Digestibility of casein, formaldehyde-treated casein and soya-bean protein in relation to their effects on serum cholesterol in rabbits

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Adult male rabbits were fed on semi-purified diets containing soya-bean protein isolate, casein or formaldehyde-treated casein as the protein source and 1 g cholesterol and 5 g of the non-absorbable marker chromic oxide/kg diet. The concentration of cholesterol in serum and in liver was increased on both the casein and formaldehyde-treated-casein diets. Excretion of bile acids and their concentration in faeces were lower in rabbits fed on casein or formaldehyde-treated casein when compared with rabbits fed on soya-bean protein. Apparent digestibility of nitrogen was lowest when formaldehyde-treated casein was fed, and highest on the casein diet. In rabbits fed on casein treated with formaldehyde, higher proportions of N were found in the water-soluble and trichloroacetic acid-insoluble protein fractions of the gastrointestinal tract contents compared with rabbits on the other two diets. Absorption of phosphate from the gastrointestinal tract was higher in rabbits fed on casein than in rabbits fed on soya-bean protein or formaldehyde-treated casein. The results indicate that, in rabbits, protein digestibility may not be an important determinant of serum cholesterol.

Cholesterol: Protein digestibility: Rabbit

Formaldehyde treatment of proteins causes cross-linking of the protein chains by coupling the free amino groups of lysine (Walker, 1964). Such treated proteins, so-called protected proteins, have been introduced in ruminant nutrition in order to protect the dietary protein against destruction by micro-organisms in the rumen and thus making amino acids available to the intestines. The protein adducts formed with formaldehyde are stable at pH 6–7 in the rumen but are broken down at pH 3 in the stomach, thereby generating protein which then becomes available to the ruminant. However, intense treatment with relatively high amounts of formaldehyde produces proteins that are poorly digested in both ruminants and non-ruminants (Kaufmann & Lüpping, 1980). West *et al.* (1984) have shown that excessive formaldehyde treatment of casein reduces its hypercholesterolaemic effect in rabbits fed on cholesterol-free diets. They suggested that this effect may be due to the observed reduced digestibility of the protein caused by formaldehyde treatment. Since undigested protein is able to bind bile acids, as has been shown in in vitro experiments

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(Sklan *et al.* 1979; Woodward & West, 1984), bile acid re-absorption in the distal part of the intestine would be partly inhibited. Thus, theoretically the lower serum cholesterol levels in rabbits fed on formaldehyde-treated casein would be the result of increased faecal excretion of bile acids (West *et al.* 1984). Since cholesterol is the substrate for the synthesis of the acids, an increased loss of bile acids in faeces would deplete body cholesterol. A similar mechanism is most likely responsible for the hypocholesterolaemic action of soya-bean protein which causes increased rates of bile acid excretion in rabbits (Huff & Carroll, 1980; Kuyvenhoven *et al.* 1986; Van der Meer *et al.* 1988).

In rats fed on either cholesterol-free or high-cholesterol diets the hypocholesterolaemic effect of soya-bean protein, when compared with casein, is also associated with increased rates of faecal bile acid excretion (Beynen *et al.* 1986). Surprisingly, feeding rats on cholesterol-enriched diets containing formaldehyde-treated casein produced higher concentrations of serum cholesterol than when native casein was given (Beynen *et al.* 1985). Formaldehyde treatment of casein markedly reduced its apparent digestibility (Beynen *et al.* 1985).

In the present experiment rabbits were fed on semi-purified, cholesterol-enriched diets containing soya-bean protein, casein or formaldehyde-treated casein. The objectives were as follows. First, we wanted to see whether the lack of cholesterol-lowering effect of formaldehyde-treated casein in rats fed on high-cholesterol diets (Beynen *et al.* 1985) extends to rabbits, as in this animal species fed on cholesterol-free diets, formaldehyde treatment of casein reduces its hypercholesterolaemic action (West *et al.* 1984). Second, we wished to test further whether the hypercholesterolaemic action of casein resides in its high degree of digestibility.

MATERIALS AND METHODS

Feeding trial and collection of samples

In the present experiment, thirty adult male SPF rabbits of the New Zealand White strain were used (Broekman Institute, Helmond, The Netherlands). The rabbits were kept individually in cages with wire-mesh bases in a room with controlled lighting (12 h/d), constant temperature (18°) and relative humidity (60%). Up until 2 weeks before the experimental period when the animals were 36 weeks of age, they were maintained on commercial rabbit pellets (Trouw & Co. B. V., Putten).

Two weeks before the experimental period, the rabbits were transferred to a cholesterol-free semi-purified diet containing soya-bean protein isolate (Ralston Purina Co., St Louis, MO 63188, USA). The rabbits were allocated to one of the experimental diets (ten animals per diet) on the basis of their serum cholesterol concentrations measured 6 d before the start of the experimental period. The experimental diets contained soya-bean protein isolate, acid casein (DMV, 5466 BA Veghel, The Netherlands) or acid casein treated with formaldehyde (Table 1). Formaldehyde-treated casein was prepared as described by West *et al.* (1984). Food was provided on a restricted basis (70 g/d) for a period of 3 h each day in order to achieve uniformity in the degree of digestion at the time the rabbits were killed. Tap water was provided *ad lib.*

The animals were weighed weekly. Blood samples were taken before feeding between 08.00 and 09.00 hours from a marginal ear vein into tubes without anticoagulant. Faeces were collected from all animals on days 31–35 of the experiment. On day 37, five randomly chosen animals per dietary group were killed by cervical dislocation. The other fifteen animals were kept for another 2 weeks. In the final week, these rabbits were fitted with a collar to prevent coprophagy. Faeces of rabbits fitted with a collar were also collected over days 45–49 and over the 4 d before fitting the collar (days 38–42). All rabbits were killed by cervical dislocation immediately after consuming their last meal. Liver and the contents

Table 1. *Composition of diets**

Diet... Ingredients	Soya-bean protein	Casein	Formaldehyde-treated casein†
Soya-bean protein	307	—	—
Casein	—	310	—
Formaldehyde-treated casein‡	—	—	350
Methionine	3	—	—
Sawdust	175	172	132
Sodium chloride	5	8	8
Chromic oxide§	5	5	5
Cholesterol‖	1	1	1
Constant components¶	504	504	504
Total	1000	1000	1000

* Expressed as g/kg feed. Weight after freeze-drying: 940, 940 and 930 g/kg feed for the three diets respectively.

† Based on the lower in vivo digestibility of the formaldehyde-treated casein preparation determined earlier (West *et al.* 1984); the proportion of protein in the formaldehyde-treated casein diet was increased at the expense of sawdust.

‡ The concentration of total and bound formaldehyde was found to be 17·6 and 11·6 g/kg treated casein respectively. Formaldehyde measurement was performed as described by West *et al.* (1984).

§ The concentration, determined by analysis was 5·1, 5·1 and 5·2 g/kg feed for the three diets respectively.

‖ The concentration, determined by analysis, was 0·9, 1·0 and 1·0 g/kg feed for the three diets respectively.

¶ The constant components consisted of (g/kg diet): starch 130, glucose 150, coconut fat 90, soya-bean oil 10, molasses 50, dicalcium phosphate 29, potassium bicarbonate 18, magnesium carbonate 3, magnesium oxide 2, vitamin premix 12, mineral premix 10. The composition of the vitamin and mineral premixes has been described previously (West *et al.* 1984).

of the stomach and colon, and a sample of the contents of the caecum were collected and weighed. The small intestine was removed and divided into four segments of equal length, which were emptied, washed with cold water and dried. Each segment was weighed before emptying and after drying to determine the wet weight of the contents.

Food, faeces and contents of stomach, caecum and colon were lyophilized and ground. Livers were homogenized with a tissue homogenizer (model PT 10/35; Polytron Kinematica GmbH, Switzerland) and made up to a total volume of about 200 ml with distilled water. Contents of intestinal segments were homogenized in a similar way, in a total volume of about 40 ml. The homogenates of livers and intestine segments were stored at −20°.

Analytical methods

Treatment of casein with formaldehyde, determination of formaldehyde in the protein preparations and determination of serum cholesterol were carried out as described previously (West *et al.* 1984). For determination of liver cholesterol, samples of liver homogenate were extracted according to Folch *et al.* (1957). Lipid extracts were taken to dryness under a stream of nitrogen, dissolved in 100 μl of an aqueous solution containing 100 ml polyoxyethylene-9-laurylether (Sigma) and 125 ml propan-2-ol/l, and incubated at 50 ° for 30 min. Total cholesterol was then determined with the CHOD-PAP kit supplied by Boehringer Mannheim (FRG). Three samples of liver homogenates containing cholesterol in the range of concentrations expected were used as calibration standards. The concentration of cholesterol in these calibration standards was measured as follows. The lipids in the liver homogenates were saponified in methanolic sodium hydroxide (2 M) for 2 h after which the lipids were extracted with light petroleum (b.p. 60–80°). Neutral steroids were subsequently derivatized to their trimethylsilylethers in a 2:1 (v/v) mixture of *N*,*N*-dimethylformamide/bis-trimethylsilyltrifluoracetamide at 80° for 30 min, and

analysed on a gas–liquid chromatograph (model 439, Hewlett Packard) fitted with a Sil 5 capillary column (Chrompack, Middelburg, The Netherlands). Two calibrated serums were included as a control.

Bile acids were determined as described by Glatz *et al.* (1985). Since most of the bile acids in the small intestine were found by thin-layer chromatography (Glatz *et al.* 1985) to be present as their glycine conjugates, it was necessary to introduce a hydrolysis step into the method for the determination of bile acids in this material. The hydrolysis step was also used for the analysis of the caecum samples, but proved to be unnecessary for faeces. A portion of homogenized contents of small intestine (1 ml) or caecum (0·3 g) was suspended in 2 or 4 ml respectively of 100 mM-acetate buffer, pH 5·7, containing 20 mM-EDTA and 1 mM-1,4-dithiothreitol. The suspension was incubated overnight at 37 ° with 250 μg of an extract of a crude acetone powder of *Clostridium perfringens* (C3636; Sigma). At the end of the incubation period, pellets of sodium hydroxide to produce a final concentration of 5 M-NaOH were added followed by 2 vol. methanol containing an internal bile acid standard. For the analysis of bile acids in faeces, a portion of faeces (0·5 g) was suspended in 5·5 ml of 1·1 M-NaOH in aqueous methanol (760 ml/l) containing the internal bile acid standard. The suspensions of intestinal or caecal contents or of faeces were heated under reflux at 80 ° for 2 h. The neutral sterols were subsequently extracted with light petroleum (b.p. 60–80 °). The methanol in the residue was evaporated under N_2 and 12 M-hydrochloric acid was added to reduce the pH below 1. Bile acids were extracted with diethyl ether, derivatized to their trimethylsilylethers (Glatz *et al.* 1985) and analysed on a gas–liquid chromatograph (model 439; Hewlett Packard) fitted with a Sil 19 capillary column (Chrompack, Middelburg). Qualitative and quantitative analyses were performed with the use of a calibration mixture containing lithocholic acid, deoxycholic acid, cholic acid, chenodeoxycholic acid, urso-deoxycholic acid and 12-ketolithocholic acid. Initially 7-ketodeoxycholic acid was used as an internal standard, but because of the instability of keto-bile acids it was replaced by ursocholanic acid. However, no significant difference was found between the results obtained using the two standards.

Samples of intestinal contents were dried for the determination of dry matter before analysis. N was measured by the Kjeldahl method (Henry *et al.* 1974) in the intestinal contents and faeces. Analyses were carried out on the total fractions and also on the water-insoluble, trichloroacetic acid (TCA)-insoluble and TCA-soluble fractions. The TCA-insoluble fraction was obtained by centrifugation at 1500 ***g*** for 10 min after the addition of TCA to a final concentration of 100 g/l. Chromic oxide was determined as described by Williams *et al.* (1962) using an atomic absorption spectrophotometer (model 2380; Perkin Elmer). For the determination of phosphate and calcium, samples containing 25 mg faeces or 0·25 ml homogenized intestinal contents, to which 1 ml perchloric acid (700 g/l) was added, were digested at 180–200 ° for 2 h. When cool, 6 ml distilled water was added, and 70 μl of this solution was diluted with 2 ml of a solution containing TCA (50 g/l) and strontium (3 g/l) added as strontium sulphate before analysis by atomic absorption spectrophotometry. Phosphate was determined by the colorimetric assay described by Fiske & Subbarow (1925), and Ca by atomic absorption spectrophotometry.

Statistical analyses

Since most of the values were not distributed normally, the parameter-free Kruskal–Wallis rank statistics test was used to analyse for differences between groups based on ranks (Miller, 1966).

Table 2. *Effect of giving diets containing soya-bean protein, casein and formaldehyde-treated casein (F-casein) on feed consumption, body-weight and concentration of cholesterol in serum and liver of rabbits**

(Results are means with their standard errors for ten rabbits from each group, except for liver cholesterol which is based on five rabbits per group)

Diet...	Soya-bean protein		Casein		F-casein	
	Mean	SE	Mean	SE	Mean	SE
Feed consumption† (g/d)	59	4	49	3	57	4
Body-wt (kg)						
Day 0	3·38[a]	0·06	3·27[a]	0·04	3·38[a]	0·05
Day 35	3·40[a]	0·07	3·19[a]	0·07	3·31[a]	0·08
Serum cholesterol (mmol/l)						
Day	1·36[a]	0·14	1·34[a]	0·19	1·41[a]	0·22
Increase in serum cholesterol (mmol/l)‡	1·58[a]	0·30	2·97[b]	0·49	3·50[b]	0·71
Liver cholesterol (mmol/kg wet liver)	1·07[a]	0·8	13·9[b]	1·7	18·6[b]	2·0

[a, b] Values with unlike superscript letters were significantly different ($P < 0·05$) between treatments.

* For details of diets, see Table 1.

† Mean feed consumption per rabbit from day 0 to day 37.

‡ Change between days 6 and 37.

RESULTS

Table 2 shows the influence of dietary protein on feed consumption, body-weight and the concentration of cholesterol in serum and liver. Although feed consumption tended to be somewhat lower on the diet containing casein, the difference did not reach statistical significance. The body-weight of the rabbits remained constant throughout the experiment in all dietary groups. The diets containing casein and formaldehyde-treated casein induced elevated levels of serum cholesterol and these changes paralleled the concentration of liver cholesterol.

Excretion of bile acids was reduced in rabbits fed on casein or formaldehyde-treated casein when compared with rabbits fed on soya-bean protein (Table 3). This effect was accompanied by a change in the proportions of the individual bile acids. In rabbits fed on native or formaldehyde-treated casein the relative proportions of 12-ketolithocholic acid and deoxycholic acid were decreased and increased respectively.

There was a small but significant difference in apparent digestibility of N between rabbits fed on soya-bean protein and those fed on casein when the results of all rabbits not fitted with a collar were considered (Table 4). This difference was not observed in the rabbits fitted with collars to prevent coprophagy. As expected, treatment of casein with formaldehyde clearly reduced its digestibility both in the presence and absence of a collar. The fitting of a collar also resulted in significantly lower apparent digestibility of N in all dietary groups. The differences in apparent digestibility reflected differences in the in vitro digestibility as measured with pepsin. The pepsin digestibility of casein was slightly higher than that of soya-bean protein, while the formaldehyde treatment markedly reduced the digestibility of casein (Table 4).

Table 5 shows values relative to the non-absorbable marker Cr_2O_3 for dry matter, bile acids, total N, Ca and phosphate in various regions of the gastrointestinal tract and in faeces. For the contents of the various intestinal segments and of the caecum, there was no difference in the bile acids:chromic oxide ratio between the rabbits on the soya-bean-

Table 3. *Effect of giving diets containing soya-bean protein, casein and formaldehyde-treated casein (F-casein) on faecal excretion of bile acids in rabbits**

(Results are means with their standard errors for ten rabbits from each group on days 31–35)

	Relative percentage of individual bile acids								Total excretion (μmol/d)	
	Lithocholic		Deoxycholic		12-Ketolithocholic		Others†			
Diet	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Soya-bean protein	17[a]	1	47[a]	1	26[a]	2	11[a]	1	98[a]	6
Casein	21[a]	2	56[b]	1	12[b]	1	11[a]	1	61[b]	5
F-Casein	22[a]	2	54[b]	2	14[b]	1	11[a]	1	58[b]	6

[a, b] Values with unlike superscript letters were significantly different ($P < 0.01$) between treatments.
* For details of diets, see Table 1.
† Includes cholic, chenodeoxycholic, isolithocholic, isochenodeoxycholic and isodeoxycholic acids.

Table 4. *In vitro and in vivo digestibility of nitrogen in semi-purified diets containing soya-bean protein, casein or formaldehyde-treated casein (F-casein)**

(Results are means with their standard errors for no. of rabbits given in parentheses, where appropriate)

	Pepsin-digestible protein in vitro (g N/kg N)†		Apparent digestibility of N in vivo (g N/kg N)‡			
			All rabbits without collars (10)		Rabbits with collars (5)	
Diet	Mean	SE	Mean	SE	Mean	SE
Soya-bean protein	771[a]	4	897[a]	5	851[a]	8
Casein	799[b]	4	931[b]	3	820[b]§	34
F-Casein	250[c]	0	733[c]	15	504[b]	36

[a, b, c] Values with unlike superscript letters were significantly different ($P < 0.05$) between the treatments.
* For details of diets, see Table 1.
† Digestibility determined in vivo in triplicate as described earlier (West *et al.* 1984).
‡ Apparent digestibility of N was calculated by dividing the difference between the amount of N in the diet and faeces by the amount in the diet. The N content of the faeces was corrected for the recovery of dietary chromic oxide. Digestibility was determined in all rabbits without a collar on days 31–35 and in half the rabbits fitted with a collar to prevent coprophagy on days 45–49.
§ Mean from four rabbits because one rabbit refused to eat while fitted with a collar.

protein diet and those on the casein diet. However, the ratio was significantly lower in the faeces of rabbits fed on casein compared with their counterparts fed on soya-bean protein. Formaldehyde treatment of casein reduced the ratio in intestinal segments 2 and 3, in the caecum and in faeces. The decrease in the bile acids:chromic oxide ratio along the gastrointestinal tract from segment 1 to faeces was found to be similar for all dietary groups. Almost all the bile acids in the intestine consisted of deoxycholic acid and lithocholic acid, with the former making up about 80–85% of the total. These proportions were comparable for all diets (values not shown).

In all dietary groups there was a decrease in the total N:Cr_2O_3 ratio in the stomach when compared with the diet, followed by a large increase in the ratio in segment 1 of the small intestine when compared with the stomach (Table 5). This phenomenon is indicative of retention of chromic oxide in the stomach and production of a relatively large amount of

Table 5. *Dry matter, bile acids, total nitrogen, calcium and phosphate relative to chromic oxide in various regions of the gastro-intestinal tract and in faeces of rabbits fed on semi-purified diets containing soya-bean protein, casein or formaldehyde-treated casein (F-casein)**

(Results are means with their standard errors for five rabbits per sample (rabbits without collars) except for faeces for which the results refer to ten rabbits (all rabbits))

		Dry matter (g/kg wet wt)		Dry matter: Cr_2O_3 (g/mg)		Bile acids: Cr_2O_3 (μmol/mg)		Total N: Cr_2O_3 (mg/mg)		Ca: Cr_2O_3 (μmol/mg)		Phosphate: Cr_2O_3 (μmol/mg)	
Diet	Sample	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Soya-bean protein	Diet	940		0·2		—		9·4		46·9		52·6	
	Stomach	284[a]	16	0·1[a]	0·0	—		4·9[a]	0·6	—		—	
	Segment 1†	136[a]	8	1·3[a]	0·3	59·6[a]	18·4	123·4[a]	31·6	84·3[a]	14·5	—	
	Segment 2	132[a]	8	0·7[a]	0·2	36·2[a]	7·9	67·0[a]	16·2	61·1[a]	7·6	—	
	Segment 3	128[a]	5	0·3[a]	0·0	25·5[a]	4·0	24·1[a]	5·4	49·2[a]	4·4	144·5[a]	19·9
	Segment 4	174[a]	19	0·2[a]	0·0	15·4[a]	4·1	16·1[a]	4·7	45·7[a]	4·8	144·8[a]	45·4
	Caecum	236[a]	6	0·1[a]	0·0	1·0[a]	0·3	5·0[a]	0·4	—		—	
Casein	Diet	940		0·2		—		10·0		43·4		56·8	
	Stomach	360[a]	18	0·1[a]	0·0	—		4·4[a]	0·4	—		—	
	Segment 1	146[a]	9	2·3[a]	0·7	124·5[a]	35·9	238·4[a]	78·3	173·5[a]	61·5	—	
	Segment 2	148[a]	6	0·7[a]	0·1	39·0[a]	6·6	63·7[a]	13·0	82·7[a]	23·5	—	
	Segment 3	150[a]	9	0·3[a]	0·1	21·4[a]	3·9	24·6[a]	8·6	50·1[a]	8·6	145·5[a]	37·4
	Segment 4	198[a]	36	0·3[a]	0·1	17·3[a]	6·8	26·2[a]	13·2	60·5[a]	18·7	197·8[a]	78·6
	Caecum	226[a]	15	0·1[a]	0·0	1·3[a]	0·1	5·4[b]	0·7	—		—	
	Faeces					0·3[b]	0·0	0·7[b]	0·0	33·2[a]	0·9	37·1[b]	0·9
F-casein	Diet	930		0·2		—		10·3		44·2		55·5	
	Stomach	356[b]	7	0·1[a]	0·0	—		5·5[a]	0·3	—		—	
	Segment 1	146[a]	10	0·9[b]	0·2	51·2[a]	14·8	82·9[a]	20·1	134·7[a]	32·9	—	
	Segment 2	163[a]	18‡	0·4[a]	0·0	12·0[b]	2·2	30·1[a]	5·4	64·5[a]	10·7	—	
	Segment 3	164[a]	16	0·2[a]	0·0	8·8[b]	2·0	10·5[a]	1·7	43·2[a]	6·3	75·0[b]	8·1
	Segment 4	186[a]	22	0·1[a]	0·0	6·6[a]	2·2	9·0[a]	1·6	48·8[a]	8·7	67·6[b]	10·4
	Caecum	260[a]	13	0·1[a]	0·0	0·6[b]	0·2	6·2[a]	0·2	—		—	
	Faeces	626[c]	20	0·1[b]	0·0	0·2[c]	0·0	2·7[c]	0·2	34·2[a]	1·2	40·3[a]	1·1

[a,b] Values with unlike superscript letters were significantly different ($P < 0·05$) between treatments.

* For details of diets, see Table 1.

† The small intestine was divided into four parts of equal length (see text): segment 1 refers to the most proximal part and segment 4 refers to the most distal part.

‡ Mean from three rabbits.

Table 6. *Effect of giving diets containing soya-bean protein, casein and formaldehyde-treated casein (F-casein) on the absorption of calcium and phosphate from the diet**

(Results are means with their standard errors for ten rabbits from each group)

	Intake (mmol/d)				Excretion† (mmol/d)				Percentage absorbed			
	Ca		Phosphate		Ca		Phosphate		Ca		Phosphate	
Diet	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Soya-bean protein	12·5[a]	0·7	14·0[a]	0·9	8·9[a]	0·4	10·8[a]	0·5	27·2[a]	2·5	22·2[a]	1·4
Casein	9·9[b]	0·4	13·0[a]	0·5	7·6[b]	0·4	8·5[b]	0·5	23·6[a]	1·9	34·8[b]	1·7
F-casein	12·0[a]	0·7	15·1[a]	0·9	9·3[a]	0·6	11·0[a]	0·7	22·4[a]	2·8	27·7[a]	2·0

[a, b] Values with unlike superscript letters were significantly different ($P < 0·05$) between treatments.

* For details of diets, see Table 1.

† Ca and phosphate excretions were calculated on the basis of the excretion of chromic oxide which had been added to the diet.

Table 7. *Effect of fitting a collar on dry matter, dry matter: chromic oxide and total nitrogen: Cr_2O_3 and the percentage of N fractions in several regions of the gastrointestinal tract and in faeces of rabbits fed on formaldehyde-treated casein**

(Results are means with their standard errors; values for dry matter in feed and faeces are expressed as g/d)

		Dry matter (g)		Dry matter: Cr_2O_3 (g/mg)		Total N: Cr_2O_3 (mg/mg)		N_a		N_b		N_c	
	Sample	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rabbits without collars	Feed	56		0·2		10·3		—		—		—	
	Stomach	51[a]	8	0·1[a]	0·0	5·5[a]	0·3	94[a]	0	0[a]	0	5[a]	0
	Segment 1†	0·94[a]	0·13	0·9[a]	0·2	83[a]	20	12[a]	2	62[a]	4	26[a]	3
	Segment 2	1·37[a]	0·17	0·4[a]	0·0	30[a]	5·4	20[a]	2	49[a]	3	30[a]	2
	Segment 3	1·99[a]	0·09	0·2[a]	0·0	11[a]	1·7	43[a]	4	25[a]	4	32[a]	4
	Segment 4	1·89[a]	0·23	0·1[a]	0·0	9·0[a]	1·6	50[a]	4	21[a]	3	28[a]	3
	Faeces	16·5[a]	1·2	0·1[a]	0·0	2·7[a]	0·2	—		—		—	
Rabbits with collars	Feed	48		0·2		10·3		—		—		—	
	Stomach	57·3[a]	5·6	0·1[a]	0·0	7·1[b]	0·4	96[b]	0	0[a]	0	4[a]	0
	Segment 1	0·97[a]	0·17	3·2[a]	0·8	317[b]	83	6[a]	1	57[a]	2	36[a]	2
	Segment 2	0·98[a]	0·18	3·2[b]	1·4	310[b]	149	15[a]	5	55[a]	4	30[a]	5
	Segment 3	1·22[a]	0·17	0·8[b]	0·4	74[b]	37	21[b]	4	42[b]	4	38[a]	4
	Segment 4	1·15[a]	0·28	0·6[b]	0·3	47[b]	23	26[a]	6	32[a]	1	42[a]	4
	Faeces	13·7[a]	1·2	0·1[a]	0·0	5·1[b]	0·3	—		—		—	

[a, b] Values with unlike superscript letters were significantly different ($P < 0·05$) between treatments.

N_a, N_b, N_c, N in the water-insoluble, trichloroacetic acid-insoluble and water-soluble fractions respectively.

* For details of diets, see Table 1.

† The small intestine was divided into four parts of equal length (see text): segment 1 refers to the most proximal part and segment 4 refers to the most distal part.

endogenous N in the first segment of the small intestine. There were no significant differences in the total N:Cr_2O_3 ratio in the various regions of the digestive tract between the rabbits fed on soya-bean protein and the rabbits fed on casein. The ratios seemed to be somewhat lower when formaldehyde-treated casein was fed, but the difference did not reach statistical significance. As would be expected from the differences in apparent digestibility of N, the total N:Cr_2O_3 ratios in the three dietary groups were significantly different in faeces.

The faecal excretions of both phosphate and Ca were lower on the casein diet when compared with the other two diets (Table 6). However, the difference in Ca excretion on the casein diet may have been due to the lower feed intake, as the proportion of Ca absorbed (Table 6) and the Ca:Cr_2O_3 ratio excreted in the faeces (Table 5) were similar on all three diets. In contrast, the proportion of phosphate absorbed was higher and the phosphate:Cr_2O_3 ratio excreted in the faeces was lower on the casein diet compared with the soya-bean protein diet (Table 5). Treatment of casein with formaldehyde resulted in an intermediate rate of phosphate absorption (Table 6) and phosphate:Cr_2O_3 ratio in the faeces (Table 5).

The proportions of N in the insoluble and TCA-insoluble fractions were combined because this combined fraction is representative of the material of larger particle size and protein of higher molecular weight. The proportions of N in the combined fraction in various regions of the gastrointestinal tract were similar when the diets containing soya-bean protein or casein were fed. Apart from segment 1, the proportions were significantly higher in rabbits fed on the formaldehyde-treated casein diet (results not shown).

When soya-bean protein or casein was given, the fitting of collars did not affect the dry matter:chromic oxide ratio, the N:Cr_2O_3 ratio, or the proportion of N in the various fractions (values not shown). However, there were several differences between the rabbits without collars and the rabbits with collars on the diet containing formaldehyde-treated casein (Table 7).

DISCUSSION

Increases in serum cholesterol concentrations were observed in rabbits given high-cholesterol diets containing either native or formaldehyde-treated casein compared with rabbits given soya-bean protein (Table 2). The elevated levels of serum cholesterol were accompanied by reduced excretion of bile acids (Tables 3 and 5), and by a lower bile acids:Cr_2O_3 ratio in faeces (Table 5). The reduced excretion of bile acids may be responsible for the hypercholesterolaemic effects of both native and formaldehyde-treated casein (Beynen *et al.* 1986).

Thus formaldehyde treatment of casein did not reduce its hypercholesterolaemic effect. In fact, levels of cholesterol in serum and liver showed a tendency to be somewhat higher in rabbits fed on formaldehyde-treated casein, when compared with rabbits fed on casein. This is contrary to findings reported previously using cholesterol-free diets (West *et al.* 1984). However, the present results in rabbits fed on diets fortified with cholesterol are in perfect agreement with findings reported for rats fed on high-cholesterol diets containing formaldehyde-treated casein (Beynen *et al.* 1985). In rats, formaldehyde-treated casein also produced higher concentrations of serum cholesterol than did native casein. Thus, it could be suggested that the hypocholesterolaemic effect of treatment of casein with formaldehyde disappears on the addition of cholesterol to the diet. The cholesterol-lowering effect of soya-bean protein, when compared to casein, will be observed irrespective of whether the diet is cholesterol-free or contains added cholesterol (Beynen *et al.* 1986). This implies that the mechanism underlying the hypocholesterolaemic action of soya-bean protein and that of formaldehyde-treated casein in a cholesterol-free diet cannot be similar.

We and others have hypothesized earlier (West *et al.* 1984; Woodward & Carroll, 1985)

that the higher degree of digestibility of casein, when compared with soya-bean protein, could be responsible for the hypercholesterolaemic activity of casein. Proteins that are not completely digested would interfere with the absorption of bile acids, and may interrupt the enterohepatic circulation of bile acids. This in turn would result in an enhanced loss of steroids in the faeces, and consequently in lower levels of cholesterol. The present results would disprove this hypothesis. Indeed, casein was somewhat more digestible than soya-bean protein, both in vitro and in vivo (Table 4). However, the digestibility of fomaldehyde-treated casein was reduced to much less than that of soya-bean protein (Table 4). The faecal ratio of total N:Cr_2O_3 in rabbits fed on formaldehyde-treated casein was greater than that of rabbits fed on soya-bean protein or casein (Table 5). This may have been due to the presence of a larger amount of undigested protein. In order to examine the course of protein digestion in the gastrointestinal tract, the protein in the various segments of the gut was fractionated. No differences in the total N:Cr_2O_3 ratio and in the proportion of water-insoluble and TCA-insoluble N fractions were observed between the soya-bean-protein diet and the casein diet (Table 5). This may have been due to the presence of a relatively large amount of endogenous N. However, when diets containing formaldehyde-treated casein were fed, the major proportion of the protein was found in the water-insoluble and TCA-insoluble N fractions. As these fractions represent protein fractions of higher molecular weight, this also points to reduced digestibility of formaldehyde-treated casein. Thus the digestibility of soya-bean protein is reduced, when compared with native casein, but formaldehyde-treated casein is even less digestible. Since both native and formaldehyde-treated casein caused an increase in serum cholesterol concentrations, the present study suggests that protein digestibility may not be a determinant of serum cholesterol levels in rabbits.

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