Lowering effect of dietary milk-whey protein *v.* casein on plasma and liver cholesterol concentrations in rats

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The effect of dietary whey protein *v.* casein on plasma and liver cholesterol concentrations was investigated in female, weanling rats. Balanced, purified diets containing either whey protein or casein, or the amino acid mixtures simulating these proteins, were used. The high-cholesterol diets (10 g cholesterol/kg feed) had either 150 or 300 g protein or amino acids/kg feed. The diets were fed for 3 weeks. At the low dietary protein level, whey protein *v.* casein did not affect plasma total cholesterol, but lowered the concentration of liver cholesterol. At the high dietary protein level, whey protein significantly lowered plasma and liver cholesterol and also plasma triacylglycerols. The hypocholesterolaemic effect of whey protein was associated with a decrease in very-low-density-lipoprotein cholesterol. At the high dietary protein concentration, whey protein reduced the faecal excretion of bile acids when compared with casein. The effects of intact whey protein *v.* casein were not reproduced by the amino acid mixtures simulating these proteins. It is suggested tentatively that the cholesterol-lowering effect of whey protein in rats is caused by inhibition of hepatic cholesterol synthesis.

Whey protein: Plasma cholesterol: Liver cholesterol: Bile acids: Rat

The type of dietary protein can influence plasma cholesterol concentration (Kritchevsky, 1979; Beynen, 1990). Animal proteins generally exert a hypercholesterolaemic effect when compared with plant proteins (Carroll, 1982). However, different animal proteins may cause different degrees of hypercholesterolaemia by different mechanisms (Lovati *et al.* 1990). Milk-whey protein instead of casein in formulas fed to infants has been shown to lower plasma cholesterol concentrations (Tseng *et al.* 1990). The hypocholesterolaemic effect of dietary whey protein *v.* casein also occurs in rabbits (Lovati *et al.* 1990), whereas in rats inconsistent results have been obtained (Sautier *et al.* 1983; Choi *et al.* 1989).

In the present study using female rats we re-addressed the question of whether milk-whey protein *v.* casein in the diet influences plasma and liver cholesterol concentrations. In contrast to previous work (Sautier *et al.* 1983; Choi *et al.* 1989; Lapré *et al.* 1989) the diets containing either casein or whey protein were isonitrogenous and carefully balanced for residual fat and cholesterol in the protein preparations. Amino acid mixtures simulating either whey protein or casein were fed also to see whether any effects of the intact proteins can be explained by their amino acid composition or rather by protein structure or non-protein components. The proteins and amino acid mixtures were incorporated into the diets at low (150 g/kg diet) and high (300 g/kg diet) levels because protein-type effects on

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plasma and liver cholesterol concentrations are generally amplified by higher protein intakes (Terpstra *et al.* 1982; Zhang & Beynen, 1992). All diets used contained a high concentration of cholesterol (10 g/kg diet) to enhance the differential cholesterolaemic responses to the type of protein (Van der Meer & Beynen, 1987).

MATERIALS AND METHODS

Diets

The analysed composition and amino acid profile of the casein (Havero bv, Rotterdam, The Netherlands) and milk-whey protein (DMV Campina, Veghel, The Netherlands) preparations are shown in Table 1. Either the protein preparations or amino acid mixtures simulating either casein or whey protein (Table 1) were used as the only N source in the experimental diets. There were two levels of N source: 150 or 300 g protein or amino acid mixture/kg diet. The diets containing intact proteins were isonitrogenous and balanced for residual fat, cholesterol, Ca, P and Mg in the protein preparations. Dietary cholesterol concentration was 10 g/kg. Table 2 gives the ingredient composition of the experimental diets. The analysed composition of the diets (Table 3) agreed well with the calculated composition. The diets, which were in powdered form, were stored at 4° until feeding. Food and demineralized water were provided on an *ad lib.* basis.

Animals and experimental procedures

Weanling, female Wistar rats (Hsd/Cpb:WU), aged about 3 weeks, were used. On arrival the rats were given free access to a commercial, pelleted non-purified diet (RMH-B®; Hope Farms, Woerden, The Netherlands) and tap water. They were housed in groups of five rats in Macrolon III cages (UNO BV, Zevenaar, The Netherlands) with a layer of sawdust as bedding.

After 1 week all animals were transferred to the purified, pre-experimental diet and demineralized water. The powdered, pre-experimental diet consisted of the following (g/kg diet): casein 175·3, soya-bean oil 30, coconut fat 87·2, cholesterol 9·96, cellulose 30, glucose 614·79, $CaCO_3$ 12·45, $NaH_2PO_4.2H_2O$ 8·93, $MgCO_3$ 1·37, KCl 1·0, $KHCO_3$ 7·0, mineral premix 10, vitamin premix 12. The composition of the mineral and vitamin premix has been described (Grooten *et al.* 1991). After another week, on day 0 of the experiment, the rats were divided into eight groups of twelve animals each on the basis of plasma cholesterol concentration and body weight. The eight groups were randomly assigned to the experimental diets (Table 2). The rats had *ad lib.* access to food and demineralized water. During the experimental period the rats were housed individually in metabolism cages (Tecniplast Gazzada, Buguggiate, Italy). The cages were placed in a room with controlled temperature (20–24°), relative humidity (40–45%) and lighting (light 06.00–18.00 hours).

The experiment lasted 21 d. The rats were weighed weekly and feed intake was recorded. On day 21 the rats were anaesthetized with xylazine (6·86 mg/kg, administered intraperitoneally) and ketamine (60 mg/kg, administered intramuscularly) and exsanguinated by aortic puncture. Livers were removed and weighed. The heparinized blood was centrifuged to collect plasma. Livers and plasma were stored at −20° until analysis.

Chemical analyses

N in protein preparations and diets was determined by the Kjeldahl method (Joslyn, 1970). Cholesterol in proteins and diets was determined by gas–liquid chromatography (Nordby & Nagy, 1973). Crude fat was determined by extraction according to the Soxhlet method (Joslyn, 1970), and fatty acid composition by the method of Metcalfe *et al.* (1966). Ca and Mg in the protein preparations were analysed by atomic absorption spectroscopy after dry ashing and dissolving the ash in 6 mol HCl/l as described previously (Hoek *et al.* 1988).

Table 1. *Analysed composition (g/kg) of the protein preparations*

Dietary protein...	Casein	Milk-whey protein
Components		
N	136·9	120·3
Crude fat	16·0	71·0
Cholesterol	0·21	2·1
Ca	0·12	3·17
Mg	0·02	0·55
P	1·30	3·40
Amino acids*		
Alanine	19·8	39·0
Arginine	30·6	21·0
Aspartate	46·3	86·0
Cysteine	2·6	19·0
Glutamate	175·5	138·0
Glycine	25·6	15·0
Histidine	23·2	16·0
Isoleucine	50·4	55·0
Leucine	95·2	86·0
Lysine	62·1	74·0
Methionine	24·0	17·0
Phenylalanine	36·4	29·0
Proline	80·3	49·0
Serine	28·1	46·0
Threonine	35·6	60·0
Tryptophan	11·6	17·0
Tyrosine	47·2	24·0
Valine	58·8	52·0

* Values provided by manufacturers.

Table 2. *Ingredient composition (g/kg) of the experimental diets*

Protein or amino acid mixture (g/kg feed...)	Casein		Whey protein		Casein amino acids		Whey-protein amino acids	
	150	300	150	300	150	300	150	300
Ingredient								
Casein	175·3	350·6	—	—	—	—	—	—
Whey protein	—	—	199·5	399·0	—	—	—	—
Casein amino acids*	—	—	—	—	150	300	—	—
Whey-protein amino acids*	—	—	—	—	—	—	150	300
Soya-bean oil	30	30	30	30	30	30	30	30
Coconut fat	87·2	84·4	75·8	61·67	90	90	90	90
Cholesterol	9·96	9·93	9·58	9·16	10	10	10	10
Glucose	604·83	433·54	596·56	416·92	626·12	476·12	626·12	476·12
$CaCO_3$	12·45	12·39	10·92	9·34	12·5	12·5	12·5	12·5
$NaH_2PO_4.2H_2O$	18·89	17·78	16·64	13·28	20	20	20	20
$MgCO_3$	1·37	1·36	1·00	0·63	1·38	1·38	1·38	1·38
Constant components†	60	60	60	60	60	60	60	60

* For composition, see Table 1.

† Constant components consisted of the following (g/kg feed): cellulose 30, KCl 1·0, $KHCO_3$ 7·0, mineral premix 10, vitamin premix 12. The composition of the mineral and vitamin premix has been described (Grooten *et al.* 1991).

Table 3. *Analysed composition (g/kg) of the experimental diets*

Protein or amino acid mixture (g/kg feed)...	Casein		Whey protein		Casein amino acids		Whey-protein amino acids	
	150	300	150	300	150	300	150	300
Component								
Dry matter	953	946	958	955	963	972	966	962
N	24·2	49·1	23·9	47·5	19·6	36·9	18·1	36·0
Fat	125	123	116	101	124	127	126	125
Cholesterol	12·5	10·4	10·7	10·9	10·5	9·8	9·7	10·2
Ca	5·2	4·5	4·9	4·8	4·9	4·3	4·9	4·7
Mg	0·4	0·4	0·4	0·4	0·4	0·4	0·4	0·4
P	4·8	5·4	4·2	4·3	4·9	4·3	4·3	4·4
Selected fatty acids (g methylester/kg methylesters)								
8:0	55	52	49	48	53	55	52	57
10:0	40	38	37	34	39	40	38	41
12:0	326	320	312	287	329	331	327	330
14:0	135	134	132	122	137	137	137	136
16:0	102	105	105	107	103	101	103	100
18:0	40	41	41	41	40	40	40	37
18:1	115	118	121	130	115	113	116	114
18:2	159	163	173	196	157	156	158	157
18:3*n*−3	15	16	17	19	15	15	15	15

P in the same samples was analysed enzymically using a commercial kit (MA-KIT Phosphate; Roche, Basel, Switzerland).

Cholesterol and triacylglycerols in plasma were measured enzymically using kits (Monotest and Test-Combination) supplied by Boehringer Mannheim GmbH, Mannheim, Germany. Plasma lipoproteins were isolated by density-gradient ultracentrifugation (Terpstra *et al.* 1981). The following fractions were obtained (density, d; g/ml): very-low-density lipoprotein (VLDL; $d < 1{\cdot}006$), intermediate-density lipoprotein (IDL; $1{\cdot}006 < d < 1{\cdot}019$), low-density lipoprotein (LDL; $1{\cdot}019 < d < 1{\cdot}063$), and high-density lipoprotein 2 (HDL_2; $1{\cdot}063 < d < 1{\cdot}125$) and high-density lipoprotein 3 (HDL_3; $1{\cdot}125 < d < 1{\cdot}210$). Lipoprotein cholesterol was measured as plasma total cholesterol.

Total faecal 3-α bile acids were determined with the use of a test combination based on an enzymic, spectrofluorimetric method (Sterognost 3-α Flu; Nyegaard & Co, Oslo, Norway).

Statistical analyses

All statistical analyses were carried out with the SPSS/PC$^+$ programme (SPSS Inc., 1986). The data were subjected to three-way ANOVA with type of N source (casein *v.* whey protein), form of N source (intact protein *v.* amino acid mixture), and amount of N source (150 *v.* 300 g protein or amino acid mixture/kg diet) as main effects. The probability of a type I error $< 0{\cdot}05$ was taken as criterion of statistical significance. The main effects were also evaluated in selected, direct comparisons with the use of Student's *t* test. When testing for effects of type, form and amount of N source in the diet, a *P* value of 0·017 was used to take into account the increased probability of a type I error because of multiple comparisons.

RESULTS

Final body weights were similar between dietary groups (Table 4). Whey protein feeding slightly reduced food intake when compared with casein. Rats given amino acid mixtures

Table 4. *Growth performance and liver weight in the rats fed on the experimental diets for 21 d*†

(Values are means with pooled standard errors for twelve rats per dietary group)

Protein or amino acid mixture (g/kg feed...)	Casein		Whey protein		Casein amino acids		Whey-protein amino acids		Pooled SE	Statistical significance (ANOVA)* of effects of:
	150	300	150	300	150	300	150	300		
Body wt (g)										
Day 0	93	93	94	94	93	95	95	95	2·6	
Day 21	176	187	174	174	165	181	173	170	5·0	T × A
Feed intake (g/d, days 18–20)	13·9	13·2	12·1	11·9	13·4	12·9	13·1	12·3	1·6	T
Liver wt (g/kg body weight)	52	55[af]	50	47[t]	48[f]	46[f]	52	47[a]	1	F, A, T × F, T × A, A × F

T, type of N source (whey protein *v.* casein); A, amount of N source (150 *v.* 300 g protein or amino acid mixture/kg diet); F, form of N source (intact protein *v.* amino acid mixture); T × A, T × F, A × F, T × A × F, interactions; t, a, f group comparisons for two groups with one dietary variable ($P < 0·017$): t, significant N type effect (intact whey protein *v.* casein or whey protein amino acids *v.* casein amino acids); a, significant N amount effect (150 *v.* 300 g protein or amino acid mixture/kg diet); f, significant N form effect (casein amino acids *v.* intact casein or whey protein amino acids *v.* intact whey protein).

* $P < 0·05$.

† For details of diets, see Tables 1–3.

simulating either casein or whey protein consumed similar amounts of feed. Increasing N intake reduced feed intake irrespective of the nature of the N source. Feeding intact whey protein caused significantly lower liver weight than feeding casein. The amino acid mixture corresponding to casein produced lower liver weight than intact casein. Such an effect was not seen with whey protein amino acids. Apart from the groups given intact casein, increasing N intake lowered relative liver weight.

At the low dietary protein level, plasma cholesterol concentrations were similar for rats given either intact whey protein or casein (Table 5). An increased intake of casein raised the group mean plasma cholesterol concentration, whereas an increased intake of whey protein had the opposite effect. Thus, an increment in protein intake resulted in a significant protein-type effect: whey protein *v.* casein significantly reduced plasma cholesterol concentration by about 35%. The plasma cholesterol lowering as induced by extra whey protein in the diet was most clearly reflected in the VLDL fraction (Table 6). The influence of the intact proteins on plasma free cholesterol mirrored that on plasma total cholesterol.

At the high dietary N concentration the amino acid mixture simulating casein lowered plasma cholesterol concentrations when compared with intact protein (Table 5). The casein amino acids produced lower cholesterol concentrations in VLDL and IDL. Such an effect was not seen at the low dietary N concentration. The amino acid mixture simulating whey protein did not systematically influence plasma cholesterol concentration when compared with intact whey protein. The whey protein amino acids in the diet did not significantly alter plasma cholesterol concentrations when compared with casein amino acids.

Dietary whey protein produced lower group mean plasma triacylglycerol concentrations than casein, but a corresponding N source effect was not found in rats fed on amino acid mixtures simulating either whey protein or casein (Table 5). The amino acid mixtures generally lowered triacylglycerols when compared with the intact proteins. Increasing intakes of intact proteins, but not of amino acid mixtures, reduced plasma triacylglycerols.

Whey protein *v.* casein significantly reduced liver cholesterol concentrations, this effect being greater when dietary protein level was increased (Table 5). Such an effect of N source

Table 5. *Plasma and liver cholesterol concentrations in rats fed on the experimental diets for 21 d*†

Protein or amino acid mixture (g/kg feed...)	Casein		Whey protein		Casein amino acids		Whey-protein amino acids		Pooled SE	Statistical significance (ANOVA)* of effects of:
	150	300	150	300	150	300	150	300		
Plasma cholesterol (mmol/l)										
Day 0	3·05	3·03	3·04	3·02	3·04	3·05	2·96	3·05	0·14	
Day 21	2·63	3·00	2·64	2·04^{a,t}	2·49	2·26^{f}	2·07^{f,t}	2·35	0·14	T, F, T × A × F
Plasma free cholesterol (mmol/l)	0·38	0·46	0·37	0·26^{a,t}	0·33	0·34^{f}	0·32	0·33^{f}	0·02	T, F, T × F, T × A, T × F × A
Plasma triacylglycerols (mmol/l)	1·37	1·22	1·14	0·85	1·08	1·07	1·00	1·00	0·14	
Liver cholesterol (mmol/g liver)	92·4	90·4	57·1^{t}	38·6^{t}	56·8^{f}	43·2^{f}	59·0	52·7	6·80	T, A, F, T × F
Faecal 3-α bile acids (μmol/d)	49·2	50·7	53·7	41·9^{a,t}	43·9	41·1	39·2^{f}	42·4	2·78	F, T × A × F

T, type of N source (whey protein *v.* casein); A, amount of N source (150 *v.* 300 g protein or amino acid mixture/kg diet); F, form of N source (intact protein *v.* amino acid mixture); T × A, T × F, A × F, T × A × F, interactions; t, a, f group comparisons for two groups with one dietary variable ($P < 0·017$): t, significant N type effect (intact whey protein *v.* casein or whey protein amino acids *v.* casein amino acids); a, significant N amount effect (150 *v.* 300 g protein or amino acid mixture/kg diet); f, significant N form effect (casein amino acids *v.* intact casein or whey protein amino acids *v.* intact whey protein).

* $P < 0·05$.

† For details of diets, see Tables 1–3.

Table 6. *Distribution of cholesterol between plasma lipoproteins in rats fed the experimental diets for 21 d*†

Protein or amino acid mixture (g/kg feed)...	Casein		Whey protein		Casein amino acids		Whey-protein amino acids		Pooled SE	Statistical significance (ANOVA)* of effects of:
	150	300	150	300	150	300	150	300		
Lipoprotein cholesterol (mmol/l whole plasma)										
VLDL cholesterol	0·98	0·94	0·66	0·46^{t}	0·50^{f}	0·39^{f}	0·35^{t}	0·37	0·08	T, F, T × F
IDL cholesterol	0·25	0·32	0·19	0·19	0·24	0·15	0·12^{t}	0·18	0·04	T, F, T × A × F
LDL cholesterol	0·22	0·40^{a}	0·36	0·37	0·44^{f}	0·43	0·38	0·43	0·05	F
HDL_2 cholesterol	0·84	0·85	1·04	0·91	0·92	1·04	0·95	0·95	0·07	
HDL_3 cholesterol	0·10	0·14	0·10	0·11	0·10	0·10	0·10	0·11	0·02	

VLDL, very-low-density lipoprotein; IDL; intermediate-density lipoprotein.

T, type of N source (whey protein *v.* casein); A, amount of N source (150 *v.* 300 g protein or amino acid mixture/kg diet); F, form of N source (intact protein *v.* amino acid mixture); T × A, T × F, A × F, T × A × F, interactions; t, a, f group comparisons for two groups with one dietary variable ($P < 0·017$): t, significant N type effect (intact whey protein *v.* casein or whey protein amino acids *v.* casein amino acids); a, significant N amount effect (150 *v.* 300 g protein or amino acid mixture/kg diet); f, significant N form effect (casein amino acids *v.* intact casein or whey protein amino acids *v.* intact whey protein).

* $P < 0·05$.

† For details of diets, see Tables 1–3.

was not seen in rats fed on diets containing the amino acid mixtures. At the two dietary N concentrations the amino acid mixture simulating casein lowered liver cholesterol concentrations when compared with the intact protein. For whey protein at the high dietary N concentration there was an opposite tendency.

At the high dietary protein level intact whey protein *v.* casein significantly reduced the faecal excretion of bile acids (Table 5). The two amino acid mixtures did not differently influence bile acid excretion. Apart from the diet containing the high concentration of whey-protein amino acids, the other diets with amino acids produced lower rates of bile acid excretion than the corresponding diets with intact proteins.

DISCUSSION

Conflicting results have been reported as to the effect of dietary whey protein *v.* casein on plasma and liver cholesterol concentrations in rats. Sautier *et al.* (1983), using non-balanced diets without added cholesterol, found that dietary whey protein produced lower plasma cholesterol concentrations than casein, while liver cholesterol concentration was not significantly reduced. Choi *et al.* (1989) did not show any effect of whey protein *v.* casein on plasma and liver cholesterol. In those two studies dietary protein concentration was about 200 g/kg feed. In the present study whey protein reduced liver cholesterol, but not plasma cholesterol, when given at a dietary concentration of 150 g/kg feed. This corroborates the work of Lapré *et al.* (1989). However, 300 g whey protein/kg feed instead of an identical amount of casein markedly lowered both plasma and liver cholesterol levels. In rats fed on whey protein at the high level, cholesterol in VLDL was reduced. Since this lipoprotein fraction is a major carrier of plasma triacylglycerols, it is not surprising that group mean total triacylglycerol concentrations in plasma were decreased in rats given the high amount of whey protein.

Thus, whey protein *v.* casein had cholesterol-lowering activity and, by definition, casein *v.* whey protein had cholesterol-elevating activity. To see whether the hypocholesterolaemic effect of whey protein can be explained by its amino acid composition, amino acid mixtures simulating either whey protein or casein were also fed. It is appreciated, however, that the effects of amino acid mixtures may not give information about the intact proteins. An intact protein cannot be simply replaced by an amino acid mixture resembling its composition because the order in which peptides or amino acids are released during digestion is characteristic for the intact protein. The differential effect of intact whey protein and casein on plasma and liver cholesterol levels was not seen with the amino acids simulating either whey protein or casein. The amino acid mixtures produced significantly different plasma cholesterol concentrations when compared with the intact corresponding proteins. The results could imply that the cholesterol-lowering activity of whey protein *v.* casein resides in different protein structure or non-protein components rather than in different amino acid composition *per se*. In contrast, the hypocholesterolaemic effect of dietary soya-bean protein *v.* casein in rats has been reproduced by feeding amino acid mixtures formulated to simulate these proteins (Yadav & Liener, 1977; Nagata *et al.* 1981).

The hypocholesterolaemic effect of soya-bean protein *v.* casein may be explained by stimulation of faecal excretion of bile acids and neutral steroids (Beynen, 1990). In contrast, the mechanism underlying the hypocholesterolaemia produced by the feeding of whey protein instead of casein is not clear. In rabbits, whey protein *v.* casein slightly lowered plasma total cholesterol concentration, which was associated with increased rates of faecal excretion of neutral steroids and bile acids (Lovati *et al.* 1990). In rats, however, whey protein did not change the excretion of neutral steroids and bile acids in faeces (Sautier *et al.* 1983). In the present study whey protein at the high dietary N concentration significantly reduced the faecal excretion of bile acids. Thus, it is unlikely that in rats whey protein *v.*

casein inhibits re-absorption of bile acids as has been shown for soya-bean protein *v.* casein (Beynen, 1990).

It is possible that, in rats fed on the high level of whey protein, cholesterol synthesis was inhibited when compared with their counterparts fed on casein. This is indicated by the observation that whey protein induced lower VLDL-cholesterol levels. The lower liver cholesterol concentrations in rats given whey protein could also point to a depressed *de novo* cholesterolgenesis, but the fact that cellular cholesterol usually acts as a feedback inhibitor of cholesterol synthesis speaks against this. Since cholesterol synthesized *de novo* in the liver is a major precursor of bile acids, the depressed excretion of bile acids in rats given whey protein instead of casein could indicate that whey protein inhibits cholesterol synthesis. Whether dietary whey protein *v.* casein indeed blocks hepatic cholesterol synthesis or, in other words, whether casein stimulates cholesterol synthesis compared with whey protein can only be proved by direct measurement of cholesterol synthesis.

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