bile acid synthesis, whether estimated using washout or isotopic measurements, was essentially the same in pigs fed on either the C16 or the S16 diet. It was, however, three times higher with soyabean-protein concentrate than with casein when no β-cyclodextrin was added to the diet.

Moreover, we found that biliary lecithin species varied in pigs fed on the different protein diets. As far as we know, there have previously been four other demonstrations of biliary phospholipid species being modified by dietary means, with a concomitant modification in the formation of cholesterol crystals or stones (Booker *et al.* 1989, 1990; Scobey *et al.* 1991; Juste *et al.* 1997*b*). However, whether differences in lecithin species were partly responsible for the variations in cholesterol precipitation in our study is uncertain, since (1) the respective proportions of the two *sn*-1-palmitoyl species exhibited changes between groups that did not coincide with crystallization values, and (2) the relevance of the specific variations in the 18:0–18:2 and 18:2–18:2 lecithin species cannot be inferred from the hitherto fragmentary information derived from model systems (Halpern *et al.* 1993; Konikoff *et al.* 1994; Tazuma *et al.* 1994). On the whole, among the qualitative crystallization effectors that we have investigated to date in the relevant biles, bile acid molecular species appears to be the best candidate for explaining the diet-induced differences in the crystallization rate.

Lastly, we found that a high protein intake (diet CS32) had a hypercholesterolaemic effect in pigs compared with a normal level of protein intake, and that soyabean-protein concentrate, either alone (S16) or mixed with casein (CS16), tended to decrease the plasma cholesterol level as compared with casein as the only protein source (C16). This corroborates other demonstrations of the hypercholesterolaemic action of high-protein diets in rabbits (Newburgh & Clarkson, 1923; Terpstra *et al.* 1981) and of the hypocholesterolaemic action of soyabean protein as compared with animal protein in man and a variety of animal models including pigs (Kim *et al.* 1978, 1980; van der Meer & Beynen, 1987; Erdman & Fordyce, 1989; Beynen *et al.* 1990; Carroll, 1991; Terpstra *et al.* 1994).

In conclusion, this study demonstrates the inhibitory influence of soyabean-protein concentrate as the only protein supply on cholesterol crystallization from bile and on cholesterol level in plasma. The effectiveness of a mixed casein–soyabean-protein (70 : 30, w/w) diet in delaying and reducing biliary cholesterol precipitation was also high, provided that the diet was not overly high in protein. This finding could be of interest in clinical nutrition. Substituting soyabean protein or other vegetable proteins for part of the normal animal protein supply in human Western diets could be an application, or at least a therapeutic adjuvant, for primary and recurrence prevention of cholelithiasis at the earliest stage of crystal formation.

Acknowledgements

We thank Roquette Frères for the generous gift of Kleptose. We are indebted to Dominique Besnard, Georgette Brachet, Francis Cointepas and Gérard Lebonnois for excellent animal management. We are grateful to Claire Chabanet for her precious assistance in statistical analysis, to Yvon Le Coz for preparation and maintenance of the sterile isolator and to Françoise Marcille and Jean Dabard for sterility controls. We thank Marc Weber for preparing figures and Michael Dever and Kirsteen Lynch for their advice on the English version of the text.

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