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Influence of dietary casein and soy protein isolate on intestinal cholesterol and bile acid concentration

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Summary

This study reports quantitative and qualitative differences in intestinal bile acids and cholesterol in miniature pigs following dietary casein or soy protein isolate. The total amount of bile acids in the small intestine was significantly higher when soy protein isolate was fed in comparison to casein. The values were (mean ± SEM) 4.51 ± 0.39 mmol and 2.43 ± 0.08 mmol, respectively, when the proteins were given as the sole component of the diet. When the proteins were given as part of a semi-purified diet, these values were 6.44 ± 1.04 mmol and 3.95 ± 0.39 mmol, respectively. Hyocholic acid amounted to 39.6 %, hyodeoxycholic acid to 31 %, and chenodeoxycholic acid to 27.6 % of total bile acids in the small intestine when casein was fed. The soy-fed animals tended to have more secondary bile acids.

The total small bowel chymus content, on a wet weight basis, was 63 % higher in the soy group. In all experimental conditions studied, there was a close correlation between small bowel chyme content and bile acid content. The distribution of bile acids in the small intestine showed that the soy fed animals tended to have more bile acids in the distal parts of the jejunum.

The intestinal cholesterol contents were not significantly different between dietary groups.

Zusammenfassung

In der vorliegenden Studie werden quantitative und qualitative Unterschiede im Gehalt an Gallensäuren und Cholesterin beschrieben, die im Dünndarm von Miniaturschweinen nach Diäten beobachtet wurden, die entweder Casein oder Sojaproteinisolat enthielten. Die intestinale Gesamtmenge an Gallensäuren war signifikant höher, wenn Sojaproteinisolat gefüttert worden war. Wurde das Protein als einzige Komponente verfüttert, so lagen die Werte bei 4.51 ± 0.39 mmol, während die Caseingruppe 2.43 ± 0.08 mmol aufwies. Wurde das Protein als Teil einer semisynthetischen Diät gegeben, waren die Werte für die Sojagruppe 6.44 ± 1.04 mmol und für die Caseingruppe 3.95 ± 0.39 mmol. Bei der Caseingruppe lagen die prozentualen Anteile an der Gesamtmenge der Gallensäuren im Dünndarm bei 39.6 % Hyocholsäure, 31 % Hyodeoxycholsäure und 27.6 % Chenodeoxycholsäure. Die Tiere, die Sojaproteinisolat bekamen, hatten in der Tendenz mehr sekundäre Gallensäuren.

Das Chymusfrischgewicht im Dünndarm lag in der Sojagruppe um 63 % über dem der Caseingruppe. Unter allen experimentellen Bedingungen wurde eine enge Beziehung zwischen dem Gehalt an Chymus und Gallensäuren im Dünndarm beobachtet. Die Sojagruppe wies in der Tendenz höhere Mengen an Gallensäuren im distalen Jejunum auf.

Die Soja- und Caseingruppe wiesen keine Unterschiede im Cholesteringehalt des Dünndarms auf.

Key words: pig, dietary protein, intestine, bile acids, cholesterol

Introduction

The hypercholesterolaemic effect of casein in comparison with soy protein is very species-dependent. In contrast to rodents, such as rabbits and rats (3, 32), hypercholesterolaemic effects of casein have been reported in pigs only when high amounts of cholesterol had been added to the diet (5, 11).

Several factors contribute to the manifestation of different serum cholesterol levels when soy protein or casein are fed (31, 28). Huff et al. (8) and Nagata et al. (18) have reported that the amino acid composition of the dietary protein plays a role. An influence of dietary protein on hormonal status, which might lead to changes in cholesterol metabolism, has been demonstrated by Sugano et al. (27). West et al. (33) have recently found that the structure of the protein is also involved.

There is evidence that soy protein and casein affect the enterohepatic circulation of steroids differently. In rabbits (9, 1), rats (19) and pigs (12) the cholesterol-lowering action of soy protein is accompanied by an increased fecal excretion of neutral and acidic steroids. Huff and Carroll (9) have shown a higher intestinal absorption of cholesterol, when casein was fed. Kritchevsky et al. (15) have reported that biliary steroid secretion in hamsters is affected by dietary protein.

In the present study we have focussed on the amount and distribution of neutral and acidic steroids in the small intestine of casein or soy-fed miniature pigs.

Materials and methods

Experiment I

Animals and diets

Six adult Göttingen miniature pigs, 4 male and 2 female, aged between 6 and 10 months and weighing between 15 and 30 kg, were used. They were kept individually in cages and maintained on a commercial pig diet (Sauen N, Raisdorfer Mühle, 2313 Raisdorf, West Germany) before they were transferred to a semipurified diet (Cas 22) for one week. The composition of Cas 22 was as follows (g/kg): Casein, 220; corn starch, 489; lard, 75; margarine, 75; cellulose, 60; mineral and vitamin mixture, 80; cholesterol, 0.6. The supply of vitamins and minerals with the diet was: vitamins (mg/kg): A, 17.5; D, 0.165; E, 300; K, 22.5; thiamin, 10; riboflavin, 20; pyridoxine, 19; B_{12}, 0.15; C, 875; pantothenic acid, 70; nicotinic acid, 83; choline 2750; folic acid, 6.25; biotin, 1.13 and of minerals (g/kg): Ca, 12.25; P, 5.75; Na, 3; Mg, 4,5; K, 4.25; Cl, 4.25 and as (mg/kg): Fe, 250; Zn, 250; Mn, 125; Cu, 75; I, 1.5; Se, 0.5; Co, 0.75. The pigs were then divided into 2 groups, which received 110 g casein (Biogen-Salzsäure-casein 5023, 15.5 % N, Bayerische Milchversorgungs GmbH, Nürnberg, West Germany) or 130 g soy isolate (Purina protein 610, 14.5 % N, Ralston Purina Company, USA) plus 150 g lactose, each, for 6 days. On day 7, the animals only received the protein plus 6 g polyethylene (Hostalen powder GM 2250 P, Ruhrchemie AG, Oberhausen, West Germany), an unabsorbable marker, which served to follow the

chyme flow. Water was provided *ad libitum*, food was provided twice a day, equivalent to an energy supply of 0.29 MJ/kg body weight and day.

Experimental procedure

One animal per group was anesthetized at 30, 180 and 310 min after feeding, using a combination of 0.1 ml/kg Stresnil (azaperone) and 0.25 ml/kg Hypnodil (metomidate) (Jansen GmbH, Düsseldorf, West Germany). The abdomen was opened; the whole small intestine was ligated *in situ* at each end. Then the duodenum was isolated by ligatures distal to the bile duct and at the flexura duodenojejunalis. Next, the ileum was isolated by ligatures at the beginning and end of the ligamentum ileocaecale. The jejunum was divided into 8 parts of approximately equal length. The parts were ligated as quickly as possible during the operation. This procedure was finished within 10 min. Subsequently, the animals were sacrificed. The intestinal contents were removed and each segment was washed twice with distilled water. The samples obtained after combining the three fractions were lyophilized before analysis.

Analyses

Bile acids were extracted from 0.2–0.5 g lyophilized material with 100 ml hot methanol in a Soxhlet apparatus for 5 hours. This time was found to give maximum bile acid extraction. Methanol was preferred instead of ethanol because of its higher extraction capacity (Fig. 1). The samples were evaporated to dryness and dissolved in 8 ml methanol. An aliquot was used for the enzymatic analysis of bile acids, using 3α-hydroxy-steroid dehydrogenase (3α-HSD, No. H-1506, Sigma Chemical Company, USA) according to (13). The coefficient of variation of 5 repeated samples was 10 %. Recovery of 26–260 µmol cholic acid (Serva, Heidelberg, W. Germany), added to the lyophilized chyme was better than 91 %, independent of whether the chyme was taken from soy or casein-fed animals. Cholesterol in the methanol extracts of small intestinal contents was determined enzymatically (20). Polyethylene (PE) was quantified in the freeze-dried chyme. The samples were boiled in a mixture of

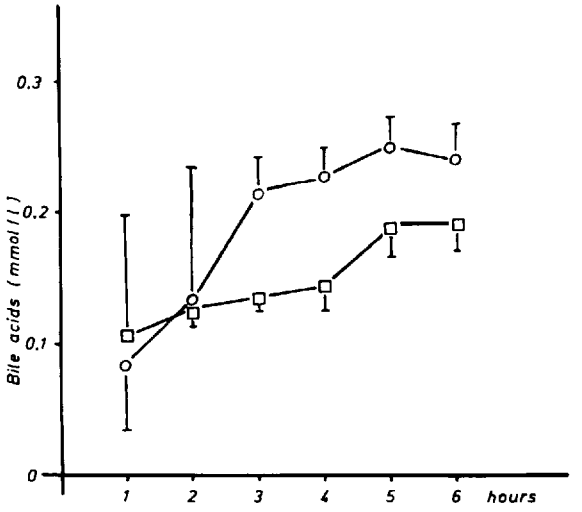

Fig. 1. Extraction of bile acids with methanol (0) or ethanol (□) as a solvent. Concentration of bile acids in the solvent after 1–6 hours of refluxing ($\bar{x} \pm SD$; n = 5).

concentrated HNO_3 and H_2SO_4 (2.5:1, v/v) for 4 h in order to dissolve all biological material. Subsequently, PE was separated from the acid by repeated washing with chloroform in a separating funnel. PE particles were washed with acetone and dried for 15 h at 105 °C. Polyethylene was calculated as the difference in weight before and after ashing.

Experiment II

Animals and diets

Ten noncastrated male Göttingen miniature pigs, aged between 6 and 8 months, with an initial body weight between 15 and 30 kg were used. The pigs were fed on the commercial pig diet before they were divided into 2 groups on the basis of their serum cholesterol concentrations and body weights. They were then maintained on the semipurified diet "Cas 22" (see Experiment I) for an adaptation period of 15 days. Subsequently, the animals were transferred to the experimental semipurified diets containing either casein (Cas C) or soy isolate (Soy C) for 4 weeks. The composition of the diets was the same as for Cas 22 except that 1 % (w/w) cholesterol was added. The housing and the feeding regimen were as described above.

Experimental procedure

Blood samples were taken from the jugular vein using a syringe after an overnight fast on days –15, –7, –1 (adaptation period), 14, 21 and 28 (experimental period). For the analysis of serum- and lipoprotein cholesterol, plasma was used. On the morning of day 28 of the experimental period, the animals received 50 % of their daily feed, but without cholesterol. 310 minutes after feeding, 3 animals per group were anesthetized. The experimental procedure and the analytical methods for the estimation of bile acids, cholesterol in chyme, serum and in lipoprotein fractions was as described above. The separation of very low density lipoproteins (VLDL) was achieved by flotation at 1.006 g/ml in an airfuge (2). High density lipoprotein (HDL) cholesterol was determined in the supernatant after adding 1.3 g/l of dextrane sulfate plus 0.13 mol/l $MgCl_2$ to whole plasma (17, 14).

In Experiment II the enzymatic analysis of bile acids in the whole small intestine was complemented by a quantification of individual bile acids by gas chromatography (4). For this the methanolic extracts were hydrolyzed enzymatically and purified (21) and the bile acids were derivatized for gas chromatography (4). 5β-cholanic acid and 7,12-diketolithocholic acid from Serva (Heidelberg, West Germany) were used as internal standards. Since almost no lithocholic acid and deoxycholic acid were detected in the chromatograms, the procedure was repeated with glycolithocholic and glycodeoxycholic acid (Sigma, St. Louis, MO, USA) as internal standard. A Carlo Erba model 4200 gas chromatograph equipped with a flameionisation detector and a 12-meter fused-silica capillary column coated with trifluoropropylsilicone as stationary phase were used. The carrier gas was hydrogen. The temperatures (°C) were: injector 240, oven 220 isotherm, and detector 262. To confirm qualitative and quantitative results, chromatograms were repeated on a 15 meter Durabond-225 fused silica column (J. u. W. Scientific, Rancho Cordova, CA, USA) and on a 2 meter column (2 mm diameter) packed with 3 % SP 2401 on Supelcoport 100/120 mesh (Supelco Inc., Bellefonte, PA, USA). The temperatures (°C) were: injector 248, oven 230 isotherm, and detector 272. Recoveries were 65 % for chenodeoxycholic acid and cholic acid, 72 % for lithocholic acid and 70 % for hyodeoxycholic acid.

Statistics

Student's two-tailed t-test was used (26). Results are given as means ± S.E.M.

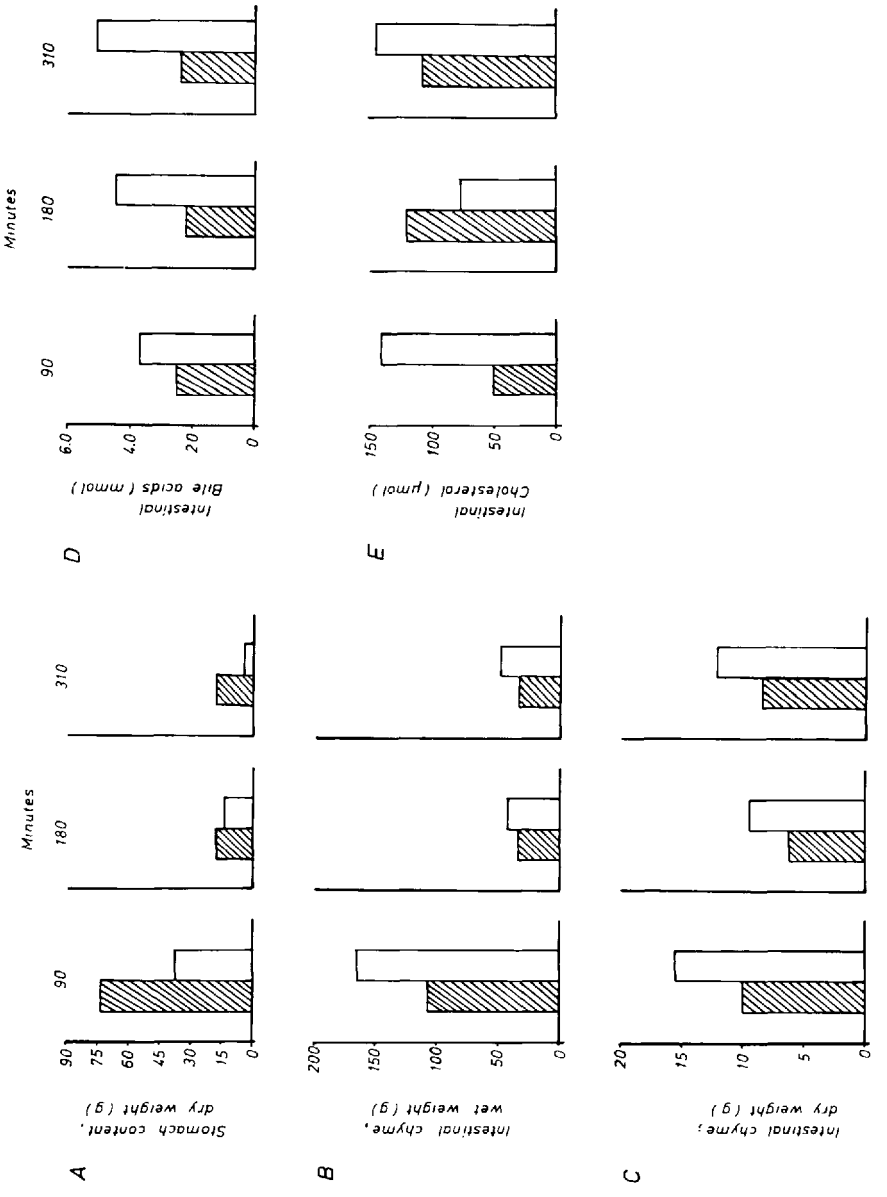

Fig. 2. Stomach contents (A), wet weight of chyme (B) and dry weight of chyme (C), bile acids (D) and cholesterol (E) in the small intestine of individual miniature pigs fed on a semipurified diet containing either casein (▨) or soy isolate (□). One animal per group was sacrificed 90, 180 and 310 minutes after feeding. For details see Exp. I.

Results

Experiment I

Intestinal contents: The stomach content (A), and the contents in the small intestine of chyme wet weight (B), chyme dry weight (C), bile acids (D) and cholesterol (E) for individual animals are shown in Fig. 2. The animals of the soy group had less chyme in the stomach but more in the intestine than the corresponding animals in the casein group. The amount of polyethylene (not shown) in the intestine of soy-fed animals was 38 % of the intake, whereas only 23 % was found when casein was fed. These findings indicate a more rapid passage of gastric contents to the intestine when soy protein is fed.

Cholesterol and bile acids: There was no difference in intestinal cholesterol content between both groups (Fig. 2 E). The intestinal bile acid content was higher in the soy-fed animals than in the corresponding animals of the casein group. This difference between soy protein and casein was true for every time point but most pronounced 310 min after feeding (Fig. 2 D).

It was typical for all 6 animals that not more than 9 ± 3 % of the total bile acids were found in the first third of the small intestine, whereas the amounts of bile acids in the second and last third were similar with 47 ± 9 % and 41 ± 17 %, respectively.

Experiment II

Body weight: The body weight gain was similar in both dietary groups, the initial body weight being 22.8 ± 4.9 kg in the casein group and 22.9 ± 5.3 in the soy group. At the end of the experiment the casein-fed animals weighed 24.9 ± 5.6 kg and the soy-fed animals 25.3 ± 5.2 kg.

Cholesterol in serum and lipoproteins: The cholesterol concentrations in serum and lipoproteins are presented in Table 1. Seven days after transfer of the animals to the cholesterol-containing diet, serum cholesterol concentrations increased in both the casein and soy group, but the rise was less pronounced in the soy group. On day 14 and on day 21 the difference between the casein-fed and the soy-fed animals was most marked. However, the difference in increase between the casein and soy group was not statistically significant.

The increment of serum cholesterol after feeding the experimental diets was mainly transported in the LDL fraction, to a lesser extent in the VLDL fraction, and to a slight extent in the HDL fraction (Table 1).

Intestinal contents: As described for Experiment I, the diet containing soy protein again caused higher amounts of chyme wet weight (79.27 ± 14.40 g) and dry weight 18.02 ± 3.10 g) in comparison to the casein diet (47.82 ± 6.61 g and 13.82 ± 1.42 g, respectively). These differences, although very pronounced, were not statistically significant.

Bile acids in intestinal contents: Figure 3 illustrates that miniature pigs fed on the diet containing casein had a much lower amount of intestinal bile acids with 3.95 ± 0.39 mmol than the animals on soy with 6.44 ± 1.04 mmol. This difference was statistically significant even with three animals ($p < 0.1$).

Table 1. Serum cholesterol concentration and lipoprotein cholesterol concentration in miniature pigs fed on a semisynthetic diet containing either casein or soy isolate ($\bar{x} \pm$ S. E. M.; n = 5).

		Cas 22	Cas 22 or Soy 22 + 1 % Cholesterol				
		day –7	day –1	day 7	day 14	day 21	day 28
					(mmol/l)		
Soy	Serum	2.48 ± 0.17	2.60 ± 0.18[1])	3.88 ± 0.99	4.02 ± 1.20	5.96 ± 1.61	5.40 ± 1.70
	VLDL		0.09 ± 0.02[1])		0.34 ± 0.24[1])		0.72 ± 0.44
	LDL		1.02 ± 0.23[1])		2.24 ± 0.91[1])		3.08 ± 1.58[1])
	HDL		1.26 ± 0.07[1])		1.40 ± 0.25[1])		1.31 ± 0.16[1])
Casein	Serum	2.10 ± 0.29	2.22 ± 0.15	4.96 ± 0.72	6.14 ± 1.10	7.34 ± 1.19	6.91 ± 1.65
	VLDL		0.08 ± 0.01		0.70 ± 0.17		1.07 ± 0.34
	LDL		0.80 ± 0.13		3.16 ± 0.76		3.18 ± 0.89[1])
	HDL		1.07 ± 0.07		1.53 ± 0.18		1.31 ± 0.25[1])
					Cas 22		
Control[2])	Serum		2.37 ± 0.20		1.80 ± 0.14		1.97 ± 0.25

[1]) n = 4
[2]) n = 3; For details see Exp. II.

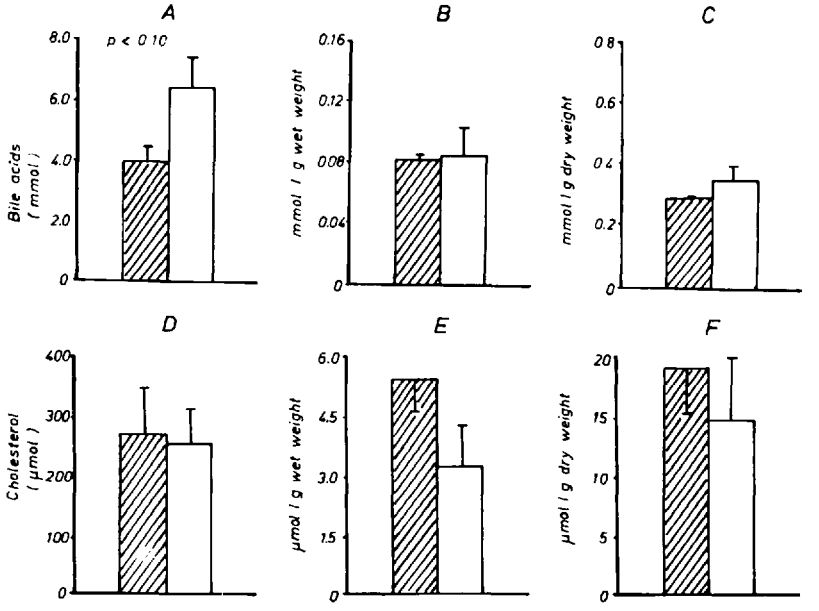

Fig. 3. Bile acids (A, B, C) and cholesterol (D, E, F) in intestinal contents of miniature pigs fed on a semipurified diet containing either casein (▨) or soy isolate (□). A and D: total content in the small intestine; B and E: concentration in chyme (wet weight). C and F: concentration in chyme (dry weight) (n = 3, $\bar{x} \pm$ S.E.M.). The animals were sacrificed 310 minutes after feeding. For details see Exp. II.

The chyme bile acid concentration was not influenced by the kind of protein: the increase in intestinal bile acid content of the soy group paralleled the increase in chyme wet weight.

As shown in Table 2 quantitative results of bile acids analyzed enzymatically or by gas chromatography were similar and the ratio of both with values between 0.85 and 1.10 was satisfactory. The main bile acids in casein- or soy-fed miniature pigs were chenodeoxycholic acid (27.6 and 30.5 % (w/w), respectively), hyodeoxycholic acid (30.0 and 39.6 %, respectively) and hyocholic acid (39.6 and 28.8 %, respectively). These three bile

Table 2. Intestinal bile acids for individual animals.

Method	Animal No. C_3	C_6	C_8	S_4	S_7	S_9
				mmol		
3 α-HSD (1)	4.64	3.28	3.94	4.38	7.66	7.27
Gaschromatography (2)	5.10	3.38	4.14	4.51	6.51	6.32
Ratio (2):(1)	1.10	1.03	1.05	1.03	0.85	0.87

C_3, C_6, C_8: Casein-fed animals
S_4, S_7, S_9: Soy-fed animals

Table 3. Composition of intestinal bile acids in casein- or soy-fed miniature pigs.

Bile acids	Casein (n = 3)	Soy (n = 3)
	% of total	
Chenodeoxycholic acid	27.6	30.5
Hyodeoxycholic acid	31.0	39.6
Hyocholic acid	39.6	28.8
Cholic acid	1.8	1.1

acids make up more than 98% of the bile acids. The soy-fed animals tended to have more secondary bile acids.

The distribution of bile acids in the small intestine is given in Fig. 4. In both groups a marked peak of the amount of bile acids was obvious. This peak was more pronounced in the distal parts of the small intestine when soy was fed. The concentration of bile acids was highest in the middle of the jejunum in both dietary groups. Independent of the diet, in each animal the first third of the gut contained only 10% of the total bile acids.

Cholesterol in intestinal contents: There was no statistically significant difference in total intestinal cholesterol (Fig. 3 D), cholesterol concentration on a wet weight basis (Fig. 3 E) nor cholesterol concentration on a dry weight basis (Fig. 3 F) between casein- and soy-fed animals.

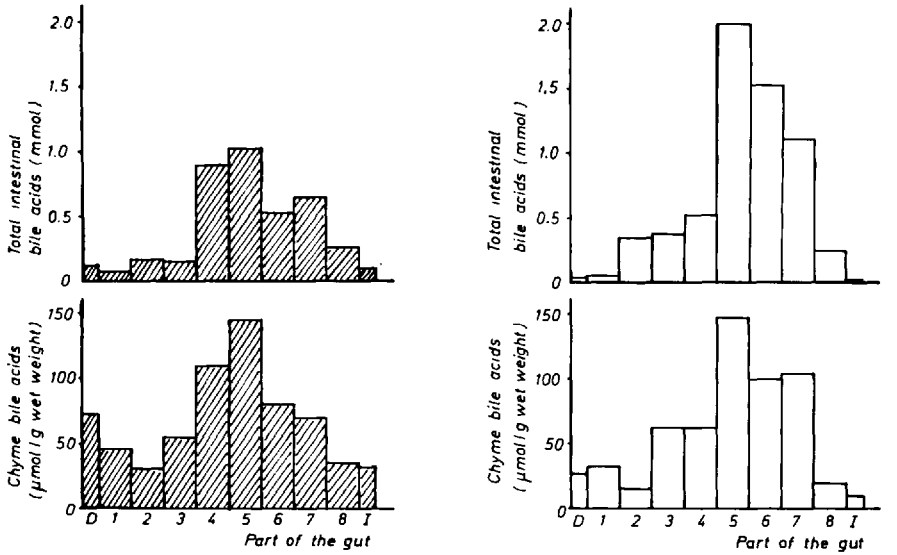

Fig. 4. Distribution of total bile acids and chyme bile acids in the small intestine of miniature pigs fed on a semipurified diet containing either casein (▨) or soy isolate (□). The parts of the gut indicate: D: duodenum; 1–8: parts of the jejunum of about equal length; I: ileum. Mean of 3 animals. The animals were sacrificed 310 minutes after feeding. For details see Exp. II.

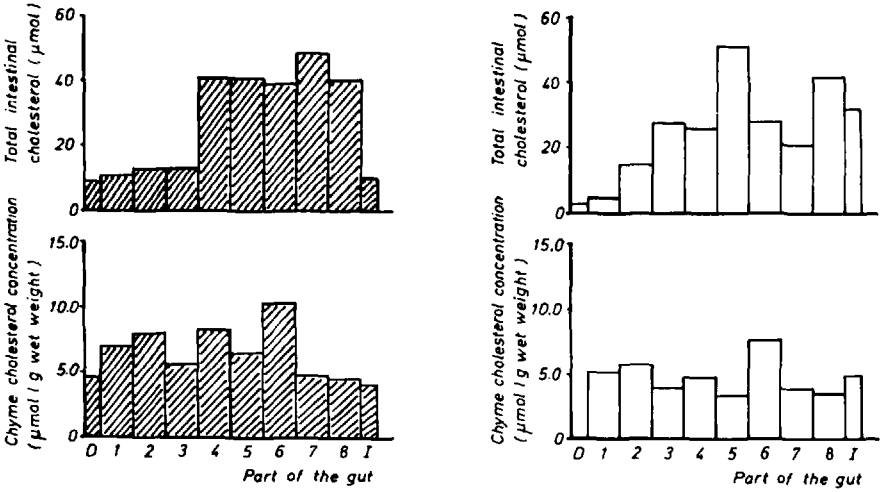

Fig. 5. Distribution of total cholesterol and chyme cholesterol concentration in the small intestine of miniature pigs fed on a semipurified diet containing either casein (▨) or soy isolate (□). The parts of the gut indicate: D: Duodenum; 1–8: parts of the jejunum of about equal length; I: ileum. Mean of 3 animals. The animals were sacrificed 310 minutes after feeding. For details see Exp. II.

As for bile acids (Fig. 4) low amounts of cholesterol were found in the first third of the small intestine (Fig. 5), independent of whether casein of soy protein isolate was fed. The concentration of cholesterol in the chyme on a wet weight basis did not differ very much in the different segments, either in the soy protein or in the casein group (Fig. 5).

Discussion

Several attempts have been made to explain serum lipid changes induced by different dietary proteins, through changes of fecal steroid excretion (32, 28). This assumption was supported by several investigators reporting a rise of fecal bile acids (12) and neutral steroids (9, 1) following an exchange of dietary casein by plant protein.

Such an assumption agrees with the general observation that steroid losses induced either by ileal by-pass, biliary diversion, anion exchange resin (6) or dietary fibre (10) are paralleled by lowered serum lipid levels. It was the purpose of this study to clarify the gastro-intestinal mechanisms mediating such changes of steroid metabolism following intake of casein or soy protein.

Our results clearly demonstrate that lower serum cholesterol levels, as found in soy-fed animals, are accompanied by increased amounts of chyme and bile acids in the small intestine, independent of whether the protein is fed as an isolated component (Experiment I) or in a semisynthetic diet supplemented with 30 energy % fat and 1% (w/w) cholesterol (Experiment II). This observation is similar to findings in mice where

more chyme and bile acids have been found in the small intestine after feeding of soy protein compared with casein (22).

Several explanations may be considered to explain this increase in the amount of chyme and bile acids in the small intestine produced by soy protein. It is unlikely to be produced by lower pre-caecal nitrogen digestibility because observations in fistulated pigs showed no statistically significant difference in pre-caecal nitrogen digestibilities (7).

There may be a higher secretion of biliary bile acids into the intestine. Three other findings do not support this assumption. Schneeman (23) did not observe a higher rate of biliary bile acid secretion following intraduodenal infusion of soy isolate. Similar findings were observed in our laboratory showing that bile flow and biliary bile acids and cholesterol were not statistically different 90 min postprandially in pigs fed either soy or casein (7). Thirdly in rats, Tanaka et al. (29) found no differences in biliary bile acid excretion and rate of bile flow when animals were fed cholesterol-free diets containing soy isolate or casein (29). The increase in the amount of chyme in the small intestine as observed in this paper suggests that the emptying of the stomach is enhanced by feeding soy protein. Moreover a higher secretory activity of either the intestinal mucosa and/or the excretory pancreas (22, 23) may be visualized as factors to contribute to higher chyme contents following soy. Morphological

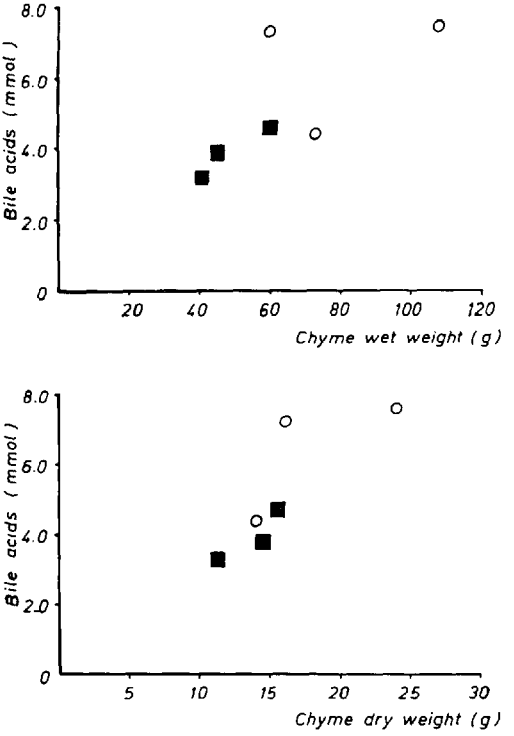

Fig. 6. Relation between amount of intestinal bile acids and chyme content of casein (■) or soy (0) fed miniature pigs. For details see Exp. II.

changes of intestinal mucosa in calves fed on a diet containing soy have indeed been observed (24). Such a mechanism is consistent with the higher dry and wet chyme content observed in our study caused by soy.

We therefore propose the following mechanism: more chyme in the small intestine caused either by the hypersecretory pancreas and mucosa or by an enhanced emptying of the stomach, would function as a trap for bile acids. Either this larger amount of chyme would then cause a diffusion barrier for the bile acids, or the ileal bile acid concentrations are less than is necessary to saturate the active transport system (16). Both alternate mechanisms would cause a limited ileal reabsorption velocity so that more bile acids by-pass the ileal salvage and show up in the feces. Which of these alternate modes of action is correct is presently under investigation in this laboratory.

There was a close correlation between chyme wet and dry weight and the intestinal bile acid pool in our experiments, independently of whether casein or soy was fed (Fig. 6). This does not support the assumption that specific binding of bile acids to chyme constituents, including dietary protein, is important for the higher intestinal trapping of bile acids following soy. This is in agreement with reports of Woodward and West (34) and Sklan et al. (25). These investigators did not find evidence that *in vitro* binding of cholate to soy is stronger than to casein or that the former has a particular inhibitory effect on cholate uptake by avian duodenal loops.

In contrast to the bile acids, total intestinal cholesterol content was not statistically different (Fig. 3). Similar results have been reported by Tanaka et al. (30), who have investigated the effect of soy protein or casein on cholesterol and coprostanol excretion and their contents in the gut of rats. There was no difference between the 2 types of protein when total neutral steroids were compared in different parts of the gut. These findings indicate that the lower serum cholesterol values of the soy-fed animals must have been mediated by other mechanisms than only lower absorption of dietary cholesterol.

In conclusion, the data reported are in full agreement with the proposal that alterations of intestinal bile acid physiology are involved in the mechanism mediating the different response of serum lipids to dietary protein. The most significant finding needing further investigation was the parallel rise of chyme wet and dry weight and small intestinal bile acid pool size following the feeding of soy protein isolate.

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