Influence of dietary proteins on cholesterol metabolism and nephrocalcinosis

Invloed van voedingseiwit op cholesterolstofwisseling en nierverkalking

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Proefschrift

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Dedicated to:

my dear wife Shi Juqin, son Zhang Yao and our parents!

献给我亲爱的妻子史菊琴、儿子张遥
以及我们的父母亲。

Zhang Xizhong

BIBLIOTHEEK
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STELLINGEN

1. Plaice meal has greater cholesterol lowering activity than whiting meal (This thesis).

2. Dietary phytate raises the fecal excretion of endogenous magnesium (Defence of Ph.D. thesis by Lisette Brink, Wageningen Agricultural University, April 1, 1992).

3. Chain length of fatty acids affects tissue calcium concentrations (J.M. Huang et al., Proc. Natl. Acad. Sci. USA, 1992, 89:6452-6456).

4. Dietary cholesterol does not effectively inhibit liver cholesterol synthesis in analbuminemic rats (This thesis).

5. Mice and flies head to head is more likely than back to back (P. Holland et al. Nature, 1992, 358:627-628).

6. The term fish protein is meaningless to predict its metabolic effect after ingestion.

7. When carrying out nutritional experiments, the use of diets balanced for components other than those of interest is critically important.

8. The word human is easy to write, but humane is difficult to be.

9. Fly [flai] not always refers to an insect.

10. Overexpression of LDL receptors prevents diet-induced hypercholesterolemia in transgenic mice that express the human LDL receptor gene (M. Yokode et al. Science, 1991, 250:1273-1275).

11. Dieticians often forget their own luncheons.

12. Commercial, natural-ingredient rodent diets vary considerably in their fatty acid composition (A.C. Beynen and J. Ritskes-Hoitinga, Scand. J. Lab. Anim. Sci. 1992, 19:93-94).

Stellingen behorende bij het proefschrift "Influence of dietary proteins on cholesterol metabolism and nephrocalcinosis" van Xizhong Zhang.
Wageningen, 30 September, 1992

CONTENTS

SCOPE OF THIS THESIS

This thesis consists of two parts. The first part deals with the effects of type and amount of various animal proteins on plasma and liver cholesterol concentrations in female, weanling rats. The second part focusses on the nephrocalcinogenic effects of dietary proteins in female rats.

Chapter 1 presents an overview of published studies on the effects of dietary fish proteins on cholesterol metabolism. Chapter 2 describes the cholesterolemic effects of various dietary fish proteins in comparison with the effects of casein and soybean protein. Chapter 3 reports the hypocholesterolemic effect of whey protein versus casein. In Chapter 4, the cholesterolemic effect of casein versus soybean protein in analbuminemic rats is described.

The second part of this thesis attempts to identify the possible mechanisms underlying the different degrees of nephrocalcinosis as induced by type and amount of dietary protein. First, a brief review is given (Chapter 5). Chapter 6 then describes nephrocalcinogenesis in rats fed different amounts and/or types of either soybean protein, casein or cod meal. The anti-nephrocalcinogenic effect of whey protein versus casein is described in Chapter 7. Chapter 8 presents evidence that female analbuminemic rats are less sensitive to dietary-casein-induced nephrocalcinosis than Sprague-Dawley rats.

PART I

DIETARY PROTEIN AND CHOLESTEROL METABOLISM

Chapter 1

Dietary fish proteins and cholesterol metabolism

Xizhong Zhang and Anton C. Beynen

Monographs on Atherosclerosis (1990) 16:148-152

INTRODUCTION

The type of dietary protein influences cholesterol metabolism in experimental animals and humans. In general, animal proteins are more hypercholesterolemic than vegetable proteins. However, this is an oversimplified statement because for certain animal and plant proteins there is no clear distinction between their cholesterolemic effects. For instance, different sources of fish proteins vary in their composition and may have different effects on serum cholesterol concentrations, when compared with a given plant protein.

There is controversy about the effect of dietary fish protein on the concentration of serum and liver cholesterol. The conflicting results are partly due to poor experimental design. Frequently, the experimental diets used were not balanced for components other than protein in the fish protein preparations. In addition, various authors probably assumed that all fish proteins are identical and did not report either the chemical composition or the source of the fish proteins used. In this communication we refer to several studies so as to illustrate the variation of the effects of different dietary fish proteins on cholesterol metabolism.

Type of Fish Protein

Dietary fish proteins influence serum cholesterol concentrations in rabbits. Partial replacement of a mixture of casein and gelatin by fish protein of undefined source decreased serum cholesterol concentrations [1]. When compared with casein, noncharacterized fish protein as the sole source of protein in the diet also reduced serum cholesterol concentrations [2, 3]. Cod fish protein has been shown to lower serum cholesterol concentrations when compared with casein [4]. However, in another study using high-cholesterol diets that were carefully balanced for cholesterol and for the amount and type of fat in the protein preparations, a mixture of protein prepared from cod and whiting increased serum cholesterol

Table 1. ***Effects of dietary fish protein preparations on group mean of serum and liver cholesterol concentrations in rats in comparison with the effects of soybean protein and casein***

Type of fish protein	Diet composition, % w/w		Percentage change in serum and liver cholesterol				Ref.
	Protein	Cholesterol	serum vs.		Liver vs.		
			soy	casein	soy	casein	
Cod	15	-	+12	- 8			7
Cod	15	1	- 22	- 40			7
Cod	25	1	+33	- 16	- 15	- 3	8
Whiting	18	0.15	- 14	- 18	+ 8	-18	9
Pollack	18	-	+12	- 30	+10	-12	10
Pollack	20	-	+36	+ 0	+ 5	- 1	11
Sardine	20	-	+28	- 3	+ 4	-10	11

when compared with casein [5]. Such a hypercholesterolemic effect was also reported for whitefish meal protein [6]. Thus, in rabbits, fish protein preparations have been found to be hypocholesterolemic in some studies [1-4] and hypercholesterolemic in others [5, 6], when compared with casein.

Dietary fish protein may affect the distribution of cholesterol between serum lipoproteins. In one study with rabbits [4], cod fish protein reduced cholesterol in very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) but increased cholesterol in high-density lipoprotein (HDL), when compared with casein. In another study [5], rabbits fed a mixture of cod and whiting meal showed lower VLDL and higher LDL cholesterol concentrations when compared with rabbits fed casein.

Table 1 summarizes the results of studies with rats by comparing the effects of fish protein preparations on serum and liver cholesterol concentrations with

those of soybean protein and/or casein. Apart from one study [9], the experimental diets were not balanced for residual fat and cholesterol in the protein preparations. Cod fish and whiting protein were less hypercholesterolemic than casein. The effect of cod protein versus casein became more pronounced when high-cholesterol diets were used. In rats fed cholesterol, in which cholesterol in serum is carried mainly by VLDL, cod fish protein induced a decrease of VLDL cholesterol, and an increase of HDL cholesterol when compared with casein [12]. When compared with soybean protein, cod fish protein did not systematically influence serum cholesterol concentrations.

The cholesterolemic effect of pollack versus casein is not clear, but it increased serum cholesterol when compared with soybean protein (Table 1). Sardine fish protein produced similar serum cholesterol concentrations as did casein. However, it cannot be excluded that differential cholesterolemic effects of the two proteins will appear after the feeding of high-cholesterol diets. In studies with rats fed various fish proteins, we used purified diets that contained 1% (w/w) of cholesterol and 2.4% nitrogen, and were balanced for cholesterol and fat in the protein preparations. We found that cod meal and plaice meal reduced group mean serum cholesterol by 8 and 15%, when compared with casein, whereas whiting meal increased serum cholesterol by 6% [unpubl.].

Amount of Fish Protein

Table 2 illustrates that increasing the amount of cod fish protein in the diets further reduced serum and liver cholesterol concentrations when compared with casein. No such dose-response was seen in rats fed casein as dietary protein source. Liver cholesterol concentrations were decreased in rats fed cod meal, the effect being most pronounced when the diets contained 7.2% nitrogen.

Table 2. ***Effect of the amount of cod fish protein in the diet on serum and liver cholesterol concentrations in rats***

Protein amount	Dietary nitrogen concentration, % w/w		
	2.4	4.8	7.2
Plasma cholesterol, mmol/l			
Cod protein	4.5 ± 0.7	4.1 ± 0.7	3.4 ± 0.5
Casein	5.1 ± 1.0	5.3 ± 1.0	5.1 ± 1.2
Liver cholesterol, μmol/g			
Cod protein	49.0 ± 9.8	39.9 ± 5.3	31.2 ± 8.9
Casein	56.8 ± 15.2	50.1 ± 12.4	58.1 ± 20.6

Means ± SD for 6 rats. Diets contained 1% cholesterol and were balanced for residual cholesterol, fat and minerals in the protein preparations. Extra protein was added at the expense of the glucose component. Dietary groups were stratified for initial plasma cholesterol concentrations. Diets were fed for 21 days.

Table 3. ***Influence of dietary fish protein on serum cholesterol level and degree of atherosclerosis in rabbits***

Type of dietary protein	Serum cholesterol, mmol/l	Aortic atherosclerosis[1]	
		arch	thoracic
Fish protein[2]	6.2 ± 1.0	1.6 ± 0.2	1.0 ± 0.2
Casein	13.7 ± 2.0	2.1 ± 0.2	1.1 ± 0.3
Whole milk	12.0 ± 1.6	2.6 ± 0.2	1.6 ± 0.2

Diets contained 25% of protein preparation and were fed for 8 months. After Kritchevsky *et al.* [2].

[1] On a 0-4 scale. [2] Source unknown.

Table 4. ***Influence of dietary fish protein on serum cholesterol level and degree of atherosclerosis in rabbits***

Type of dietary protein	Serum cholesterol, mmol/l	Aortic atherosclerosis, % atheroma	
		arch	thoracic
Soy protein	3.0 ± 0.2	33 ± 10	2 ± 2
Milk	4.6 ± 0.7	40 ± 9	4 ± 2
Whitefish	13.4 ± 2.4	77 ± 11	75 ± 14

Means ± SE for 10-17 rabbits. Diets contained 31% of protein and were fed for 12 months. After Goulding *et al.* [6].

Fish Protein and Atherosclerosis

The type of fish protein preparation influences the severity of atherosclerosis in rabbits. Compared with casein or milk protein, fish protein either reduced (Table 3), or aggravated the development of atherosclerosis (Table 4). Differences in source of fish protein and/or nonprotein components in the preparations may explain this discrepancy.

Effects of Fish Proteins on Cholesterol Metabolism

Pollack fish protein in the diet of rats increased the rate of fecal excretion of radioactive bile acids and neutral steroids after intraperitoneal injection of [4-^{14}C] cholesterol, when compared with casein [10]. Similar effects, though not as pronounced, were seen for steroid excretion as measured by gas-liquid chromatography [10]. However, in another study with rats [11], pollack protein did enhance the excretion of neutral steroids in feces, but depressed that of bile acids, when compared with casein. Sardine protein versus casein induced higher excretion rates

of both neutral steroids and bile acids [11]. In rats fed high-cholesterol diets, whiting protein versus casein did not affect fecal bile acid excretion [9]. In rabbits, a mixture of cod and whiting protein in the diet produced increased rates of fecal excretion of neutral steroids and bile acids when compared with casein [5]. Thus, when all studies are considered there is no general, systematic effect of fish protein on steroid excretion.

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Chapter 2

Influence of dietary fish proteins on plasma and liver cholesterol concentrations in rats

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ABSTRACT The effects of amount and type of dietary fish proteins on plasma and liver cholesterol concentrations were evaluated in female rats. The isonitrogenous diets used contained 10 g cholesterol/kg and were carefully balanced for residual fat, cholesterol, calcium, magnesium and phosphorus in the protein preparations. Either cod meal, soya-bean protein or casein was incorporated into the diets as sole source of dietary protein at three levels: either 24, 48 or 72 g nitrogen/kg diet. Extra protein was added to the diet at the expense of the glucose component. In a second experiment, either soya-bean protein, casein, cod, whiting or plaice meal was added to the diet at a level of 24 g nitrogen/kg. When compared with casein, cod meal and soya-bean protein decreased plasma and liver cholesterol concentrations. A further cholesterol-lowering effect was achieved by increasing the proportion of either soya-bean protein or cod meal in the diet. Substitution of casein for glucose did not influence plasma and liver cholesterol concentrations. Plaice meal in the diet produced lower group mean plasma cholesterol concentrations than did whiting meal. In rats fed the diet containing plaice meal, liver cholesterol concentrations were significantly lower than in their counterparts fed either cod meal or whiting meal. This study demonstrates that different fish proteins in the diet have different effects on cholesterol metabolism and that the cholesterol-influencing properties of cod meal can be enhanced by the incorporation of higher proportions of this protein in the diet.

INTRODUCTION

The nature of dietary protein can influence cholesterol metabolism in experimental animals and humans (Kritchevsky, 1979; Carroll, 1982; Beynen, 1991). Most studies have focused on the hypercholesterolaemic effect of casein versus soya-bean protein. In general, animal proteins are considered to be hypercholesterolaemic when compared with plant proteins (Carroll, 1981). This concept is based on the comparison of a limited number of proteins. For instance, little is known about the effects of various types of fish proteins. Moreover, as to

the effects of dietary fish protein on serum cholesterol, controversial results have been reported (Bergeron & Jacques, 1989; Goulding *et al.* 1983; Iritani *et al.* 1985; Jacques *et al.* 1986; Jacques *et al.* 1987; Kritchevsky *et al.* 1982; Peifer *et al.* 1961; Sugano *et al.* 1984; Wexler, 1983). The different experimental results may relate to the use of poorly characterized fish proteins and experimental diets that were not balanced for components other than protein in the fish protein preparations (Zhang & Beynen, 1990). In the present experiments using rats fed carefully balanced diets, the effects of cod, whiting and plaice meal on serum and liver cholesterol concentrations were determined. Dietary casein and soya-bean protein isolate were used as reference proteins.

MATERIALS AND METHODS

Diets. Cod meal (Institute for Fishery Products, CIVO-TNO, Ijmuiden, The Netherlands), whiting and plaice fillet (local fish shop), casein (Havero BV, Rotterdam, The Netherlands) and soya-bean protein isolate (Ralston Purina Co., St. Louis, MO) were used. The fillets of whiting and plaice were cut into small pieces, freeze-dried, and powdered. The protein preparations were analysed for nitrogen, cholesterol, fat, calcium, magnesium and phosphorus. The results are given in Table 1. The amino acid compositions of the protein preparations are shown in Table 2. The fish proteins and casein had higher levels of lysine and methionine but lower levels of arginine, when compared with soya-bean protein. On the other hand, when compared with casein, both fish proteins and soya-bean protein had higher levels of cysteine and glycine.

In experiment 1, both type and concentration of protein preparation in the diets were varied: soya-bean protein, casein and cod meal were used at three levels of nitrogen (24, 48 and 72 g /kg diet). Extra protein was added to the diets at the expense of the glucose component. Experiment 2 involved variation in the

Table 1. ***Analysed composition of the protein preparations***

Type of protein ...	Soya-bean Protein	Casein	Cod meal	Whiting meal	Plaice meal
Component (g/kg protein preparation)					
Nitrogen	134	141	130	147	146
Crude fat	53	17	46	46	45
Cholesterol	0.00	0.26	7.15	3.43	2.66
Calcium	2.8	0.7	9.6	1.7	3.0
Magnesium	0.7	0.1	1.1	1.4	1.3
Phosphorus	7.9	2.2	10.0	2.0	10.5

Table 2. ***Amino acid composition of the protein preparations***

Type of protein ...	Soya-bean protein	Casein	Cod meal	Whiting meal	Plaice meal
Amino acid (g/kg protein preparation)					
Alanine	35.5	28.4	47.0	52.5	51.0
Arginine	66.0	33.5	54.0	58.0	56.0
Aspartate	97.5	66.0	82.5	89.0	88.5
Cysteine	10.3	4.5	8.3	9.0	9.8
Glutamate	165.5	205.0	126.0	139.0	133.5
Glycine	34.5	16.9	41.5	47.0	46.0
Isoleucine	43.0	49.5	39.5	41.5	42.0
Leucine	69.5	88.5	64.0	69.0	68.0
Lysine	53.5	74.5	72.5	81.5	80.5
Methionine	11.4	25.7	26.3	27.5	26.3
Serine	47.0	56.0	40.5	41.5	41.0
Threonine	32.5	40.5	38.0	39.5	39.5
Valine	45.0	65.0	45.0	45.5	45.5

Table 3. ***Ingredient composition of the experimental diets***

Dietary nitrogen (g/kg) ...	24			48			72			24	
Dietary protein ...	Soya-bean* protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Whiting meal	Plaice meal
Ingredient	Expt 1/2	Expt 1/2	Expt 1/2	Expt 1	Expt 1	Expt 1	Expt 1	Expt 1	Expt 1	Expt 2	Expt 2
Soya-bean protein	178.7	-	-	357.4	-	-	536.1	-	-	-	-
Methionine	1.5	-	-	3.0	-	-	4.5	-	-	-	-
Casein	-	170.7	-	-	341.4	-	-	512.1	-	-	-
Cod meal	-	-	184.3	-	-	368.6	-	-	552.9	-	-
Whiting meal	-	-	-	-	-	-	-	-	-	163.3	-
Plaice meal	-	-	-	-	-	-	-	-	-	-	164.0
Soybean oil	20.5	30	21.5	11.1	30	13	1.6	30	4.6	22.5	22.6
Coconut fat	90	87.1	90	90	84.2	90	90	81.3	90.0	90.0	90.0
Cholesterol	10	9.96	8.68	10	9.91	7.36	10	9.87	6.05	9.44	9.56
Glucose	599.8	596.24	601.62	437.8	430.69	441.44	275.9	265.23	281.35	609.56	616.14
$CaCO_3$	13.7	14.7	10.6	12.5	14.4	6.2	11.2	14.1	1.7	14.3	13.8
$NaH_2PO_4.2H_2O$	23.1	28.3	20.9	16.0	26.4	11.7	8.9	24.5	2.4	28.6	21.5
$MgCO_3$	2.0	2.3	1.7	1.5	2.3	1.0	1.1	2.2	0.3	1.6	1.7
Constant components+	60.7	60.7	60.7	60.7	60.7	60.7	60.7	60.7	60.7	60.7	60.7

* This diet was also used as pre-experimental diet.

\+ Constant components consisted of (g/kg diet): cellulose, 30; KCl, 1.0; $KHCO_3$, 7.7; mineral premix, 10; vitamin premix, 12. The compositions of the mineral and vitamin premix are described elsewhere (Lovati *et al.* 1990).

Table 4. ***Analysed composition of the experimental diets***

Dietary nitrogen (g/kg) ...	24			48			72			24	
Dietary protein ...	Soya-bean* protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Whiting meal	Plaice meal
Ingredient	Expt 1/2	Expt 1/2	Expt 1/2	Expt 1	Expt 1	Expt 1	Expt 1	Expt 1	Expt 1	Expt 2	Expt 2
Component (g/kg diet)											
Nitrogen	24.4/24.6	24.2/24.3	23.6/24.5	48.4	48.8	48.8	74.4	72.6	72.2	23.4	25.1
Crude fat	131/123	136/127	129/126	135	137	136	135	136	128	124	123
Cholesterol	13.5/10.0	9.1/11.2	8.3/10.2	10.2	9.6	9.6	10.2	9.2	10.2	10.7	11.0
Calcium	5.9/5.5	5.8/5.4	6.0/4.9	5.9	5.7	6.3	5.0	5.5	6.2	5.2	5.4
Magnesium	0.7/0.7	0.7/0.6	0.7/0.6	0.7	0.6	0.8	0.7	0.6	0.8	0.6	0.7
Phosphorus	6.2/5.2	7.3/7.5	6.5/5.2	6.4	8.2	6.4	6.1	8.5	6.6	6.0	7.2
Fatty acid (g methylester/kg methylesters)**											
C12:0	352/346	333/317	359/344	361	321	371	368	320	380	340	343
C14:0	140/147	133/137	138/134	140	127	144	141	129	146	147	147
C16:0	100/104	103/107	97/92	102	101	104	104	106	104	107	106
C18:0	35/41	37/41	32/35	34	35	35	33	38	34	41	40
C18:1	108/116	119/124	103/95	104	117	101	97	120	90	114	113
C18:2	129/124	141/170	121/111	124	153	86	119	149	46	137	137
Sat.	738/743	716/683	744/723	752	694	776	764	706	802	726	726
Mono.	110/117	121/125	111/97	105	118	125	98	121	124	115	114
Poly.	142/138	155/189	133/155	138	175	95	133	164	50	154	154
Unknown	9/2	8/3	12/25	5	13	24	4	9	53	5	6

** Selected fatty acids in shorthand notation. Sat.=saturated fatty acids; Mono.=monounsaturated fatty acids; Poly.=polyunsaturated fatty acids.

type of dietary protein only; soya-bean protein, casein, cod meal, whiting meal and plaice meal were compared at a fixed nitrogen concentration of 24 g/kg diet. The experimental diets were balanced for nitrogen, cholesterol, the amount and type of fat, calcium, magnesium and phosphorus. Table 3 shows the ingredient composition of the diets. The analysed composition of the diets is given in Table 4, which indicates that the diets were reasonably well balanced. This is supported by the analysed fatty acid composition of the diets (Table 5). Separate batches of diets were made for each experiment. The diets were in meal form and kept at 4°C until feeding.

Animals and experimental procedures. The interval between the two experiments was about 2 months. Female Wistar rats (Cpb/Hsd, Harlan-CPB, Zeist, The Netherlands) were used throughout. On arrival, when they were aged 3 (expt 2) or 4 (expt 1) weeks, the animals were housed 4 in a Macrolon type III cage (UNO BV, Zevenaar, The Netherlands) with a layer of sawdust as bedding. The rats were fed ad libitum a commercial, pelleted natural-ingredient diet (RMH-B^{R}, Hope Farms, Woerden, The Netherlands) and tap water for 3 d. Then, they were transferred to the pre-experimental, purified diet containing soya-bean protein at a level of 24 g nitrogen/kg (Table 3) and demineralized water. After 1 week, (d 0 of the experimental period), the rats were divided into either 9 (expt 1) or 5 groups (expt 2), each comprising 6 rats. The groups within each experiment had similar distributions of plasma cholesterol concentration and body weight. Each group was randomly assigned to one of the experimental diets. One group remained on the pre-experimental diet. Feed and demineralized water were provided ad libitum. The animals were weighed weekly, and feed intakes were recorded. Both experiments lasted 3 wk.

During the experiment (d 0-21), the rats were housed individually in metabolic cages (Techniplast Gazzada, Buguggiate, Italy). The cages were placed

in a room with controlled temperature (20-24 °C), relative humidity (40-45%) and lighting (light, 06.00-18.00 h).

Heparinized blood samples were taken in the non-fasting state by orbital puncture while under light diethyl-ether anaesthesia. At the end of each experiment, the anaesthetized animals were killed by cervical dislocation. Livers were removed and weighed; they were stored at -20 °C until analysis.

Chemical analyses. Crude protein contents of protein preparations and diets were analysed by the Kjeldahl method (Joslyn, 1970). Cholesterol was determined by gas-liquid chromatography (Nordby & Nagy, 1973). Crude fat was determined by the Soxhlet method (Joslyn, 1970) and fatty acid composition according to Metcalfe *et al.* (1966). Amino acids in the protein preparations were analysed by the method of Moore (1963) as modified by Slump & Bos (1985). Calcium and magnesium in protein preparations and diets were analysed as described (Hoek *et al.* 1988). Phosphorus was analysed by the method of Taussky and Shorr (1953) in experiment 1, and with the use of the kit (MA-kit Phosphate) from F. Hoffmann-La Roche Co. Ltd. Diagnostica (Basel, Switzerland) in experiment 2.

The concentrations of plasma cholesterol and triglycerides were measured enzymatically using the kits (Monotest and Test-Combination) supplied by Boehringer Mannheim Gmbh (Mannheim, FRG). Control sera (Precinorm U and LIPIDS) from Boehringer Mannheim Gmbh were used as standards. Liver cholesterol was extracted and analysed according to Abell *et al.* (1952).

Statistical analyses. Statistical analysis of the data was done using the SPSS statistical package (SPSS Inc., 1986). Two-way ANOVA (analysis of variance) was used to determine the influence of source and level of dietary protein in experiment 1. Group means of dietary groups in experiment 2 were evaluated for statistically significant differences using Duncan's test. The level of significance

Table 5. ***Body and liver weight and feed intake in rats fed the experimental diets in experiment 1***

Dietary nitrogen (g/kg) ..	24			48			72				
Dietary protein ...	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Pooled SE	ANOVA*
Body weight (g)											
d 0	144	142	143	142	142	142	144	142	145	4.92	
d 21	203	196	204	194	203	197	193	200	198	6.92	
Feed intake (g/d)	12.3	11.4	12.3	11.5	11.6	11.6	10.7	11.0	11.6	0.35	a
Liver weight (g)	8.9	9.9	9.8	8.6	9.7	9.6	8.2	9.8	9.0	0.47	T

Values are means and pooled SE's for 6 rats per dietary group.

* ANOVA significance: T ($P<0.01$) or t ($P<0.05$) = significant effect of dietary protein type; A ($P<0.01$) or a ($P<0.05$) = effect of amount of protein in the diet; T x A ($P<0.01$) or a x t ($P<0.05$) = significant effect of interaction.

Table 6. ***Plasma and liver cholesterol concentrations in rats fed experimental diets in experiment 1***

Dietary nitrogen (g/kg) ..	24			48			72				
Dietary protein ...	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Soya-bean protein	Casein	Cod meal	Pooled SE	ANOVA*
Plasma cholesterol (mmol/L)											
d 0	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	0.16	
d 21	3.4	5.1	4.5	2.9	5.3	4.1	2.5	5.1	3.4	0.33	T,a
Liver cholesterol (μmol/g)	28.5	58.6	49.0	12.5	50.1	39.9	7.0	58.1	31.2	4.50	T,A
Plasma triglycerides (mmol/L)	2.17	2.10	1.80	1.66	1.55	1.57	1.04	1.46	0.97	0.26	A

Values are means and pooled SE's for 6 rats per dietary group.

* ANOVA significance: T ($P<0.01$) or t ($P<0.05$) = significant effect of dietary protein type; A ($P<0.01$) or a ($P<0.05$) = effect of amount of protein in the diet; T x A ($P<0.01$) or a x t ($P<0.05$) = significant effect of interaction.

was pre-set at $P < 0.05$.

RESULTS

Experiment 1. The type and amount of dietary protein in experiment 1 did not significantly influence body weight (Table 5). Higher intakes of protein at the expense of glucose significantly lowered feed intake. Liver weights fell with increasing amounts of dietary protein in rats fed either soya-bean protein or cod meal, but not in rats fed diets containing casein. Soya-bean protein produced lower liver weights than did either casein or cod meal.

A decrease of cholesterol concentrations in plasma and liver was observed in rats fed increasing amounts of either soya-bean protein or cod meal (Table 6). An increase in dietary soya-bean protein concentration from 24 to 72 g nitrogen/kg diet lowered plasma and liver cholesterol concentration by 26 and 75 %, respectively. No such dose response was found in rats fed diets with casein as protein source; the concentration of casein in the diet did not influence plasma and liver cholesterol concentrations. Plasma triglyceride levels were significantly reduced when dietary protein level increased, regardless of the source of protein.

Experiment 2. In the second experiment, body weight and feed intake did not differ significantly between the dietary groups (Table 7). Soya-bean protein induced significantly lower liver weights than did either casein, cod meal or whiting meal.

Soya-bean protein produced lower group mean plasma cholesterol concentrations than casein, cod meal or whiting meal, but only the difference with whiting meal reached statistical significance (Table 8). Rats given the diet with plaice meal had lower group mean plasma cholesterol concentrations than rats fed diets containing either casein or whiting meal. As expected, liver cholesterol concentration was significantly higher in rats fed casein than in those fed soya-bean protein.

Table 7. ***Body and liver weight and feed intake in rats fed the experimental diets in experiment 2***

Dietary protein	Soya-bean protein	Casein	Cod meal	Whiting meal	Plaice meal	Pooled SE
Body weight (g)						
d 0	88.6	86.4	89.4	89.1	87.3	2.56
d 21	151.9	153.2	158.9	166.4	152.6	4.97
Feed intake (g/d)	10.6	10.4	11.1	11.2	13.9	0.38
Liver weight (g)	6.7^{a}	7.8^{b}	8.1^{b}	8.3^{b}	$7.4^{a,b}$	0.29

Results expressed as means and pooled SE's for 6 rats per dietary group. Group means in the same row not sharing a common superscript are significantly different ($P< 0.05$).

Table 8. ***Plasma and liver cholesterol concentrations in rats fed the experimental diets in experiment 2***

Dietary protein	Soya-bean protein	Casein	Cod meal	Whiting meal	Plaice meal	Pooled SE
Plasma cholesterol (mmol/L)						
d 0	3.6	3.5	3.6	3.6	3.5	0.16
d 21	3.6^{a}	$5.2^{a,b}$	$4.8^{a,b}$	5.5^{b}	$4.4^{a,b}$	0.51
Liver cholesterol (μmol/g)						
	44.7^{a}	105.0^{b}	65.8^{c}	69.7^{c}	43.6^{a}	6.33
Plasma triglycerides (mmol/L)						
	1.0	1.3	1.6	1.3	1.3	0.29

Results expressed as means and pooled SE's for 6 rats per dietary group. Group means in the same row not sharing a common superscript are significantly different ($P< 0.05$).

Dietary cod meal and whiting meal also caused significantly higher liver cholesterol concentrations than soya-bean protein but produced significantly lower values than casein. Plaice meal in the diet produced liver cholesterol concentrations similar to those induced by soya-bean protein. Plasma triglyceride concentrations were not significantly influenced by the type of protein in the diet.

DISCUSSION

The present studies clearly show that dietary cod meal versus either soya-bean protein or casein alters plasma and liver cholesterol concentrations. Cod meal produced lower cholesterol concentrations than casein but higher concentrations than soya-bean protein. The cholesterol-lowering effect of dietary cod meal and soya-bean protein, when compared with casein, was greater with increasing dietary protein levels. In rabbits, cod fish protein has also been shown to lower serum cholesterol concentrations when compared with casein (Bergeron & Jacques, 1989). Earlier work carried out with rats has yielded variable results. Cod meal did consistently lower serum cholesterol concentrations when compared with casein, but when compared with soya-bean protein it either lowered or raised serum cholesterol (Jacques *et al.* 1986; Sugiyama *et al.* 1986). However, in those studies the experimental diets were not balanced for residual fat and cholesterol in the protein preparations. In the present studies, the diets were isonitrogenous and balanced for selected non-protein components of the protein preparations. Table 3 illustrates that this balancing of the diets had been quite successful. Cod meal versus soya-bean protein in the diet consistently raised group mean plasma cholesterol concentrations (Table 6 and 8).

Increasing intakes of soya-bean protein at the expense of glucose lowered plasma and liver cholesterol concentrations, whereas with casein such an effect was not seen (Table 6). This dose-dependent effect of soya-bean protein has been reported earlier (Terpstra *et al.* 1982a, 1982b). However, in those studies

increasing casein were found to elevate plasma and liver cholesterol concentrations in rats. This discrepancy with the present studies might relate to the use of maize starch instead of glucose as replacer of protein. The type of carbohydrate in the diet of rats affects plasma and liver cholesterol concentrations, and this effect is influenced by the background composition of the diet (Beynen & Lemmens, 1987; Meijer & Beynen, 1988; Herman *et al.* 1991). A new finding is that increasing intakes of cod meal at the expense of glucose reduced plasma and liver cholesterol concentrations. Thus, the cholesterol-lowering activity of both soya-bean protein and cod meal, when compared with casein, can be enhanced by increasing the proportion of the proteins in the diet.

Whiting meal produced similar plasma cholesterol as did casein, but liver cholesterol concentrations were significantly lower in rats fed whiting meal. This corroborates earlier work (Lapré *et al.* 1989). In rabbits, dietary whiting meal has been shown to elevate serum cholesterol concentrations and to lower liver cholesterol concentrations when compared with casein (Lovati *et al.* 1990). Thus, in both rabbits and rats whiting meal versus casein appears to lower liver cholesterol concentrations. We are not aware of other studies in which serum and liver cholesterol concentrations were determined in rats fed diets containing plaice meal. Our results indicate that plaice meal has cholesterol-lowering activity when compared with either cod meal or whiting meal, and that this activity is similar to that of soya-bean protein.

The plasma and liver cholesterol concentrations as produced by the dietary protein sources tend to be associated with the amino acid composition of the proteins. In experiment 1, the concentrations of cysteine and glycine in the diet were negatively associated with plasma and liver cholesterol concentrations. In experiment 2, the concentration of methionine in the diet tended to be positively associated with plasma and liver cholesterol concentration. These associations are compatible with results of experiments in which rats were fed diets containing

various amino acid mixtures. In those studies, methionine is hypercholesterolaemic and glycine and cysteine are hypocholesterolaemic (Muramatsu & Sugiyama, 1990). There is evidence that an increase in the glycine-to-taurine ratio in conjugated bile acids enhances the hypercholesterolaemic effect of casein versus soya-bean protein (Van der Meer & Beynen, 1987). Since cysteine is a precursor of taurine, high intakes of cysteine might enhance taurine conjugation and thus have cholesterol-lowering activity. However, the hypocholesterolaemic properties of glycine cannot be readily explained in the light of formation of bile acid conjugates. Thus the metabolic basis for the observed relationship between amino acid composition of the dietary proteins and degree of cholesterolaemia remains obscure.

Animals fed diets containing soya-bean protein excrete more bile acids and neutral steroids in faeces than their counterparts fed casein (Beynen, 1990). This effect of protein type probably determines its cholesterolaemic activity. It could be suggested that cod meal and plaice meal enhance faecal excretion of bile acids and neutral steroids when compared with casein in the diet. Further work may test this suggestion.

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Chapter 3

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

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ABSTRACT. The effect of dietary whey protein versus 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 of protein or amino acids/kg feed. The diets were fed for three weeks. At the low dietary protein level, whey protein versus casein did not affect plasma total cholesterol, but lowered the concentration of liver cholesterol. At the high protein level in diet, whey protein significantly lowered plasma and liver cholesterol and also plasma triglycerides. 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 versus 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.

INTRODUCTION

The type of dietary protein can influence plasma cholesterol concentration (Kritchevsky, 1979; Beynen, 1990). Animal proteins generally exert a hypercholesterolaemic effect when compared with plants 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 versus casein also occurs in rabbits (Lovati *et al.* 1990), whereas in rats inconsistent results have been obtained (Choi *et al.* 1989; Sautier *et al* 1983).

In the present study using female rats, we re-addressed the question whether milk-whey protein versus casein in the diet influences plasma and liver

cholesterol concentrations. In contrast to previous work (Choi *et al.* 1989; Sautier *et al.* 1983), 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 a low (150 g/kg diet) and high level (300 g/kg diet) because protein type effects on 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 sole nitrogen source in the experimental diets. There were two levels of nitrogen 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, calcium, phosphorus and magnesium 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°C until feeding. Food and demineralized water were provided on an ad libitum basis.

Table 1. Analysed composition of the protein preparations

Dietary protein ...	Casein	Milk-whey protein
Components (g/kg)		
Nitrogen	136.9	120.3
Crude fat	16.0	71.0
Cholesterol	0.21	2.1
Calcium	0.12	3.17
Magnesium	0.02	0.55
Phosphorus	1.30	3.40
*Amino acids** (g/kg)		
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

* Data provided by manufacturers.

Animals and experimental procedures. Weanling, female Wistar rats (Hsd/Cpb:WU), aged about three weeks, were used. On arrival, the rats were given free access to a commercial, pelleted nonpurified diet (RMH-B^R, 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 one week, all animals were tranferred 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 (Hoek *et al.* 1988). After another week, on day 0 of the experiment, the rats were divided into eight groups of 12 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 libitum access to food and demineralized water. During the experimental period the rats were housed individually in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy). The cages were placed in a room with controlled temperature (20-24 °C), relative humidity (40-45%) and lighting (light on:06.00-18.00 hours).

The experiment lasted 21 days. 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 °C until analysis.

Chemical analyses. Nitrogen in protein preparations and diets was determined by the Kjeldahl method (Joslyn, 1970). Cholesterol in proteins and diets was

TABLE 2. Ingredient composition of the experimental diets

Protein or amino acid mixture (g/kg) ...	Casein		Whey protein		Casein amino acids		Whey protein amino acids	
	150	300	150	300	150	300	150	300
Ingredient (g/kg diet)								
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	1.25
$NaH_2PO_4.2H_2O$	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 (Hoek *et al.* 1988).

TABLE 3. Analysed composition 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 (g/kg)								
Dry matter	953	946	958	955	963	972	966	962
Nitrogen	24.2	49.1	23.9	47.5	19.6	36.9	18.1	36.0
Fat		125	123	116	101	124	127	126125
Cholesterol	12.5	10.4	10.7	10.9	10.5	9.8	9.7	10.2
Calcium	5.2	4.5	4.9	4.8	4.9	4.3	4.9	4.7
Magnesium	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Phosphorus	4.8	5.4	4.2	4.3	4.9	4.3	4.3	4.4
Selected fatty acids (g methylester/kg methylesters)								
C 8:0	55	52	49	48	53	55	52	57
C 10:0	40	38	37	34	39	40	38	41
C 12:0	326	320	312	287	329	331	327	330
C 14:0	135	134	132	122	137	137	137	136
C 16:0	102	105	105	107	103	101	103	100
C 18:0	40	41	41	41	40	40	40	37
C 18:1	115	118	121	130	115	113	116	114
C 18:2	159	163	173	196	157	156	158	157
C 18:3 (n-3)	15	16	17	19	15	15	15	15

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). Calcium and magnesium in the protein preparations were analysed by atomic absorption spectroscopy after dry ashing and dissolving the ash in 6 mol/L HCl as described (Hoek *et al.* 1988). Phosphorus in the same samples was analysed enzymatically using a commercial kit (MA-KIT Phosphate, Roche, Basel, Switzerland).

Cholesterol and triglycerides in plasma were measured enzymatically 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, in g/ml): very-low-density lipoprotein (VLDL, $d < 1.006$), intermediate-density lipoprotein (IDL, $1.006 < d < 1.019$), low-density lipoprotein (LDL, $1.019 < d < 1.063$), and high-density lipoprotein 2 (HDL_2, $1.063 < d < 1.125$) and high-density lipoprotein 3 (HDL_3, $1.125 < d < 1.210$). 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 enzymatic, 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 nitrogen source (casein versus whey protein), form of nitrogen source (intact protein versus amino acid mixture), and amount of nitrogen source (150 versus 300 g protein or amino acid mixture/kg diet) as main effects. The probability of a type I error <0.05 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 nitrogen source in the diet, a P value of 0.017 was used to

TABLE 4. Growth performance and liver weight in the rats fed the experimental diets for 21 days

Protein or amino acid mixture (g/kg feed) ...	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled	
	150	300	150	300	150	300	150	300	SE	ANOVA*
Body weight (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	TxA
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 weight (g/100 g body weight)										F,A,TxF,
	5.2	5.5[a,+]	5.0	4.7[t]	4.8[f]	4.6[f]	5.2	4.7[a]	0.1	TxA,AxF

Values are means and pooled SE for 12 rats per dietary group.

* ANOVA significance ($P<0.05$): T = significant effect of type of nitrogen source (whey protein versus casein); A = significant effect of amount of nitrogen source (150 versus 300 g protein or amino acid mixture/kg diet); F = significant effect of form of nitrogen source (intact protein versus amino acid mixture); significant effect of interactions: TxA; TxF; AxF; TxAxF.

\+ Group comparisons for two groups with one dietary variable ($P<0.017$): t = significant nitrogen type effect (intact whey protein versus casein or whey protein amino acids versus casein amino acids); a = significant nitrogen amount effect (150 versus 300 g protein or amino acid mixture/kg diet); f = significant nitrogen form effect (casein amino acids versus intact casein or whey protein amino acids versus intact whey protein).

TABLE 5. Plasma and liver cholesterol concentrations in rats fed the experimental diets for 21 days

Protein or amino acid mixture (g/kg) ...	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled	
	150	300	150	300	150	300	150	300	SE	ANOVA*
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,TxAxF
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,TxF, TxA,TxFxA
Plasma triglycerides (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,TxF
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,TxAxF

Values are means and pooled SE for 12 rats per dietary group.

* ANOVA significance ($P<0.05$): T = *significant effect of type of nitrogen source (whey protein versus casein)*; A = significant effect of amount of nitrogen source (150 versus 300 g protein or amino acid mixture/kg diet); F = significant effect of form of nitrogen source (intact protein versus amino acid mixture); significant effect of interactions: TxA; TxF; AxF; TxAxF.

\+ Group comparisons for two groups with one dietary variable ($P<0.017$): t = significant nitrogen type effect (intact whey protein versus casein or whey protein amino acids versus casein amino acids); a = significant nitrogen amount effect (150 versus 300 g protein or amino acid mixture/kg diet); f = significant nitrogen form effect (casein amino acids versus intact casein or whey protein amino acids versus intact whey protein).

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 simulating either casein or whey protein consumed similar amounts of feed. Increasing nitrogen intake reduced feed intake irrespective of the nature of the nitrogen 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 nitrogen 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 group mean plasma cholesterol concentration, whereas an increased intake of whey protein had an opposite effect. Thus, an increment of protein intake resulted in a significant protein type effect: whey protein versus 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 mirrorred that on plasma total cholesterol.

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

TABLE 6. Distribution of cholesterol between plasma lipoproteins in rats fed the experimental diets for 21 days

Protein or amino acid mixture (g/kg feed) ...	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled	
	150	300	150	300	150	300	150	300	SE	ANOVA*
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[f]	0.37	0.08	T,F,TxF
IDL cholesterol	0.25	0.32	0.19	0.19	0.24	0.15	0.12[t]	0.18	0.04	T,F,TxAxF
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	

Values are means and pooled SE for 12 rats per dietary group.

* ANOVA significance ($P<0.05$): T = significant effect of type of nitrogen source (whey protein versus casein); A = significant effect of amount of nitrogen source (150 versus 300 g protein or amino acid mixture/kg diet); F = significant effect of form of nitrogen source (intact protein versus amino acid mixture); significant effect of interactions: TxA; TxF; AxF; TxAxF.

\+ Group comparisons for two groups with one dietary variable ($P<0.017$): t = significant nitrogen type effect (intact whey protein versus casein or whey protein amino acids versus casein amino acids); a = significant nitrogen amount effect (150 versus 300 g protein or amino acid mixture/kg diet); f = significant nitrogen form effect (casein amino acids versus intact casein or whey protein amino acids versus intact whey protein).

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 triglyceride concentrations than casein, but a corresponding nitrogen source effect was not found in rats fed amino acid mixtures simulating either whey protein or casein (Table 5). The amino acid mixtures generally lowered triglycerides when compared with the intact proteins. Increasing intakes of intact proteins, but not of amino acid mixtures, reduced plasma triglycerides.

Whey protein versus casein significantly reduced liver cholesterol concentrations, this effect being greater when dietary protein level was increased (Table 5). Such an effect of nitrogen source was not seen in rats fed diets containing the amino acid mixtures. At the two dietary nitrogen concentrations, the amino acid mixture simulating casein lowered liver cholesterol concentrations when compared with the intact protein. For whey protein at the high dietary nitrogen concentration there was an opposite tendency.

At the high dietary protein level, intact whey protein versus 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 versus 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 versus casein on plasma and liver cholesterol. In those two studies, dietary protein concentration was about 200 g/kg feed. In this study, whey protein reduced liver cholesterol, but not plasma cholesterol, when given at a dietary concentration of 150 g/kg feed. 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 whey protein at the high level, cholesterol in VLDL was reduced. Since this lipoprotein fraction is a major carrier of plasma triglycerides, it is not surprising that group mean total triglyceride concentrations in plasma were decreased in rats given the high amount of whey protein.

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 was not seen with the amino acids simulating either whey protein or casein. This could imply that the cholesterol lowering activity of whey protein versus 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 versus casein in rats has been reproduced by feeding amino acid mixtures formulated to simulate these proteins (Nagata *et al.* 1981; Yadav & Liener, 1977).

The hypocholesterolaemic effect of soya-bean protein versus 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 versus 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 this study, whey protein at the high dietary nitrogen concentration significantly reduced the faecal excretion of bile acids. Thus, it unlikely that in rats whey protein versus casein inhibits re-absorption of bile acids as has been shown for soya-bean protein versus casein (Beynen, 1990).

It is possible that in rats fed the high level of whey protein cholesterol synthesis was inhibited when compared with their counterparts fed 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 feed back inhibitor of cholesterol synthesis speaks against this. Since cholesterol *de novo* synthesized 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 indeed blocks hepatic cholesterol synthesis can only be proved by direct measurement of cholesterol synthesis.

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Chapter 4

Excessive cholesterolemic response in analbuminemic rats fed a cholesterol-rich diet containing casein

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ABSTRACT Female Nagase analbuminemic rats and Sprague-Dawley rats were fed purified diets with or without 1% cholesterol and containing either soybean protein or casein. After consuming the cholesterol-free diets, Nagase analbuminemic rats had significantly higher plasma cholesterol and triglyceride concentrations than Sprague-Dawley rats. The higher plasma cholesterol in the Nagase analbuminemic rats were essentially in the low density and high density lipoproteins. Based on the fact that Nagase analbuminemic rats excreted more bile acids in feces it is possible that the higher baseline plasma cholesterol concentrations in Nagase analbuminemic rats may be partly caused by overproduction of cholesterol. The Nagase analbuminemic rats displayed a greater cholesterolemic response to cholesterol feeding than Sprague-Dawley rats, but only if casein was the protein source in the diet. Casein vs. soybean protein in either cholesterol-free or high-cholesterol diets reduced bile acid excretion in Sprague-Dawley rats but not in Nagase analbuminemic rats. The increased sensitivity to casein plus cholesterol feeding in Nagase analbuminemic rats may be caused by a lack of inhibition of *de novo* cholesterol synthesis.

INTRODUCTION

The Nagase analbuminemic rat is a mutant Sprague-Dawley rat characterized by an almost absolute deficiency of plasma albumin (1). With increasing age, Nagase analbuminemic rats develop hyperlipidemia (2-4). When Nagase analbuminemic and Sprague-Dawley rats are transferred from a commercial to a purified diet containing casein, the increase of plasma cholesterol concentrations is more pronounced in Nagase analbuminemic rats (unpublished data). Possibly, Nagase analbuminemic rats are more sensitive to the hypercholesterolemic effect of casein than Sprague-Dawley rats. When compared with such plant proteins as soybean protein, dietary casein causes increased plasma cholesterol concentrations in various animal species, including rats (5). The

hypercholesterolemic effect of casein becomes more pronounced when this protein is incorporated into diets high in cholesterol (6).

To determine whether Nagase analbuminemic rats more sensitive than Sprague-Dawley rats to the hypercholesterolemic effect of casein, female Nagase analbuminemic and Sprague-Dawley rats were fed cholesterol-free purified diets containing either casein or soybean protein. To increase the cholesterolemic response to the type of protein, diets enriched with cholesterol were also used.

MATERIALS AND METHODS

The experimental protocol was approved by the animal ethical committee of the State University, Utrecht, The Netherlands.

Animals and diets. Female 3-wk old rats of the Sprague-Dawley (SD/Hsd-Ola, Harlan-CPB, Zeist, The Netherlands) and Nagase analbuminemic strain (bred from rats kindly supplied by S. Nagase, Tokyo, Japan) were used. The animals were given free access to a commercial, pelleted nonpurified diet (RMH-B®, Hope Farms, Woerden, The Netherlands) and tap water. They were housed in groups of four rats in wire-topped polycarbonate cages (37.5 x 22.5 x 15.0 cm) with sawdust as bedding. The animal room had controlled temperature (20-24°C), relative humidity (40-45%) and lighting (light, 06.00-18.00 h).

After 1 wk of the commercial diet, all animals were transferred to the cholesterol-free, purified diet containing soybean protein (Table 1); demineralized water was supplied instead of tap water. After another 2 wk, the rats were divided into four dietary groups per strain (d 0) and housed three per cage. Within each strain, there were four dietary groups with similar distributions of plasma cholesterol concentrations and body weight. The groups within each strain were randomly assigned to one of the four experimental, purified diets (Table 1).

The experimental diets were in pelleted form and contained either soybean

TABLE 1. ***Ingredient composition of the experimental diets***

Ingredient	Cholesterol-free diets		High-cholesterol diets	
	Soy protein	Casein	Soy protein	Casein
	g/100 g diet			
Soy isolate[1]	17.87	-	17.87	-
Methionine	0.15	-	0.15	-
Casein[2]	-	17.07	-	17.07
Soybean oil	2.05	3.00	2.05	3.00
Coconut fat	9.00	8.71	9.00	8.71
Cholesterol	0.004	-	1.0	0.996
Glucose	60.976	60.62	59.98	59.624
$CaCO_3$	1.37	1.47	1.37	1.47
$NaH_2PO_4.2H_2O$	2.31	2.83	2.31	2.83
$MgCO_3$	0.20	0.23	0.20	0.23
Constant components[3]	6.07	6.07	6.07	6.07

1 Analyzed composition (g/100 g): nitrogen, 13.43; fat, 5.3; cholesterol, 0.0001; calcium, 0.28; magnesium, 0.07; phosphorus, 0.79.

2 Analyzed composition (g/100 g): nitrogen, 14.06; fat, 1.7; cholesterol, 0.026; calcium, 0.07; magnesium, 0.01; phosphorus, 0.22.

3 Constant components consisted of the following (g/100 g diet): cellulose, 3.0; KCl, 0.1; $KHCO_3$, 0.77; mineral premix, 1.0; vitamin premix, 1.2. The composition of the mineral and vitamin premix has been described (9).

protein or casein as protein source with (1 g/100 g) or without added cholesterol (Table 1). The diets were isonitrogenous and balanced for residual fat, cholesterol, calcium, phosphorus and magnesium in the protein preparations.

The analyzed composition of the diets (Table 2) agreed well with the calculated composition. The diets were stored at 4°C until feeding. The feeding period lasted 21 d. Food and demineralized water were freely available.

During the last week of the experiment (d 15-21) the rats were housed individually in metabolic cages (20 x 12 x 10 cm). The animals were weighed weekly and food intake was recorded from d 19-21.

The experiment was carried out with two cohorts of animals due to limited availability of the Nagase analbuminemic rats. Each cohort consisted of three rats per strain per dietary treatment. The interval between the start of the two cohorts was 1 week.

Collection of samples On d 21, the rats were anesthetized with diazepam (2.5 mg/kg, administered intraperitoneally) and fentanyl-fluanisone (0.5 ml/kg, administered intramuscularly) and exsanguinated by aortic puncture into tubes containing EDTA. Livers were removed and weighed. Blood was immediately centrifuged at 4°C (2000 x g, 10 min) to collect plasma. Plasma samples and livers were stored at -20°C until analysis. Plasma samples for apolipoprotein determination were stored at -80°C.

Chemical analyses. Nitrogen in protein preparations and diets was determined by the Kjeldahl method. Cholesterol in proteins and diets was determined by gas-liquid chromatography (7). Crude fat was determined by extraction according to the Soxhlet method (8). Calcium and magnesium in protein preparations and diets were analyzed by atomic absorption spectroscopy as described (9). Phosphorus in the ashed samples dissolved in 6 mol/L HCl was analyzed with a commercial test combination (MA-KIT Phosphate, Roche, Basle, Switzerland).

Cholesterol and triglycerides in plasma were measured enzymatically using Monotest and Test-Combination kits (Boehringer Mannheim GmbH, Mannheim, Germany). To determine plasma free cholesterol concentrations, cholesterol

TABLE 2. ***Analyzed composition of the experimental diets***

Component	Cholesterol-free diets		High-cholesterol diets	
	Soy protein	Casein	Soy protein	Casein
	g/100 g diet			
Dry matter	91.3	91.5	91.6	91.4
Nitrogen	2.47	2.44	2.36	2.41
Fat	11.0	11.7	11.5	12.7
Cholesterol	0.003	0.004	0.93	0.94
Calcium	0.70	0.71	0.71	0.67
Magnesium	0.09	0.09	0.09	0.08
Phosphorus	0.69	0.75	0.61	0.75
Fatty acids	g methylester/100 g methylesters			
C 8:0	6.7	5.8	6.3	5.6
C 10:0	4.9	4.3	4.7	4.2
C 12:0	37.9	33.5	36.7	33.5
C 14:0	14.6	13.3	14.5	13.5
C 16:0	10.6	9.8	9.7	10.0
C 18:0	3.4	3.7	3.6	3.6
C 18:1	9.8	11.3	10.6	11.3
C 18:2	10.8	14.8	11.1	14.6
C 18:3 (n-3)	0.8	1.1	0.8	1.1

esterase was omitted from the reaction mixture. Liver cholesterol was extracted and analyzed as described (10). Plasma apolipoprotein A-I was measured by electroimmunoassay as described (11). Plasma apolipoprotein B was determined by radial immunodiffusion (12), using a specific antiserum raised in rabbits

against purified rat LDL (13). Plasma apolipoprotein B concentration was calculated as percentage of a rat serum standard pool assayed simultaneously and expressed in arbitrary units.

Plasma lipoproteins were isolated by density-gradient ultracentrifugation (14). The following fractions were obtained: very low density lipoproteins, $d <$ 1.006 kg/L; intermediate density lipoproteins (IDL), $1.006 < d < 1.019$ kg/L; low density lipoproteins, $1.019 < d < 1.063$ kg/L; and high density lipoproteins in the form of HDL_2 ($1.063 < d < 1.125$ kg/L) and HDL_3 ($1.125 < d <$ 1.21 kg/L). Lipoprotein cholesterol was measured as plasma total cholesterol.

Plasma total protein concentrations were measured colorimetrically using a commercially available kit (Bio-Rad Lab, Munich, Germany). Albumin in plasma was determined as described (15).

Total 3-α bile acids in feces were determined with the use of an enzymatic, spectrofluorimetric test-combination (Sterognost-$3\alpha^R$ Flu, Nyegaard & Co, Oslo, Norway).

Statistical analyses. The data were subjected to three-way ANOVA with strain, type of dietary protein and amount of cholesterol in the diet as main effects. The probability of a type I error < 0.05 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. To test for strain effects, a P value of <0.05 was used. When testing for effects of protein type and amount of cholesterol in the diet, a P value of 0.025 was used to take into account the increased probability of a type I error because of multiple comparisons. All tests were done with the SPSS/PC$^+$ program (16).

RESULTS

Characteristics of Nagase analbuminemic rats. As expected, the Nagase analbuminemic rats were almost completely deficient in plasma albumin

TABLE 3. ***Growth performance, liver weight and plasma albumin concentrations of Sprague-Dawley (SD) and Nagase analbuminemic (NA) rats fed the experimental diets***[1]

Measure	Cholesterol-free diets		High-cholesterol diets		Pooled SEM	ANOVA[2]
	Soy protein	Casein	Soy protein	Casein		
Body weight, g (d 0)						
SD rats	109	108	108	109	6.8	S
NA rats	90	81	89	89		
Body weight, g (d 21)						
SD rats	181	187	182	183	9.3	S
NA rats	157[s,3]	153[s]	164	162		
Feed intake, g/d (d 19-21)						
SD rats	13.1	14.1	13.0	13.9	1.7	
NA rats	13.0	13.1	14.6	12.9		
Liver weight, g/100 g body weight (d 21)						
SD rats	3.54	3.92	5.11	4.97[c]	0.4	S,C,SxC
NA rats	5.60[s]	5.36[s]	5.57	5.85[s]		
Plasma total protein, g/L (d 21)						
SD rats	62	69	72[c]	70	2.3	S,C,SxC
NA rats	62	63	59[s]	61[s]		
Plasma albumin, g/L (d 21)						
SD rats	22	22	23	22	0.04	S
NA rats	0.06[s]	0.06[s]	0.07[s]	0.06[s]		

1 Values are means for six rats, except for the group of four NA rats fed the cholesterol-free diet containing casein.

2 ANOVA significance ($p < 0.05$); S = significant strain effect (NA vs. SD rats); P = significant effect of dietary protein type (casein vs. soybean protein); C = significant effect of amount of cholesterol in the diet (no cholesterol vs. 1% cholesterol); significant effect of interactions: PxS; CxS; CxP; CxPxS.

3 Group comparisons: s = significant strain difference ($P<0.05$; NA vs. SD rats); p = significant effect of dietary protein type within strains and within diets with identical cholesterol background ($P<0.025$; casein vs. soybean protein); c = significant effect of amount of cholesterol in the diet within strains and within diets with identical protein background ($P<0.025$; no cholesterol vs. 1% cholesterol in the diet).

(Table 3). Plasma total protein concentrations did not differ much between the two strains. The Nagase analbuminemic rats had a lower body weight, hepatomegaly (Table 3), hypercholesterolemia and hypertriglyceridemia (Table 4). The excess of cholesterol in plasma of Nagase analbuminemic rats was predominantly carried in lipoproteins with LDL and HDL_2 density, but cholesterol concentrations in other lipoprotein fractions were also greater (Table 5). Plasma apolipoprotein A-I and apolipoprotein B concentrations were markedly greater in Nagase analbuminemic rats. Liver cholesterol concentrations were similar in Nagase analbuminemic rats and Sprague-Dawley rats (Table 4). Nagase analbuminemic rats displayed higher rates of bile acid excretion in feces (Table 4). Food intake was similar in two strains (Table 3).

Growth response to diets. Dietary treatments did not significantly affect body weight and food intake of either strain (Table 3). In the second cohort, two Nagase analbuminemic rats fed the casein diet without added cholesterol died of unknown cause. Dietary cholesterol elevated liver weight and plasma total protein in Sprague-Dawley rats but not in Nagase analbuminemic rats (Table 3).

Lipemic response to diets. Dietary casein vs. soybean protein in the cholesterol-free diet did not influence plasma cholesterol concentrations in either strain (Table 4). When cholesterol was added to the diet, casein produced higher plasma total cholesterol concentrations than did soybean protein in Nagase analbuminemic rats but not in Sprague-Dawley rats. The casein-induced extra cholesterol in plasma of Nagase analbuminemic rats was located in the VLDL and IDL fractions (Table 5).

The addition of cholesterol to the diet produced a significant elevation of total and free plasma cholesterol concentrations in Sprague-Dawley rats and Nagase analbuminemic rats (Table 4). Casein in the diet amplified the plasma cholesterol response to dietary cholesterol in Nagase analbuminemic rats but not in Sprague-Dawley rats. Cholesterol feeding caused elevated cholesterol

TABLE 4. ***Plasma lipids and apolipoproteins, and fecal bile acid excretion in Sprague-Dawley (SD) and Nagase analbuminemic (NA) rats fed the experimental diets*** [1]

Measure	Cholesterol-free diets		High-cholesterol diets		Pooled SEM	ANOVA[2]
	Soy protein	Casein	Soy protein	Casein		
Plasma cholesterol, mmol/L (d 0)						
SD rats	2.4	2.4	2.5	2.4	0.2	S
NA rats	5.2[s,3]	5.5[s]	5.0[s]	5.1[s]		
Plasma cholesterol, mmol/L (d 21)						S,P,C,
SD rats	1.9	2.0	3.6[c]	3.5[c]	0.3	PxS,CxS,
NA rats	4.1[s]	4.6[s]	6.6[c,s]	9.3[c,s,p]		CxP,CxPxS
Plasma free cholesterol, mmol/L (d 21)						
SD rats	0.5	0.5	0.6[c]	0.6[c]	0.1	S,P,C,PxS,
NA rats	1.0[s]	1.2[s]	1.4[c,s]	1.8[c,s,p]		CxS,CxPxS
Plasma triglycerides, mmol/L (d 21)						
SD rats	0.6	0.7	0.5	0.7	0.3	S,CxS
NA rats	1.7[s]	1.7[s]	2.4[s]	2.5[s]		
Plasma apolipoprotein A-I, g/L (d 21)						
SD rats	0.36	0.40	0.41[c]	0.42	0.03	S,CxP
NA rats	0.89[s]	0.92[s]	1.01[s]	1.01[s]		
Plasma apolipoprotein B, arbitrary units (d 21)						
SD rats	78	93	104[c]	115	13.3	S,P,C
NA rats	227[s]	276[p,s]	276[s]	317[s]		
Liver cholesterol, μmol/g (d 21)						
SD rats	6.6	7.9	60.7[c]	77.5[c]	9.1	C,P
NA rats	6.4	7.6	40.8[c]	74.2[c,p]		
Fecal 3-α bile acid, μmol/d (d 18-20)						
SD rats	7.3	3.0[p]	27.7[c]	18.6[c,p]	2.6	S,P,C
NA rats	11.4[s]	8.7	37.7[c]	39.0[c,s]		

[1-3] See legends to Table 3.

concentrations in VLDL and IDL, while cholesterol concentrations in the other lipoprotein fractions were not significantly affected (Table 5). The high-cholesterol diet tended to lower cholesterol concentration in the LDL density range as was found earlier (17, 18).

Group mean plasma triglyceride concentrations were slightly elevated by casein vs. soybean protein in Sprague-Dawley rats, but not in Nagase analbuminemic rats (Table 4). Dietary cholesterol produced a rise of group mean plasma triglyceride concentrations in Nagase analbuminemic rats, but not in Sprague-Dawley rats.

Cholesterol feeding significantly raised apolipoprotein A-I and apolipoprotein B concentrations in Sprague-Dawley rats fed with soybean protein but not in Nagase analbuminemic rats (Table 4). Group mean apolipoprotein B concentrations in plasma were elevated in rats fed the casein diets. Dietary cholesterol also raised group mean plasma apolipoprotein B levels (Table 4).

Casein vs. soybean protein tended to elevate liver cholesterol concentrations in the two rat strains, regardless of the amount of cholesterol in the diet (Table 4). After cholesterol feeding, liver cholesterol concentrations were drastically raised.

Dietary casein in either cholesterol-free or high-cholesterol diets significantly lowered fecal bile acid excretion in Sprague-Dawley rats (Table 4) as compared with dietary soybean protein. In Nagase analbuminemic rats such a protein effect was not observed. Cholesterol feeding raised fecal bile acid excretion in both strains.

DISCUSSION

Baseline plasma cholesterol concentrations for Nagase analbuminemic rats were about two-fold higher than sor Sprague-Dawley rats. This confirms earlier work (2-4). In Nagase analbuminemic rats fed cholesterol-free diets, the

TABLE 5. ***Distribution of cholesterol between plasma lipoproteins in Sprague-Dawley (SD) and Nagase analbuminemic (NA) rats fed the experimental diets for 21 d***[1]

Lipoprotein class	Cholesterol-free diets		High-cholesterol diets		Pooled SEM	ANOVA[2]
	Soy protein	Casein	Soy protein	Casein		
	mmol cholesterol/L whole plasma					
VLDL						
SD rats	0.09	0.06	1.73[c]	1.57[c]	0.25	S,C
NA rats	0.22[s,3]	0.29[s]	2.08[c]	2.61[c]		
IDL						S,C,P,
SD rats	0.03	0.03	0.56[c]	0.80[c]	0.12	PxS,CxS,
NA rats	0.18[s]	0.14[s]	0.95[c]	1.83[c,p,s]		CxP,CxPxS
LDL						
SD rats	0.57	0.85	0.52	0.27[c]	0.20	S,C
NA rats	1.43[s]	1.86[s]	1.11[s]	1.26[s]		
HDL_2						
SD rats	0.83	0.69	0.72	0.87	0.18	S
NA rats	1.80[s]	2.20[s]	1.81[s]	1.97[s]		
HDL_3						
SD rats	0.09	0.07	0.08	0.06	0.02	S,C
NA rats	0.17[s]	0.17[s]	0.12[s]	0.13[s]		

[1-3] See legends to Table 3.

cholesterol concentration in all five lipoprotein fractions were elevated but the excess of plasma cholesterol was located essentially in the LDL and HDL_2 density ranges. Plasma triglyceride concentrations were also markedly higher in the female Nagase analbuminemic rats, which corroborates other studies (2-4).

The mechanism underlying the development of hyperlipidemia in Nagase analbuminemic rats is not clear. Takahashi et al. (2) suggested that the hyperlipidemia in Nagase analbuminemic rats is a direct consequence of the analbuminemia because intraperitoneal administration of albumin was found to lower plasma lipids in Nagase analbuminemic rats. However, they did not include a control group to take into account possible effects of handling and injection *per se*. Moreover, the hypolipidemic effect of albumin was relatively small, but this could relate to the fact that plasma albumin concentrations were not raised to the levels in normal rats.

The hypercholesterolemia in Nagase analbuminemic rats may be partly caused by overproduction of cholesterol. When fed cholesterol-free diets, Nagase analbuminemic rats excreted more bile acids than Sprague-Dawley rats. This indicates that rates of bile acid synthesis are higher in Nagase analbuminemic rats than Sprague-Dawley rats. Another line of evidence also points to such a strain difference. Serum bile acid concentrations in Nagase analbuminemic rats are markedly reduced (19). It is likely that hepatic bile acid concentrations are also lower in Nagase analbuminemic rats leading to diminished inhibition of bile acid synthesis because this pathway is subject to feed-back regulation (20). Because cholesterol is the substrate for bile acid synthesis and because the diet was cholesterol-free, basal rates of cholesterol synthesis in Nagase analbuminemic rats must be elevated.

After 21 d, the addition of cholesterol to the soybean-protein diet had caused a slightly larger increase of plasma cholesterol concentrations (d 21 vs. d 0) in Nagase analbuminemic rats than in Sprague-Dawley rats and the addition of

cholesterol to the casein diet produced a significantly higher increase of plasma cholesterol in Nagase analbuminemic rats than in Sprague-Dawley rats (Table 4). Why does casein in the diet cause a greater strain difference in the susceptibility of plasma cholesterol to cholesterol feeding? An increment of bile acid synthesis and inhibition of cholesterol synthesis after cholesterol feeding both protect against the development of hypercholesterolemia (21). Casein is known to raise intestinal cholesterol and bile acid absorption and to lower cholesterol and bile acid synthesis (22). This is reflected in a depressed fecal excretion of neutral steroids and bile acids in animals fed casein (23-25). Casein-induced inhibition of bile acid excretion was also observed in the Sprague-Dawley rats. However, bile acid excretion in the Nagase analbuminemic rats fed the high-cholesterol diet did not respond to dietary casein. This does not agree with the greater susceptibility of plasma cholesterol in Nagase analbuminemic rats to cholesterol in a casein diet. After cholesterol feeding, the rate of bile acid synthesis, and thus also that of bile acid excretion, is the net effect of increased substrate availability in the form of exogenous cholesterol and decreased substrate availability in the form of endogenously synthesized cholesterol. Possibly, in Nagase analbuminemic rats *de novo* cholesterol synthesis is relatively insensitive to inhibition by dietary cholesterol. The rise of fecal bile acid excretion on the high cholesterol diet could not ameliorate the severe accumulation of cholesterol in the liver in either strains.

Cholesterol feeding elevated group mean plasma triglyceride concentrations in Nagase analbuminemic rats but not in Sprague-Dawley rats. In cholesterol-fed both strains, plasma cholesterol concentrations in VLDL and IDL were raised. This suggests that dietary cholesterol has a specific effect on triglyceride metabolism in Nagase analbuminemic rats. Either triglyceride synthesis is elevated or triglyceride clearance is lowered. In favor of the former possibility is the fact that cholesterol synthesis in the liver is necessary for VLDL secretion

(26, 27). Thus, stimulation of *de novo* cholesterol synthesis in the Nagase analbuminemic rats may be accompanied by an increment of VLDL synthesis. It has been shown that 0.5% or 1.0% cholesterol in the diet does not affect lipoprotein lipase (LPL) activity in either normal or spontaneously hypercholesterolemic rats (28). In addition, chylomicron and VLDL clearance is not reduced in Nagase analbuminemic rats (29) despite marked reductions in post-heparin LPL (29) and adipose tissue lipase (3) activity. Thus the cholesterol-induced hypertriglyceridemia in the Nagase analbuminemic rats is probably not caused by a defective triglyceride clearance.

In conclusion, with respect to diet-induced hypercholesterolemia, Nagase analbuminemic rats were found to be more sensitive to the combination of cholesterol and casein feeding than Sprague-Dawley rats. Comparison of dietary effects in these strains of rats may result in new concepts in the etiology of hyperlipidemia.

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PART II

DIETARY PROTEIN AND NEPHROCALCINOSIS

Chapter 5

Effects of amount and type of dietary protein on nephrocalcinosis

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ABSTRACT Nephrocalcinosis in the rat refers to the deposition of calcium salts in the kidney, usually in the corticomedullary region. The condition can be demonstrated chemically by the analysis of kidney calcium, and histologically by staining kidney slides for the presence of calcium phosphate. Nephrocalcinosis is associated with enhanced urinary excretion of albumin and increased concentrations of urea in plasma. This suggests that nephrocalcinosis impairs kidney function. The composition of the diet is an important determinant of the degree of nephrocalcinosis in female rats. Diets with high concentrations of phosphorus and/or low concentrations of magnesium induce nephrocalcinosis. Increasing the amount of dietary protein in the form of either ovalbumin, soybean protein or casein at the expense of carbohydrates, counteracts the development of nephrocalcinosis seen after feeding high-phosphorus or low-magnesium diets. In these studies, the diets were balanced for magnesium, calcium and phosphorus in the protein preparations. The type of dietary protein also affects the severity of nephrocalcinosis in female rats. With the use of diets that were carefully balanced for the concentrations of calcium, magnesium and phosphorus, nephrocalcinosis tended to increase with cod meal, soybean protein and casein, in this order. Possible mechanisms by which the amount and type of dietary protein influence nephrocalcinosis will be discussed.

INTRODUCTION

The disorder of nephrocalcinosis (kidney calcification) is characterized by the deposition of calcium phosphates in the corticomedullary junction of the kidney. This condition is frequently observed in female rats fed either natural-ingredient or purified diets (1-14). Female rats develop nephrocalcinosis more readily than do males (2, 6, 9, 11, 12). Nephrocalcinosis in rats is associated with increased excretion of urinary albumin (4) and often also with increased plasma urea concentrations (1). Rats with nephrocalcinosis have prolonged tubular fluid transit

times at the single-nephron level (15). Thus, nephrocalcinosis may impair kidney function.

Diet composition is an important determinant of development of nephrocalcinosis in female rats. Nephrocalcinosis can be provoked by diets containing either low concentrations of magnesium (1, 5) or high concentrations of phosphorus (1, 3, 6, 11). Most attention has been directed towards the influence of dietary minerals on nephrocalcinosis, but there is evidence that dietary proteins also influence nephrocalcinogenesis (8, 9, 13, 14). Here we briefly review studies on the effects of amount and type of dietary protein on nephrocalcinosis in rats. The degree of nephrocalcinosis was assessed chemically, by the analysis of kidney calcium, and/or histologically, by scoring stained kidney slides for the presence of calcium phosphate deposits.

AMOUNT OF PROTEIN

Increasing the amount of dietary protein inhibits the development of nephrocalcinosis in rats fed nephrocalcinogenic diets. Tables 1 and 2 show that an increase in dietary protein concentration completely counteracted nephrocalcinogenesis induced by either increased phosphorus or decreased magnesium intake. The diets used were balanced for calcium, magnesium and phosphorus; extra protein was added at the expense of the glucose component of the diets.

It is unlikely that the effect of the amount of ovalbumin on nephrocalcinosis refers to a specific characteristic of ovalbumin. There is evidence that increased intakes of casein and soybean protein also reduce nephrocalcinosis (8, 9, 17), but the diets used were not balanced for minerals in the protein preparations. More convincing are the data presented in Table 3. Increasing intakes of soybean protein and casein were associated with decreasing concentrations of calcium in kidney. However, no such relationship was observed in rats fed diets with cod meal. In this experiment, kidney calcium concentrations were relatively high, which explains

TABLE 1

Influence of Amount of Dietary Protein on Urinary Mineral Concentrations and on Nephrocalcinogenesis as Induced by Phosphorus Loading

	Dietary variables (%, w/w)		
Phosphorus:	0.2	0.6	0.6
Nitrogen:	2.4	2.4	4.8
Kidney calcium, % dw	0.04 ± 0.00	1.04 ± 0.41	0.02 ± 0.00
Nephrocalcinosis score, on 0-3 scale	0.0	2.0	0.0
Urinary calcium, mg/L	68.2 ± 39.6	28.0 ± 12.3	39.4 ± 19.6
Urinary magnesium, mg/L	132.5 ± 31.3	61.0 ± 19.9	61.6 ± 23.7
Urinary phosphorus, mg/mL	2.97 ± 0.06	4.25 ± 1.21	3.19 ± 0.56
Urinary pH	8.7 ± 0.2	7.3 ± 0.1	7.6 ± 0.2

The diets contained 15.1% (w/w) casein, 0.5% calcium and 0.04% magnesium; indicated nitrogen concentrations were calculated. Extra protein was added in the form of ovalbumin at the expense of the glucose component of the diet. The diets were fed for 28 days. Means ± SD for 6 rats per dietary group. Data taken from Sterck *et al.* (16).

TABLE 2

Influence of Amount of Dietary Protein on Urinary Mineral Concentrations on Nephrocalcinogenesis as Induced by Magnesium Restriction

	Dietary variables (%, w/w)		
Magnesium:	0.04	0.01	0.01
Nitrogen:	2.4	2.4	4.8
Kidney calcium, % dw	0.11 ± 0.03	2.07 ± 0.51	0.07 ± 0.03
Nephrocalcinosis score, on 0-3 scale	0.4	2.6	0.0
Urinary calcium, mg/L	23.9 ± 10.3	14.6 ± 8.9	15.7 ± 4.9
Urinary magnesium, mg/L	34.8 ± 10.8	7.6 ± 2.6	16.1 ± 6.5
Urinary phosphorus, mg/mL	3.53 ± 1.27	2.63 ± 0.69	2.32 ± 0.65
Urinary pH	8.0 ± 0.1	7.6 ± 0.1	7.7 ± 0.1

The diets contained 15.1% (w/w) casein, 0.5% calcium and 0.6% phosphorus; indicated nitrogen concentrations were calculated. Extra protein was added in the form of ovalbumin at the expense of the glucose component of the diet. The diets were fed for 28 days. Means ± SD for 8 rats per dietary group. Data taken from Sterck *et al.* (16).

why nephrocalcinosis score was not affected by the amount of protein in the diet. When nephrocalcinosis is severe, the histological score does not discriminate any more (3).

TYPE OF PROTEIN

The type of dietary protein also influence the development of nephrocalcinosis in rats. Substitution of lactalbumin for casein produces disappearance of nephrocalcinosis (14, 18). Compared with casein, single-cell protein significantly promote the development of nephrocalcinosis in female rats (7).

Soybean protein or cod meal versus casein at a dietary concentration of 2.4% (w/w) nitrogen tended to reduce kidney calcium concentrations (Table 3). We have also shown that there is no clear difference between kidney calcium concentrations of rats fed either casein, whiting or plaice meal (Zhang and Beynen, unpublished). However, in these experiments nephrocalcinosis was severe, which may have masked any effect of this kind of protein.

POSSIBLE MECHANISMS

It is clear that both amount and type of dietary protein influence the development of nephrocalcinosis in female rats, but the underlying mechanism is not understood. In nephrocalcinosis, the primary lesion is an intratubular accumulation of calcium phosphates (1). It is thus likely that increased calcium and/or phosphate concentrations in urine will stimulate nephrocalcinogenesis. Indeed, high-calcium and high-phosphorus diets produced nephrocalcinosis in rats, which is accompanied by increased calcium and phosphate excretion with urine (3). Under *in vitro* conditions, the addition of magnesium depress the formation of calcium phosphate precipitates (19). This may also occur *in vivo*. The feeding of high-magnesium diets prevents nephrocalcinosis and raises urinary magnesium concentrations (16). Decreasing the pH of solutions antagonizes the precipitation

TABLE 3
Influence of Increasing Dietary Protein Concentrations on Nephrocalcinosis and on Urinary Mineral Concentrations

Measure	Type of Protein	Amount of dietary nitrogen (%, w/w) 2.4	4.8	7.2
Kidney calcium, %				
	Soy protein	3.7 ± 1.8	2.5 ± 1.1	1.8 ± 1.0
	Casein	4.7 ± 0.7	2.6 ± 1.8	2.4 ± 1.6
	Cod meal	1.4 ± 1.4	1.3 ± 1.2	1.8 ± 1.5
Nephrocalcinosis score, on 0-3 scale				
	Soy protein	2.9 ± 0.2	2.8 ± 0.4	2.7 ± 0.6
	Casein	2.9 ± 0.2	2.7 ± 0.3	2.4 ± 0.5
	Cod meal	2.3 ± 0.5	2.3 ± 0.4	2.4 ± 0.7
Urinary calcium, mg/L				
	Soy protein	29.4 ± 11.9	37.0 ± 19.0	65.1 ± 18.7
	Casein	31.5 ± 6.9	48.1 ± 23.2	80.8 ± 20.9
	Cod meal	35.4 ± 9.6	58.4 ± 14.0	104.1 ± 57.8
Urinary magnesium, mg/L				
	Soy protein	75.1 ± 17.1	71.2 ± 25.5	79.9 ± 35.7
	Casein	93.2 ± 21.9	125.7 ± 35.9	121.2 ± 33.4
	Cod meal	111.7 ± 33.8	158.8 ± 38.6	157.1 ± 28.9
Urinary phosphorus, mg/mL				
	Soy protein	2.5 ± 1.8	1.2 ± 0.9	0.9 ± 0.2
	Casein	3.1 ± 0.9	3.2 ± 1.4	3.2 ± 0.5
	Cod meal	2.5 ± 1.2	2.5 ± 0.4	1.7 ± 0.3
Urinary pH				
	Soy protein	8.1 ± 0.4	8.1 ± 0.5	7.9 ± 0.6
	Casein	7.2 ± 0.4	6.2 ± 0.1	5.8 ± 0.1
	Cod meal	7.8 ± 0.3	6.6 ± 0.2	6.2 ± 0.2

The indicated proteins were used as sole source of dietary protein; nitrogen concentrations were calculated. The diets contains 0.6% (w/w) calcium, 0.07% magnesium and 0.7% phosphorus. Extra protein was added at the expense of the glucose component of the diets. The diets were fed for 21 days. Means ± SD for 6 rats per dietary group. Data taken from Zhang and Beynen (22).

of calcium phosphate crystals (20). This corroborates the observation that feeding of the urine acidifier, ammonium chloride, instead of an equimolar amount of ammonium carbonate, reduces phosphorus-induced nephrocalcinosis in female rats (21).

Thus, it could be hypothesized that high-protein diets reduce nephrocalcinosis by either lowering urinary pH, decreasing urinary calcium and phosphate concentrations or raising urinary magnesium concentrations, or by a combination of these. Tables 1 and 2 show that increased intakes of ovalbumin did not systematically raise urinary concentrations of magnesium, tended to elevate urinary calcium concentrations and reduced urinary phosphorus concentrations. High-protein intake did not influence urinary pH. It would thus appear that high intakes of protein inhibit nephrocalcinogenesis by lowering urinary phosphorus concentrations.

The data in Table 3 illustrate that the basis for the anti-nephrocalcinogenic effect of high-protein intake may depend on the type of protein. After increased intakes of soybean protein, the decreased urinary concentration of phosphorus may have been responsible for the lower degrees of nephrocalcinosis. However, after increased intakes of casein, the increased urinary concentrations of magnesium and decreased urinary pH may have caused the inhibition of nephrocalcinogenesis. After increased intakes of cod meal, the nephrocalcinogenic effect of increased urinary concentrations of calcium may have been nullified by the anti-nephrocalcinogenic effect of increased urinary magnesium concentrations, depressed urinary phosphorus concentrations and lower urinary pH values.

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Chapter 6

Increasing Intake of Soybean Protein or Casein, but not of Cod Meal, Reduces Nephrocalcinosis in Female Rats

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ABSTRACT Female weanling rats were fed diets with either soybean protein, casein or cod meal at 171, 342 or 513 mmol nitrogen/100 g for three wk. The diets were isonitrogenous and balanced for fat, cholesterol, calcium, magnesium and phosphorus. Cod meal feeding at 171 and 342 mmol nitrogen/100 g diet produced lower kidney calcium concentrations than the feeding of either soybean protein or casein. Increasing protein intakes were associated with reduced kidney calcium concentrations in the rats fed either soybean protein or casein but not in those fed cod meal. The anti-nephrocalcinogenic effect of increasing intakes of soybean protein may relate to the lowering of urinary phosphorus concentration. Increasing intakes of casein probably inhibited nephrocalcinogenesis by lowering urinary pH and raising urinary magnesium concentration. Increasing cod meal concentrations in the diet lowered urinary pH and raised urinary magnesium and calcium concentrations, but the effects on nephrocalcinogenesis of these changes probably counteracted each other.

INTRODUCTION

Nephrocalcinogenesis is the process of deposition of calcium phosphates in the corticomedullary junction of the kidney. This process occurs in rats fed either natural-ingredient (Ritskes-Hoitinga et al. 1991) or purified diets (Hoek et al. 1988). Female rats develop nephrocalcinosis more readily than males (Cousins and Geary 1966).

The amount of dietary protein affects the degree of nephrocalcinosis in rats. Increased intakes of protein at the expense of carbohydrates reduced calcium concentrations in kidney (Eklund et al. 1973, Goulding and Malthus 1970, Hitchman et al. 1979, Kaunitz and Johnson 1976, Schneider and Menden 1988, Shah et al. 1986), but the diets used were not balanced for minerals in the protein preparations. The addition of ovalbumin to the diet, at the expense of glucose, counteracted nephrocalcinosis in female rats as induced by feeding

a diet with either high amount of phosphorus or low amount of magnesium (Sterck et al. 1992, Van Camp et al. 1990). The severity of nephrocalcinosis is also influenced by the type of protein as illustrated by work showing that casein is more nephrocalcinogenic than whey protein (Kunkel et al. 1984, Meyer et al. 1982). The mechanism underlying the influence of amount and type of dietary protein on nephrocalcinogenesis is not understood. The present study was carried out to see whether protein effects on nephrocalcinogenesis can be explained by changes in urinary pH and urinary concentrations of calcium, magnesium and phosphorus as these four measures are considered to be determinants of nephrocalcinogenesis (Ritskes-Hoitinga and Beynen 1992). Female rats were fed carefully balanced, purified diets containing three levels of either soybean protein, casein or cod meal. After feeding these diets for 3 wk, mineral concentrations in kidney and urine were determined.

MATERIALS AND METHODS

The experimental protocol was approved by the animal experiments committee of the Department of Laboratory Animal Science, Utrecht State University.

Experimental diets. Soybean protein isolate (Ralston, Purina Co, St. Louis, MO), casein (Havero B.V., Rotterdam, The Netherlands) and cod meal (Institute for Fishery Products CIVO-TNO, Ijmuiden, The Netherlands) were used as protein sources. To balance the diets for specified components, the protein preparations were analyzed for nitrogen, cholesterol, crude fat,calcium, magnesium and phosphorus. The results are given in the legend to Table 1. The experimental diets (Table 1) contained a calculated amount of either 171, 342 or 513 mmol nitrogen/100 g. Either soybean protein, casein or cod meal was the sole source of dietary protein. The diets with soybean protein contained added methionine. Extra protein was added at the expense of the glucose component. The diets were balanced for fat, cholesterol, calcium, magnesium

TABLE 1. Ingredient composition of the experimental diets

Ingredient	171 mmol nitrogen/100 g			342 mmol nitrogen/100 g			513 mmol nitrogen/100 g		
	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal
	g/100 g diet								
Soy isolate†	17.87	-	-	35.74	-	-	53.61	-	-
D,L-Methionine	0.15	-	-	0.30	-	-	0.45	-	-
Casein‡	-	17.07	-	-	34.14	-	-	51.21	-
Cod meal§	-	-	18.43	-	-	36.86	-	-	55.29
Soybean oil	2.05	3.0	2.15	1.11	3.0	1.3	0.16	3.0	0.46
Coconut fat	9.0	8.71	9.0	9.0	8.42	9.0	9.0	8.13	9.00
Cholesterol	1.0	0.996	0.868	1.0	0.991	0.736	1.0	0.987	0.605
Glucose	59.98	59.62	60.162	43.78	43.07	44.144	27.59	26.523	28.135
CaCO3	1.37	1.47	1.06	1.25	1.44	0.62	1.12	1.41	0.17
NaH2PO4.2H2O	2.31	2.83	2.09	1.60	2.64	1.17	0.89	2.45	0.24
MgCO3	0.20	0.23	0.17	0.15	0.23	0.10	0.11	0.22	0.03
Constant components*	6.07	6.07	6.07	6.07	6.07	6.07	6.07	6.07	6.07
Chemical analysis	mmol or g/100 g diet								
Nitrogen, mmol	174.2	172.8	168.5	345.5	348.4	348.4	531.2	518.3	515.5
Crude fat, g	13.1	13.6	12.9	13.5	13.7	13.6	13.5	13.6	12.8
Cholesterol, mmol	3.49	2.35	2.15	2.64	2.48	2.48	2.64	2.38	2.64
Calcium, mmol	14.72	14.47	14.97	14.72	14.22	15.72	12.48	13.72	15.47
Magnesium, mmol	2.88	2.88	2.88	2.88	2.47	3.29	2.88	2.47	3.29
Phosphorus, mmol	20.02	23.57	20.99	20.66	26.47	20.66	19.69	27.44	21.31

Legend to Table 1.

+ Analysed composition (g/100 g): nitrogen, 13.4; fat, 5.3; cholesterol, 0.000; calcium, 0.28; magnesium, 0.07; phosphorus, 0.79.

‡ Analysed composition (g/100 g): nitrogen, 14.1; fat, 1.7; cholesterol, 0.026; calcium, 0.07; magnesium, 0.01; phosphorus, 0.22.

┤ Analysed composition (g/100 g): nitrogen, 13.0; fat, 4.6; cholesterol, 0.715; calcium, 0.96; magnesium, 0.11; phosphorus, 1.00.

* Constant components consisted of (g/100 g diet): cellulose, 3.0; KCl, 0.10; KHCO3, 0.77; mineral premix, 1.0; vitamin premix, 1.2 The composition of the mineral and vitamin premix has been described (Hoek et al. 1988).

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and phosphorus. Table 1 shows the analyzed composition of the diets.

Animals and housing. Female Wistar rats (Cpb/Hsd, Harlan-CPB, Zeist, The Netherlands) were used. On arrival, when the animals were aged three wk, they were housed in groups of 4 rats in Macrolon type III cages (UNO BV, Zevenaar, The Netherlands) with a layer of sawdust as bedding. The rats were fed ad libitum a commercial, pelleted natural-ingredient diet (RMH-B^R, Hope Farms, Woerden, The Netherlands) and tap water for 3 d. Then, they were transferred to the purified, pre-experimental diet containing 171 mmol nitrogen/100 g with soybean protein as protein source. Demineralized water was supplied.

After another wk, on d 0 of the experiment, the rats were divided into 9 groups, each comprising 6 rats. Body weight distributions of the groups were similar. Each group was randomly assigned to one of the experimental diets, including the pre-experimental diet. Food and demineralized water were freely available. The animals were weighed weekly, and feed intakes were recorded. The experiment lasted 3 wk. During the experiment (d 0-21), the rats were housed individually in metabolic cages (Techniplast Gazzada, Buguggiate, Italy). The cages were placed in a room with controlled temperature (20-24°C), relative humidity (40-45%) and lighting (light, 06.00-18.00 h).

Collection of samples. From d 18-20, urine and feces were daily collected quantitatively from each rat. The tubes for collecting urine and feces had been successively washed with phosphorus-free soap, 0.1 mol/L HCl and distilled water before use. At the end of the experiment, heparinized blood samples were taken in the non-fasted state between 09:00-12:00 h by orbital puncture while under diethyl-ether anesthesia. The anesthetized animals were killed by cervical dislocation. Right kidneys were removed and weighed. All samples were stored at -20 °C until analysis.

Chemical analyses. Crude protein contents of protein preparations and diets were analyzed by the Kjeldahl method (Joslyn 1970). Cholesterol was determined by gas-liquid chromatography (Nordby and Nagy 1973). Crude fat was determined by extraction according to the Soxhlet method (Joslyn 1970). Calcium, magnesium and phosphorus in protein preparations, diets, urine, feces, plasma and kidneys were analyzed as described (Hoek et al. 1988). Urea and creatinine in plasma and urine were measured using kits (MA-kit urea UV) and (MA-kit Creatinine) purchased from F. Hoffmann-La Roche Co. Ltd. Diagnostica, Basel, Switzerland.

Statistical analyses. Two-way ANOVA was used to determine the influence of type and level of dietary proteins. Pearson correlation coefficients were calculated for individual rats concerning relations between kidney calcium concentrations and either urinary pH or urinary concentrations of calcium, magnesium and phosphorus. The level of statistical significance was pre-set at $P < 0.05$. The SPSS-PC$^+$ program was used (SPSS Inc. 1986).

RESULTS

Body weight and food intake. Type and amount of protein in the diet did not significantly influence final body weight of the rats (Table 2). Higher amounts of dietary protein slightly, but significantly, depressed feed intake. The

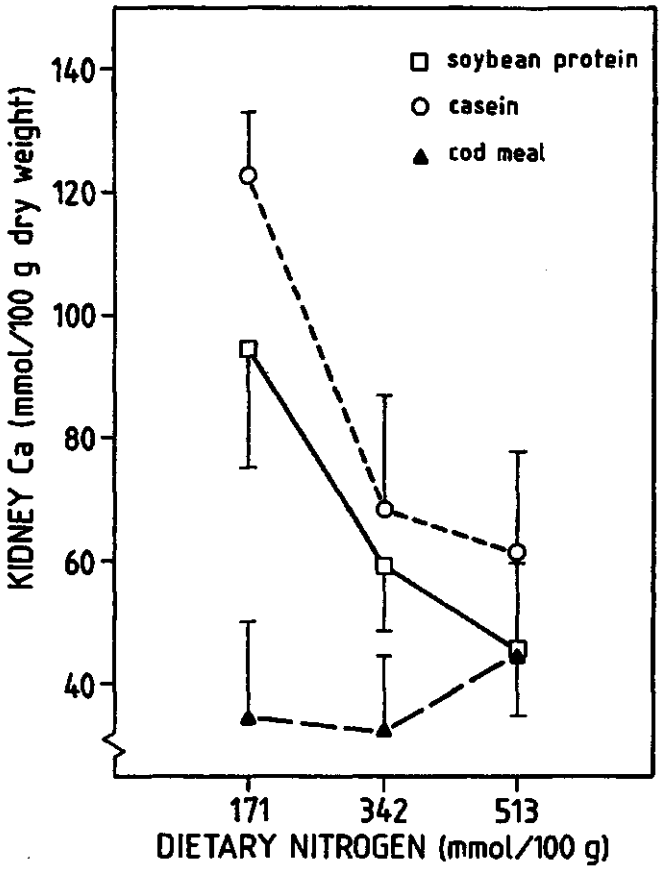

FIGURE 1. Relationship between dietary nitrogen concentration and kidney calcium concentrations in female rats fed diets containing either soybean protein, casein or cod meal at three different concentrations. ANOVA showed significant effects ($P<0.05$) of dietary protein type and amount of protein but no interaction. Results are means ± SEM for 6 rats per dietary group.

TABLE 2. Growth performance, feed intake, and nephrocalcinosis in rats fed the experimental diets

Measure	171 mmol nitrogen/100 g			342 mmol nitrogen/100 g			513 mmol nitrogen/100 g			Pooled SEM	ANOVA[1]
	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal		
Body weight, g											
d 0	144	142	143	142	142	142	144	142	145	4.92	
d 21	203	196	204	194	203	197	193	200	198	6.92	
Feed intake, g/d	12.3	11.4	12.3	11.5	11.6	11.6	10.7	11.0	11.6	0.35	A
Kidney dry weight, mg											
	204	210	190	201	241	205	216	236	231	9.04	A
Kidney phosphorus, mol/100 g dry weight											
	0.49	0.69	0.44	0.41	0.57	0.40	0.34	0.38	0.33	0.09	A
Kidney magnesium, mmol/100 g dry weight											
	5.90	6.99	6.72	5.35	8.36	7.95	4.80	6.72	10.49	1.45	T

Values are means and pooled SEM for 6 rats per group.

[1] ANOVA significance ($P < 0.05$): T = effect of dietary protein type; A = effect of amount of protein in the diet; T x A = effect of interaction.

type of dietary protein did not affect food intake.

Kidney minerals. Kidney dry weight was significantly raised with increasing dietary protein level (Table 2). This effect was clearest with cod meal in the diet, while it was essentially absent with soybean protein. Kidney calcium concentrations were significantly influenced by dietary protein level and type (Fig. 1). Diets containing either soybean protein or casein lowered kidney calcium with increasing protein intake, but diets with cod meal produced similar kidney calcium concentrations irrespective of the amount of protein in the diet. Kidney phosphorus concentrations were significantly lower with an increment of dietary protein level, irrespective of the source of protein (Table 2). The type of dietary protein significantly affected kidney magnesium concentrations. Soybean protein in the diet reduced kidney magnesium concentrations when compared with either casein or cod meal.

Urinary volume and pH. Higher protein concentrations in the diet were associated with a significantly larger urinary volume (Table 4). Urinary volume was generally greatest with soybean protein in the diet. Urinary pH was significantly affected by type and amount of protein in the diet (Fig. 2). Dietary soybean protein produced highest pH values. With casein and cod meal in the diet, but not with soybean protein, higher dietary protein concentrations lowered urinary pH. For individual rats there was no significant correlation between kidney calcium concentration and urinary pH ($r=0.03$, $n=54$).

Calcium metabolism. Plasma calcium concentrations were not significantly affected by type and amount of dietary protein (Table 3). Calcium intake was depressed with increasing protein intake. The rats fed cod meal ate more calcium than their counterparts fed either casein or soybean protein. Fecal calcium excretion was raised by cod meal in the diet. Apparent calcium absorption was reduced by increasing concentrations of either soybean protein or cod meal in the diet; the amount of dietary casein did not influence apparent

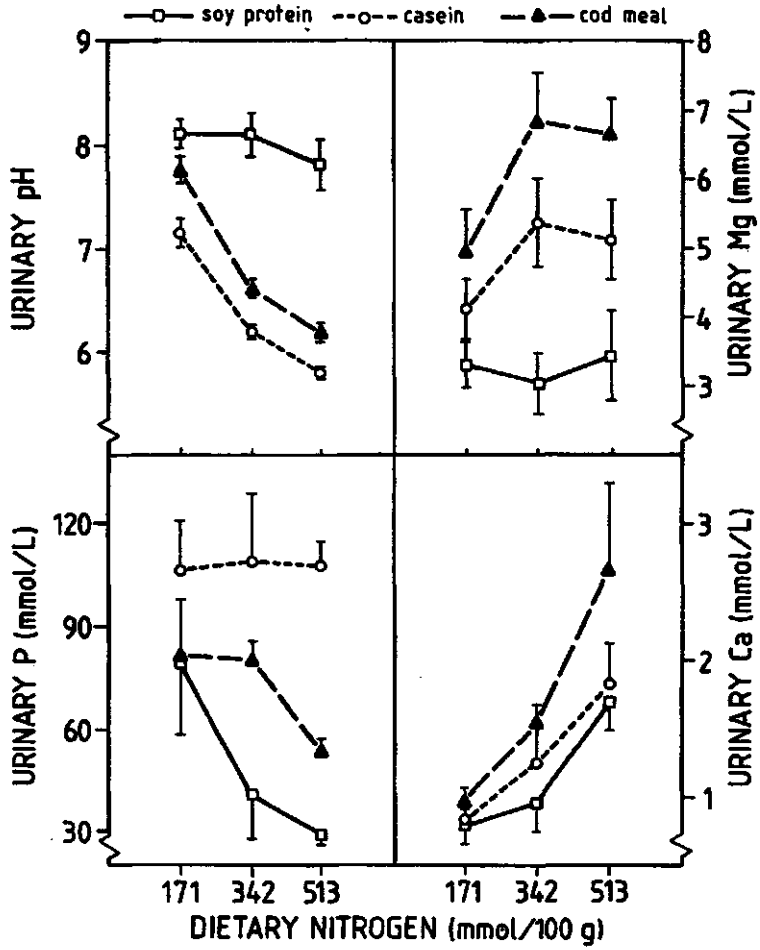

FIGURE 2. Urinary pH and urinary concentrations of phosphorus, magnesium and calcium in female rats fed diets containing either soybean protein, casein or cod meal at three different concentrations. ANOVA showed significant effects ($P<0.05$) of dietary protein type and amount of protein concerning urinary pH and calcium; there was also a significant interaction of the main effects with regard to urinary pH. Urinary phosphorus and magnesium were significantly influenced by the type of dietary protein only. Results are expressed as means ± SEM for 6 rats per dietary group.

TABLE 3. Indicators of calcium, magnesium and phosphorus metabolism in rats fed the experimental diets

Measure	171 mmol nitrogen/100 g			342 mmol nitrogen/100 g			513 mmol nitrogen/100 g			Pooled SEM	ANOVA[1]
	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal		
Calcium											
Plasma, mmol/L	9.5	9.1	9.3	9.3	9.1	8.8	8.8	9.2	9.2	0.21	
Intake, mmol/d	1.95	1.81	2.02	1.79	1.75	1.97	1.46	1.63	1.94	0.06	T,A,TxA
Feces, mmol/d	1.05	1.09	1.13	1.03	1.05	1.16	1.04	0.96	1.40	0.09	T
Urine, μmol/d	7.61	6.31	7.53	13.56	13.85	14.10	25.7	26.2	42.8	4.47	A
Apparent absorption[2], %	46.5	40.2	43.6	42.6	40.6	41.2	29.6	41.3	27.2	4.44	A
Magnesium											
Plasma, mmol/L	1.7	1.6	1.6	1.7	1.5	1.5	1.5	1.5	1.5	0.11	
Intake, mmol/d	0.38	0.31	0.39	0.35	0.30	0.41	0.34	0.29	0.41	0.01	T
Feces, mmol/d	0.24	0.22	0.23	0.20	0.19	0.22	0.19	0.16	0.27	0.02	T,TxA
Urine, μmol/d	33.67	30.86	37.17	42.79	58.15	60.41	53.97	74.40	97.51	6.07	T,A,TxA
Apparent absorption[2], %	38.3	28.7	40.9	42.7	36.5	46.8	43.0	46.3	34.9	4.48	
Phosphorus											
Plasma, mmol/L	48.8	43.9	48.0	48.4	43.9	51.0	45.6	45.4	51.0	2.23	T
Intake, mmol/d	2.65	2.95	2.83	2.51	3.26	2.59	2.31	3.25	2.67	0.10	T,TxA
Feces, mmol/d	0.91	0.96	0.99	0.96	0.90	0.92	1.05	0.80	1.11	0.07	
Urine, mmol/d	0.67	0.78	0.55	0.54	1.23	0.72	0.44	1.57	0.77	0.07	T,A,TxA
Apparent absorption[2], %	65.9	67.4	64.8	61.6	72.5	64.1	54.6	75.5	58.1	2.49	T,TxA

Legend to Table 3.

Values are means and pooled SEM for 6 rats per group.

[1] ANOVA significance ($P < 0.05$): T = effect of dietary protein type; A = effect of amount of protein in the diet; T x A = effect of interaction.

[2] Calculated as intake minus fecal excretion and expressed as percentage of intake.

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calcium absorption. Urinary calcium excretion rose markedly with higher protein intake, irrespective of the type of protein. Concentrations of calcium in urine were also raised by the enrichment of the diet with protein (Fig. 2). After feeding cod meal, urinary calcium concentration was higher than after feeding either soybean protein or casein. Kidney calcium and urinary calcium concentrations were not significantly correlated ($r=-0.18$, $n=54$).

Magnesium metabolism. Dietary treatments had no effect on plasma magnesium concentrations (Table 3). Magnesium intake was lowest in the groups fed casein. At dietary nitrogen concentrations higher than 171 mmol /100 g, cod meal produced higher rates of magnesium excretion in feces than did either soybean protein or casein. The amount of either soybean protein or cod meal in the diet did not systematically affect magnesium absorption. Increasing intakes of casein were associated with higher efficiencies of magnesium absorption. Absolute urinary excretion (Table 3) and urinary concentrations of magnesium (Fig. 2) were depressed by soybean protein when compared with either casein or cod meal. Higher amounts of either casein or cod meal in the diet were accompanied by raised excretion of magnesium in urine. When increasing dietary protein concentration from 342 to 513 mmol nitrogen/100 g, casein and cod meal did not further increase urinary magnesium concentrations (Fig. 2). Individual kidney calcium values and urinary magnesium concentration were not significantly correlated ($r=-0.13$, $n=54$).

Phosphorus metabolism. Plasma phosphorus concentrations were higher in rats fed cod meal than in rats fed either soybean protein or casein, provided

that dietary nitrogen concentration was higher than 171 mmol/100 g (Table 3). Phosphorus intake in the groups fed either soybean protein or cod meal were similar, whereas rats given casein diets consumed significantly more phosphorus. Fecal phosphorus excretion rose with increasing intakes of soybean protein, but with dietary casein the opposite was seen. The amount of cod meal in the diet did not systematically influence fecal phosphorus excretion. Apparent phosphorus absorption fell with higher dietary concentrations of either soybean protein or cod meal. In contrast, increasing the amount of casein in the diet raised apparent phosphorus absorption. Phosphorus excretion with urine dropped with increasing dietary concentrations of soybean protein. With casein, and to a lesser extent also with cod meal, the opposite was found. Concentrations of phosphorus in urine fell with higher intakes of either soybean protein or cod meal, but remained rather constant with casein in the diet (Fig. 2). Kidney calcium concentrations and urinary phosphorus concentrations of individual rats were not significantly correlated ($r=0.25$, $n=54$).

Urea and creatinine in plasma and urine. Plasma and urinary urea concentrations, and also urea clearance, rose with increasing protein intake, the type of protein not having an additional influence (Table 4). Creatinine concentrations in plasma were highest with cod meal in the diet and lowest with casein. Urinary creatinine was drastically elevated after increasing the amount of cod meal in the diet. Likewise, creatinine clearance was raised by cod meal in a dose-dependent fashion; this was not seen with either soybean protein or casein which produced lower rates of creatinine clearance than cod meal.

DISCUSSION

Nephrocalcinogenesis may be dampened by a low urinary pH, low urinary calcium and phosphorus concentrations and high urinary magnesium concentrations (Ritskes-Hoitinga and Beynen 1992). The observed anti-nephrocalcinoge-

TABLE 4. Urea and creatinine clearance, and urine excretion in rats fed the experimental diets

Measure	171 mmol nitrogen/100 g			342 mmol nitrogen/100 g			513 mmol nitrogen/100 g			Pooled SEM	ANOVA[1]
	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal	Soybean protein	Casein	Cod meal		
Urea											
Plasma, mmol/L	5.8	6.4	5.6	8.4	8.5	7.8	8.9	9.7	8.3	0.61	A
Urine, mmol/100g bw.d	3.3	3.0	2.9	8.0	7.7	7.4	12.1	13.1	13.8	0.55	A
Clearance, mL/100g bw.min	0.41	0.33	0.36	0.68	0.67	0.68	0.96	1.04	1.17	0.12	A
Creatinine											
Plasma, μmol/L	33.4	30.9	35.0	35.8	30.2	42.3	35.4	32.4	42.4	2.29	T
Urine, μmol/100 g bw.d	35.2	27.7	53.2	35.2	29.4	79.4	36.3	36.0	122.6	3.70	T,A,TxA
Clearance, mL/100 g bw.min	0.75	0.63	1.08	0.68	0.67	1.32	0.72	0.79	2.01	0.10	T,A,TxA
Urine volume, mL/d	10.8	7.8	8.4	13.6	11.8	9.1	15.6	14.7	14.9	4.59	T,A

Values are means and pooled SEM for 6 rats per group.

[1] ANOVA significance ($P < 0.05$): T = effect of dietary protein type; A = effect of amount of protein in the diet; TxA = effect of interaction.

nic effect of increasing intakes of soybean protein may essentially lie in the lowering of urinary phosphorus concentrations. Extra casein in the diets may have inhibited nephrocalcinogenesis by lowering urinary pH and raising urinary magnesium concentrations. Apparently, these two effects of increasing casein intake were not nullified by the nephrocalcinogenesis promoting effect of raised urinary calcium concentrations. Thus, inhibition of nephrocalcinogenesis seen after feeding higher amounts of either soybean protein or casein may have a different basis. Extra cod meal in the diet did not reduce kidney calcification despite the lowered urinary pH and raised urinary magnesium concentration, which may relate to the nephrocalcinogenic activity of the elevated urinary calcium concentration. The observed effects of dietary protein on urinary mineral concentrations can at least partly be traced back to differences in urinary volume. Increasing protein intake raised urinary volume, this effect being greater for casein and cod meal than for soybean protein, when expressed relative to baseline urinary volume.

Animal proteins in the diet promote urinary acidification (Schuette et al. 1981), which may be due to their relatively high concentrations of sulfur-containing amino acids. Indeed, the increment of either dietary casein or cod meal concentrations consistently lowered urinary pH. Although methionine, which was also added as such to the soybean protein diets, and cysteine intakes obviously rose with increasing intakes of soybean protein, urinary pH remained rather stable. Thus, the observed urine acidification after feeding increasing amounts of casein and cod meal must be due to components other than sulfur-containing amino acids in the protein preparations. The higher plasma and urinary urea concentrations after feeding increasing amounts of protein were most likely caused by enhanced protein catabolism. The fact that cod meal, unlike soybean protein and casein, contains creatine explains the high urinary output of creatinine after feeding the diets containing cod meal. The high

creatinine clearance in rats fed cod meal prevented a substantial rise of plasma creatinine concentrations in these animals.

An increment of dietary protein concentration produced calciuria. This was not accompanied by an increase in apparent calcium absorption, suggesting that intestinal calcium absorption did not determine urinary calcium excretion. The calciuria seen in rats fed high-protein diets is probably related to the raised excretion of sulphate after protein loading (Whiting and Draper 1980). Increasing intakes of soybean protein reduced apparent calcium absorption. Phytate, which is present in soybean protein isolate preparations (Brink et al. 1991), may have caused the lowering of calcium absorption by formation of calcium-magnesium-phytate complexes in the intestine (Champagne 1988). The fall of calcium absorption after increasing intakes of cod meal may relate to unavailable calcium in this protein preparation; the cod meal contained 24 mmol calcium/100 g, which provided up to 86% of total dietary calcium.

Urinary magnesium excretion rose after increasing dietary protein concentrations, this effect being greater with either casein or cod meal than with soybean protein. Magnesium absorption rose with higher intakes of casein, which would explain the casein-induced magnesiuria. Soybean protein versus casein reduces magnesium absorption through its phytate component (Brink et al. 1991), but this was not seen at either 171 or 342 mmol nitrogen/100 g diet which probably relates to the somewhat higher phosphorus intake in rats fed the casein diet. Dietary phosphorus effectively reduces magnesium absorption (Hoek et al. 1988, Mars et al. 1988). At 513 mmol nitrogen/100 g diet, soybean protein did lower magnesium absorption when compared with casein, pointing to an effect of phytate. The lowering of urinary pH as induced by high cod meal or casein intake may also have caused the associated magnesiuria. Lowering of urinary pH enhances magnesium excretion in urine (Greger et al. 1991). Indeed, we found for individual rats a negative relationship between urinary pH and

urinary magnesium concentration ($r=-0.63$, $n=54$, $P<0.05$).

The type of protein in the diet significantly affected phosphorus metabolism. Apparent phosphorus absorption in the rats fed either soybean protein or cod meal was lower than in those fed casein which most likely resulted from unavailable phosphorus in soybean protein and cod meal. In soybean protein isolate, about half of the phosphorus may be in the form of phytate (Brink et al. 1991). The observed lowering of phosphorus excretion in urine with increasing intakes of soybean protein may have been caused by the reduction of phosphorus absorption. In a non-steady state, urinary phosphorus excretion not only depends on intestinal absorption but also on tubular reabsorption. A low tubular pH value decreases phosphorus reabsorption (Mizgala & Quamme 1985). With dietary casein, both the lower pH of urine and enhanced absorption may have caused the rise of urinary phosphorus excretion. In rats fed cod meal, the lowering of urinary pH and fall of phosphorus absorption had opposite effects on phosphorus excretion in urine, the former effect probably being strongest.

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Chapter 7

Dietary Whey Protein Versus Casein Inhibits Nephrocalcinogenesis in Female Rats

Xizhong Zhang and Anton C. Beynen

ABSTRACT The effect of dietary whey protein versus casein on kidney calcification was studied in weanling female rats. The purified diets containing whey protein as sole source of protein significantly reduced nephrocalcinosis when compared with casein. Increasing dietary protein concentration from 15 to 30 g/100 g diet reduced kidney calcification, but casein remained more nephrocalcinogenic than whey protein. Amino acid mixtures (15 g/100 g diet) simulating either casein or whey protein did not produce different degrees of nephrocalcinosis. Increased intake of the amino acid mixtures (from 15 to 30 g/100 g diet) lowered the severity of nephrocalcinosis. The anti-nephrocalcinogenic effect of whey protein may be explained by lowering urinary phosphorus concentrations. For individual rats there was a positive correlation between degree of nephrocalcinosis and urinary phosphorus concentration. The inhibitory effect of increased nitrogen intake on nephrocalcinosis may be explained by lowering both urinary pH and urinary phosphorus concentration. The results indicate that the effect of type of protein on nephrocalcinosis, but not that of amount of protein, may relate to protein structure or non-protein components rather than amino acids in the protein.

INTRODUCTION

Nephrocalcinosis, an intratubular deposition of calcium phosphates in the corticomedullary junction of the kidney, is frequently found in female rats fed purified diets (Ritskes-Hoitinga and Beynen 1992). Type and amount of dietary protein have been shown to affect kidney calcification in female rats. Increasing intakes of protein reduce nephrocalcinosis (Eklund et al. 1973, Sterck et al. 1992, Van Camp et al. 1990, Zhang and Beynen 1992). Soybean protein (Kunkel et al. 1984, Zhang and Beynen 1992, Zhang et al. 1992) or whey protein (Kaunitz and Johnson 1976, Kunkel et al. 1984, Meyer et al. 1989) versus casein in the diet inhibits nephrocalcinogenesis.

Kidney calcification is prevented by a low urinary pH, high urinary

magnesium concentration and/or low urinary calcium and phosphorus concentrations (Ritskes-Hoitinga and Beynen 1992). The anti-nephrocalcinogenic effect of soybean protein versus casein relates to its lowering effect on urinary phosphorus concentration (Zhang and Beynen 1992, Zhang et al. 1992). The mechanism underlying the inhibitory influence of whey protein on nephrocalcinosis was unknown. We have therefore compared urinary mineral concentrations in female rats fed purified diets containing either casein or whey protein as sole source of protein. The diets were carefully balanced for minerals in the protein preparations and contained either 15 or 30 g protein/100 g. To test the hypothesis that the anti-nephrocalcinogenic activity of whey protein lies in its amino acid composition rather than in its non-protein components, diets containing amino acid mixtures (15 or 30 g/100 g diet) simulating either whey protein or casein were also fed to female rats.

MATERIALS AND METHODS

The experimental protocol was approved by the animal experiments committee of the Department of Laboratory Animal Science, State University, Utrecht.

Animals and diets. Female Wistar rats (Hsd/Cpb:WU), aged about three wk, were used. On arrival, the rats were given free access to a commercial, pelleted nonpurified diet (RMH-B^R, 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 one wk, all animals were transferred to the purified, pre-experimental diet containing the following (g/100 g diet): casein, 17.53 (171 mmol nitrogen); soybean oil, 3.0; coconut fat, 8.72; cholesterol, 0.996; cellulose, 3.0; glucose, 61.479; $CaCO_3$, 1.245; $NaH_2PO_4.2H_2O$, 0.893; $MgCO_3$, 0.137; KCl, 0.1; $KHCO_3$, 0.7; mineral premix, 1.0; vitamin premix, 1.2. The composition of the mineral and vitamin

TABLE 1. Ingredient and analyzed composition of the experimental diets[1]

	Casein		Whey protein		Casein amino acids		Whey protein amino acids	
	15	30	15	30	15	30	15	30
Ingredient	g/100 g diet							
Casein[2]	17.53	35.06	-	-	-	-	-	-
Whey protein[3]	-	-	19.95	39.90	-	-	-	-
Casein amino acids[4]	-	-	-	-	15.0	30.0	-	-
Whey protein amino acids[4]	-	-	-	-	-	-	15.0	30.0
Soybean oil	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Coconut fat	8.72	8.44	7.58	6.17	9.0	9.0	9.0	9.0
Cholesterol	0.996	0.993	0.958	0.916	1.00	1.00	1.00	1.00
Glucose	60.474	43.347	59.662	41.694	62.61	47.61	62.61	47.61
$CaCO_3$	1.25	1.24	1.09	0.93	1.25	1.25	1.25	1.25
$NaH_2PO_4.2H_2O$	1.89	1.78	1.66	1.33	2.00	2.00	2.00	2.00
$MgCO_3$	0.14	0.14	0.10	0.06	0.14	0.14	0.14	0.14
Constant components[5]	6	6	6	6	6	6	6	6
Chemical analysis	mmol or g/100 g diet							
Dry matter, g	95.3	94.6	95.8	95.5	96.3	97.2	96.6	96.2
Nitrogen, mmol	172.9	350.7	170.7	339.3	140.0	263.6	129.3	257.1
Fat, g	12.5	12.3	11.6	10.1	12.4	12.7	12.6	12.5
Calcium, mmol	13.0	11.2	12.2	12.0	12.2	10.7	12.2	11.7
Magnesium, mmol	1.64	1.65	1.64	1.64	1.64	1.64	1.64	1.64
Phosphorus, mmol	15.5	17.4	13.6	13.9	15.8	13.9	13.9	14.2

Legend to Table 1.

1 The dietary concentration of indicated protein or amino acid mixture is expressed as g/100 g diet.

2 Analyzed composition (g or mmol/100 g casein): nitrogen, 13.7 g (protein, 85.6 g); fat, 1.6 g; cholesterol, 0.055 mmol; calcium, 0.30 mmol; magnesium, 0.082 mmol; phosphorus, 4.19 mmol.

3 Analyzed composition (g or mmol/100 g whey protein): nitrogen, 12.0 g (protein, 75.2 g); fat, 7.1 g; cholesterol, 0.553 mmol; calcium, 7.93 mmol; magnesium, 2.26 mmol; phosphorus, 10.96 mmol.

4 See Table 2.

5 Constant components consisted of the following (g/100 g diet): cellulose, 3.0; KCl, 0.1; $KHCO_3$, 0.7; mineral premix, 1.0; vitamin premix, 1.2. The composition of the mineral and vitamin premix has been described (Hoek et al. 1988).

premix has been described (Hoek et al. 1988). The rats consumed food and demineralized water ad libitum.

After another wk (d 0), the rats were divided into eight groups of 12 animals each on the basis of body weight. The groups were randomly assigned to the experimental diets given in Table 1. The diets contained either 15 g or 30 g of protein or amino acid mixture/100 g diet. Dietary nitrogen was in the form of casein (Havero b.v., Rotterdam, The Netherlands), whey protein (DMV Campina, Veghel, The Netherlands) or amino acid mixtures simulating either casein or whey protein. The analyzed composition of the protein preparations is given in the legend to Table 1 and the composition of the amino acid mixtures is given in Table 2. The diets with intact proteins were balanced for nitrogen, residual fat, cholesterol, calcium, phosphorus and magnesium in the protein preparations. The analyzed composition of the diets (Table 1) agreed well with the calculated composition. The diets were in powdered form and stored at 4°C until feeding. Food and demineralized water were provided on an ad libitum basis. During the

TABLE 2. Amino acid composition of the casein and whey protein preparations

	Casein	Whey protein
	g/100 g product	
Alanine	1.98	3.9
Arginine	3.06	2.1
Aspartate	4.63	8.6
Cysteine	0.26	1.9
Glutamate	17.55	13.8
Glycine	2.56	1.5
Histidine	2.32	1.6
Isoleucine	5.04	5.5
Leucine	9.52	8.6
Lysine	6.21	7.4
Methionine	2.40	1.7
Phenylalanine	3.64	2.9
Proline	8.03	4.9
Serine	2.81	4.6
Threonine	3.56	6.0
Tryptophan	1.16	1.7
Tyrosine	4.72	2.4
Valine	5.88	5.2

Data provided by manufacturers.

experimental period (d 0-21), the rats were housed individually in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy). The cages were placed in a room with controlled temperature (20-24°C), relative humidity (40-45%), and lighting (light on: 06:00-18:00 h). The experiment lasted 21 days. The rats were weighed weekly and feed intakes were recorded.

Collection of samples. Between d 17 and 20, feces and urine were collected daily from each rat. The cages and tubes for collecting urine and feces had been washed successively with phosphorus-free soap, 0.1 mol/L HCl and distilled water. On d 21, the rats were anesthetized with xylazine (6.86 mg/kg, administered intraperitoneally) and ketamine (60 mg/kg, administered intramuscularly) and exsanguinated by aortic puncture into heparinized tubes. Blood was centrifuged to collect plasma, which was stored at -20°C until analysis. Kidneys were removed and weighed. The left kidney was fixed in 10% (v/v) neutral phosphate buffered formalin solution.

Chemical and histological analyses. Nitrogen in protein preparations and diets was determined by the Kjeldahl method (Joslyn 1970). Crude fat was determined by extraction according to the Soxhlet method (Joslyn 1970). Cholesterol in the proteins was determined by gas-liquid chromatography (Nordby and Nagy 1973). Feces were freeze-dried and ground. The determination of calcium and magnesium in feces and diet samples, protein preparations, urine and right kidney has been described (Hoek et al. 1988). Phosphate in the ashed and dissolved solid samples and in urine was analyzed enzymatically using a commercial kit (MA-KIT Phosphate, Roche, Basel, Switzerland). Histological examination of the left kidney was carried out as described (Hoek et al. 1988). Sections stained with hematoxylineosin were graded in random order and blind on a scale from 0 (no calcium phosphate deposits) to 3 (severe calcinosis).

Statistical analyses. The data were subjected to three-way ANOVA with type of nitrogen source (casein versus whey protein), form of nitrogen source (amino acid mixtures versus intact protein) and amount of nitrogen source (15 versus 30 g protein or amino acid mixture/100 g diet) as main effects. The probability of a type I error < 0.05 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. Nephrocalcinosis scores were compared with the use of Mann-

Whitney *U* test. When testing for effects of type, form and amount of nitrogen source, a P value of 0.017 was used to take into account the increased probability of a type I error because of multiple comparisons. Pearson correlation coefficients (r) were calculated for individual rats concerning possible relations between kidney calcium concentration and either urinary pH or urinary concentrations of calcium, magnesium and phosphorus. Between nephrocalcinosis scores and either kidney calcium or phosphorus concentrations, or urinary concentrations of calcium, magnesium and phosphorus, Spearman's correlation coefficients (R) were calculated. All statistical analyses were carried out with the SPSS/PC+ program (Spss Inc. 1986).

RESULTS

Growth performance. Final body weight did not differ significantly between the groups (Table 3). Whey protein versus casein in the diet depressed food intake. Increasing intake of nitrogen reduced food intake irrespective of protein type and form.

Kidney weight and nephrocalcinosis. Dietary treatments affected relative kidney weight (Table 3). Kidney weight was lower in rats fed intact whey protein at the low nitrogen level instead of casein. However, whey protein amino acids raised kidney weight when compared with casein amino acids. Increasing nitrogen intake generally produced higher kidney dry weight.

Intact whey protein versus casein significantly lowered kidney concentrations of calcium (Fig. 1), phosphorus and magnesium (Table 3). The kidney calcification preventing effect of whey protein was also seen on the basis of histological nephrocalcinosis scores (Table 3). Increasing casein intake reduced kidney calcium, magnesium and phosphorus concentrations and also the nephrocalcinosis score.

The diets containing the two amino acid mixtures at the low level produce

TABLE 3. Growth performance and nephrocalcinosis in the rats fed the experimental diets[1-3]

	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled SEM	ANOVA[4]
	15	30	15	30	15	30	15	30		
Body weight, g										
d 0	93	93	94	94	93	95	95	95	2.6	
d 21	176	187	174	174	165	181	172	170	5.0	
Feed intake, g/d (d 18-20)	13.9	13.2	12.1	11.8	13.4	12.9	13.1	12.3	0.50	T,A
Kidney weight, g/100 g body weight	0.44	0.45	0.39^{t}	0.45^{a}	0.40	0.42	$0.46^{t,f}$	0.45^{t}	0.03	A,TxF,AxF,TxFxA
Kidney dry weight, mg	166	177	145^{t}	173^{a}	141^{f}	164	156^{t}	163	4.9	F,A,TxF,TxFxA
Kidney minerals, mmol/100 g dry weight										
Magnesium	6.0	4.6^{a}	3.7^{t}	3.7	4.3^{f}	3.7^{a}	4.1^{f}	3.8^{a}	0.02	T,A,F,TxA,TxF,TxAxF
Phosphorus	84.4	53.2^{a}	40.0^{t}	38.6^{t}	52.8^{f}	$40.4^{a,f}$	51.4^{f}	40.3^{a}	0.1	T,A,F,TxA,TxF,TxAxF
Nephrocalcinosis score, on a scale of 0-3	2.9	1.9^{a}	0.2^{t}	0^{t}	2.1^{f}	$0.8^{a,f}$	2.5^{f}	$0.2^{a,t}$		

1 The dietary concentration of indicated protein or amino acid mixture is expressed as g/100 g diet.

2 Values are means and pooled SEM for 12 rats per group. For nephrocalcinosis scores only means are given.

3 Group comparisons for two groups with one dietary variable ($P<0.017$); t = significant nitrogen type effect (intact whey protein versus casein or whey protein amino acids versus casein amino acids); a = significant nitrogen amount effect (15 versus 30 g protein or amino acid mixture/100 g diet); f = significant nitrogen form effect (casein amino acids versus intact casein or whey protein amino acids versus intact whey protein).

4 ANOVA significance ($P<0.05$); T = significant effect of type of nitrogen source (whey protein versus casein); A = significant effect of amount of nitrogen source (15 versus 30 g protein or amino acid mixture/100 g diet); F = significant effect of form of nitrogen source (intact protein versus amino acid mixture); significant effect of interactions: TxA; TxF; AxF; TxAxF.

similar kidney mineral concentrations and nephrocalcinosis scores. In rats fed the diets with the high concentration of amino acids, kidney calcium concentration (Fig. 1) and nephrocalcinosis score (Table 3) were significantly lower with whey protein amino acids than with casein amino acids. An increment of the intake of the amino acids significantly lowered kidney mineral concentrations.

Kidney calcium concentrations were positively correlated with kidney phosphorus concentrations ($r=0.88$, $P<0.001$, $n=96$). Both kidney calcium and phosphorus concentration were highly correlated with nephrocalcinosis scores ($R=0.93$ and 0.80, $P<0.001$; $n=96$).

Mineral absorption. The rats fed casein in the form of either intact protein or amino acids ingested similar amounts of calcium and magnesium as their counterparts fed the diets containing whey protein (Table 4). Increasing intakes of nitrogen were associated with lower intakes of calcium and magnesium. Apparent calcium absorption did not differ significantly between the dietary groups (Table 4). Whey protein versus casein significantly raised magnesium absorption. The amino acid mixtures produced higher efficiencies of magnesium absorption than the intact proteins, but there was no effect of amino acid composition. Higher levels of nitrogen in the diets stimulated magnesium absorption (Table 4).

The rats fed intact casein ate more phosphorus than those fed whey protein (Table 4). Phosphorus intakes by rats fed the two amino acid mixtures at the same level were similar. Increment of nitrogen intake in rats fed diets other than the casein diets reduced phosphorus intake. Apparent absorption of phosphorus was not affected by the type of nitrogen source, but the amino acid mixtures produced significantly higher efficiencies than the intact proteins. Increasing nitrogen intake raised phosphorus absorption.

Urinary minerals. Irrespective of the nitrogen source, an increased intake significantly raised urinary calcium excretion (data not shown). Intact whey protein versus casein raised urinary calcium concentration (Fig. 2). The amino acid

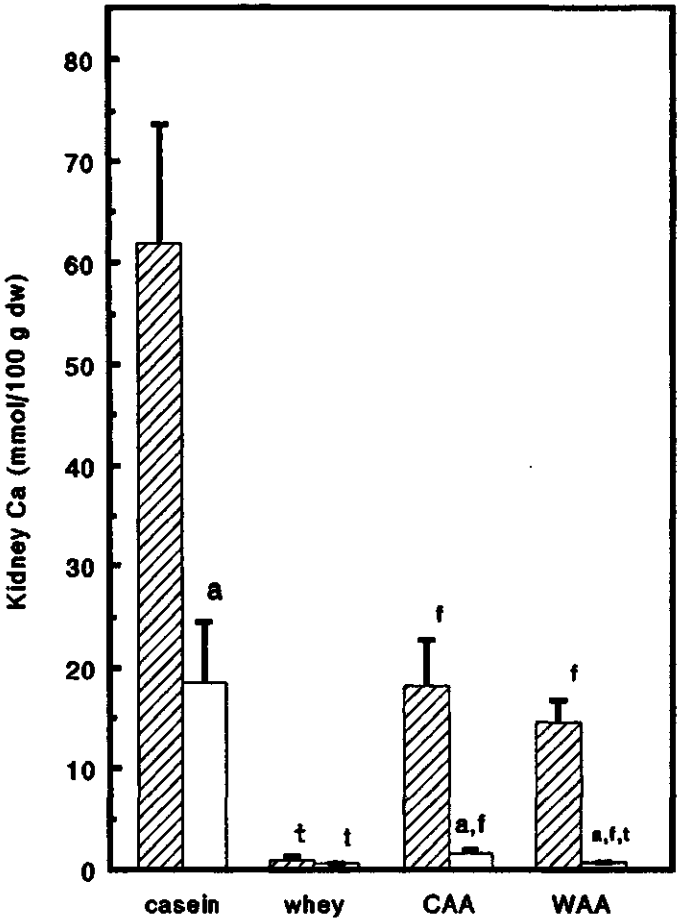

FIGURE 1 Kidney calcium concentrations in rats fed the experimental diets. CAA= casein amino acids; WAA = whey protein amino acids; hatched bars, 15 g protein or amino acid mixture/100g diet; open bars, 30 g protein or amino acid mixture/100 g diet. ANOVA revealed the following significant effects: T,F,A,TxF,TxA (for explanation of letters, see legend to Table 3). Contrast significances (see legend to Table 3) are also indicated.

TABLE 4. Apparent calcium, magnesium and phosphorus absorption in rats fed the experimental diets[1-4]

	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled	
	15	30	15	30	15	30	15	30	SEM	ANOVA[5]
Calcium,										
Intake, mmol/d	1.81	1.49[a]	1.60	1.42	1.64	1.38	1.61	1.44[a]	0.06	A,TxF
Absorption, % of intake	34.9	31.4	34.7	27.5	37.1	27.5	33.3	38.4	4.82	
Magnesium										
Intake, mmol/d	0.23	0.22	0.21	0.20	0.22	0.21	0.22	0.20	0.08	T,A
Absorption, % of intake	19.2	24.1	34.5	44.9[t]	41.2[f]	57.8[a,f]	42.0	56.9[a,f]	3.94	T,F,A,TxF
Phosphorus										
Intake, mmol/d	2.16	2.31	1.69[t]	1.57[t]	2.12	1.79[f]	1.82	1.74	0.07	T,TxF,TxAxF
Absorption, % of intake	76.4	80.9	76.1	80.7	84.6[f]	88.0[f]	83.1[f]	87.6[a,f]	1.36	F,A

1 The dietary concentration of indicated protein or amino acid mixture is expressed as g/100 g diet.

2 Apparent mineral absorption was calculated as mineral intake minus fecal excretion, and was expressed as percentage of intake.

3 Values are means and pooled SEM for 12 rats per group.

4 Group comparisons for two groups with one dietary variable ($P<0.017$); t = significant nitrogen type effect (intact whey protein versus casein or whey protein amino acids versus casein amino acids); a = significant nitrogen amount effect (15 versus 30 g protein or amino acid mixture/100 g diet); f = significant nitrogen form effect (casein amino acids versus intact casein or whey protein amino acids versus intact whey protein).

5 ANOVA significance ($P<0.05$); T = significant effect of type of nitrogen source (whey protein versus casein); A = significant effect of amount of nitrogen source (15 versus 30 g protein or amino acid mixture/100 g diet); F = significant effect of form of nitrogen source (intact protein versus amino acid mixture); significant effect of interactions: TxA; TxF; AxF; TxAxF.

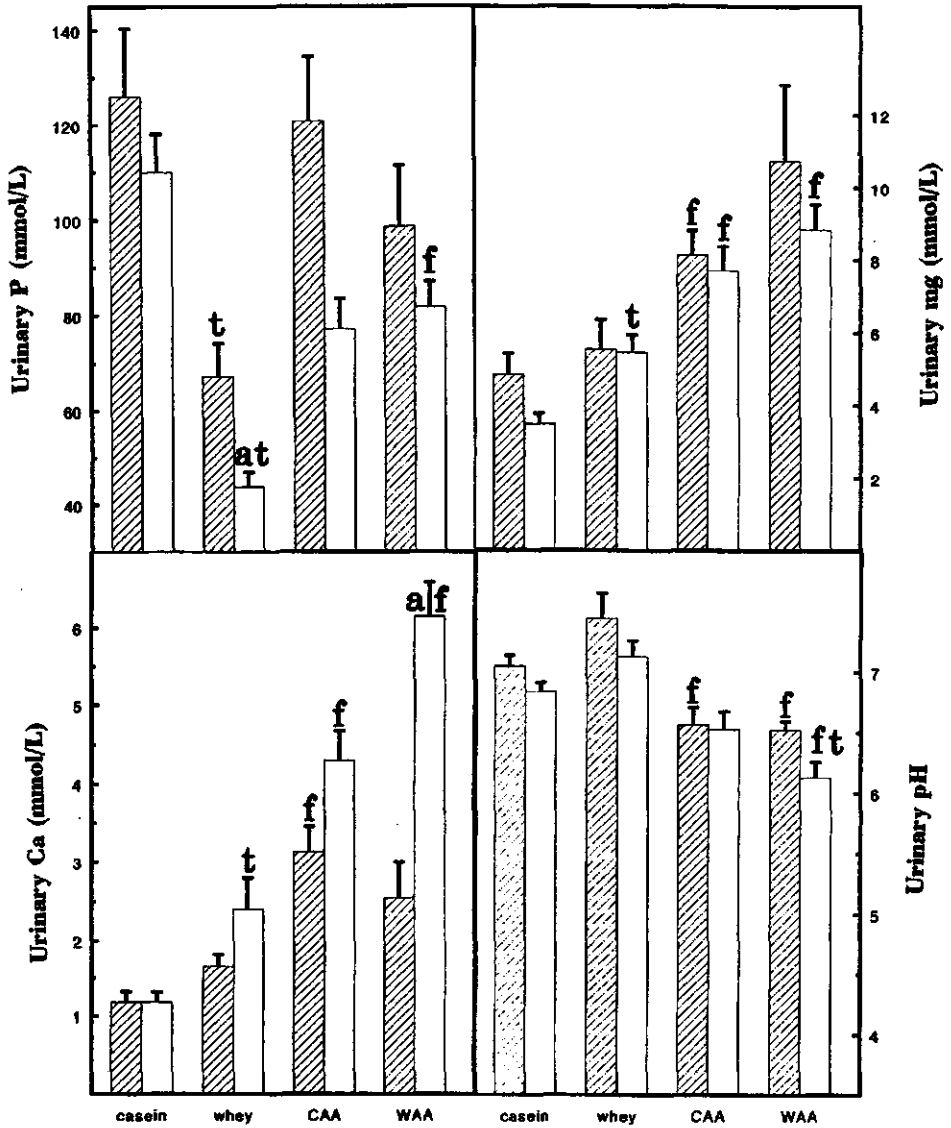

FIGURE 2 Urinary pH and urinary concentrations of calcium, magnesium and phosphorus in rats fed the experimental diets. CAA= casein amino acids; WAA = whey protein amino acids; hatched bars, 15 g protein or amino acid mixture/100g diet; open bars, 30 g protein or amino acid mixture/100 g diet. ANOVA revealed the following significant effects: urinary pH, F,A,TxF; urinary calcium, T,A,F,TxA,AxF; urinary magnesium, T,F; urinary phosphorus, T,A,TxF (for explanation of letters, see legend to Table 3). Contrast significances (see legend to Table 3) are also indicated.

mixtures produced higher urinary calcium concentrations than the intact proteins, the difference being greater with the higher nitrogen level of the diets. Urinary calcium concentrations were negatively correlated with kidney calcium concentrations ($r=-0.32$, $P<0.01$, $n=96$) and nephrocalcinosis scores ($R=-0.41$, $P<0.01$, $n=96$).

At the high dietary nitrogen level, urinary magnesium concentrations were higher in rats fed intact whey protein instead of casein, but this was not seen at the low dietary protein concentration (Fig. 2). For the amino acid mixtures simulating either casein or whey protein an opposite tendency emerged. The amount of nitrogen in the diet did not significantly influence urinary magnesium concentrations, but the amino acid mixtures produced higher concentrations than the intact proteins. Individual kidney calcium concentrations and nephrocalcinosis scores were not correlated with urinary magnesium concentration ($r=-0.098$; $R=-0.039$, $n=96$).

The rats fed intact whey protein had significantly lower urinary phosphorus concentrations than their counterparts fed casein (Fig. 2). The amino acid mixture corresponding to whey protein induced lower urinary phosphorus concentrations than that corresponding to casein, but this was seen only at the low dietary nitrogen concentration. Urinary phosphorus concentrations became lower after increasing nitrogen intake, irrespective of the nitrogen source. Both kidney calcium concentrations and nephrocalcinosis scores were positively correlated with urinary phosphorus concentrations ($r=0.44$ and $R=0.49$, $P<0.01$, $n=96$).

Urinary volume and pH. The type of nitrogen source did not influence urinary volume (Table 5). The rats fed the amino acid mixtures excreted less urine than those fed the intact proteins. Higher dietary nitrogen levels were associated with more excretion of urine. Whey protein versus casein feeding significantly elevated urinary pH (Fig. 2). The amino acid mixtures produced lower urinary pH values than the intact proteins. At the high level of dietary nitrogen whey protein

TABLE 5. Urinary volume, urea and creatinine clearance in rats fed the experimental diets[1-3]

	Casein		Whey protein		Casein amino acids		Whey protein amino acids		Pooled	
	15	30	15	30	15	30	15	30	SEM	ANOVA[4]
Urea										
Plasma, mmol/L	5.44	8.32[a]	4.74	6.35[a,t]	4.32	5.91[a,f]	3.52[f]	5.29[t]	0.32	T,A,F
Urine, mmol/L	1.15	1.61	0.93	1.65[a]	0.98	1.52[a]	0.84[f]	1.62[t]	0.12	A
Clearance, ml/(min. 100g body weight)	0.83	1.35[a]	0.83	1.74[a,t]	0.61[f]	1.43[a]	0.79	1.55[t]	0.09	T,A
Creatinine										
Plasma, mmol/L	34.2	29.7	29.7	28.8	31.4	29.0	30.0	26.7	1.67	T,A
Urine, mmol/L	9.91	5.76[a]	8.57	6.49	12.38	7.50[a,f]	10.50	7.71	0.86	F,A
Clearance, ml/(min. 100g body weight)	1.18	1.38	1.32	1.47	1.06	1.42[a]	1.19	1.44	0.08	A
Urine volume, ml/d	7.4	10.6	7.2	10.2	4.4	8.1[a]	6.4	7.4	0.94	A,F

[1] The dietary concentration of indicated protein or amino acid mixture is expressed as g/100 g diet.
[2] Values are means and pooled SEM for 12 rats per group.
[3,4] See legends to Table 3.

amino acids induced a lower pH than casein amino acids. Urinary pH values became generally lower with increased dietary nitrogen level.

Urea and creatinine clearance. Whey protein in the form of either intact protein or amino acids produced lower plasma urea concentrations than casein (Table 5). The amino acid mixtures induced lower plasma urea concentrations than the corresponding intact proteins. Increasing nitrogen intake raised plasma and urinary urea concentrations and also urea clearance. Whey protein amino acids and intact whey protein reduced plasma creatinine concentrations when compared with casein amino acids and casein, respectively. The amino acid mixtures versus intact proteins raised urinary creatinine concentrations. Plasma and urinary creatinine concentrations were lowered by increased nitrogen intake which resulted in a rise of creatinine clearance.

DISCUSSION

As assessed by histological and chemical analysis, intact whey protein versus casein markedly inhibited nephrocalcinogenesis in female, weanling rats. This observation supports earlier work (Kaunitz and Johnson 1976, Kunkel et al. 1984, Meyer et al. 1989). An increased intake of whey protein tended to further lower the degree of nephrocalcinosis, but this was not obvious because a minimum degree had already been reached with the low level of dietary whey protein. Extra casein in the diet at the expense of glucose reduced the concentrations of calcium, magnesium and phosphorus in kidney but they remained much higher than those produced by an identical amount of whey protein in the diet. The inhibitory effect on nephrocalcinogenesis of increasing casein intake is in accordance with our previous work (Zhang and Beynen 1992).

It is appreciated that drawing a comparison of effects of intact proteins and corresponding amino acid mixtures is not straight forward because the order in which amino acids as such or in the form of peptides are released during digestion

will not be the same. At the high level of nitrogen intake, the amino acid mixture simulating whey protein produced significantly lower kidney calcium concentrations and nephrocalcinosis scores than casein amino acids. This effect was relatively small when compared with the differential effect on nephrocalcinosis of the intact proteins, but there already was a low degree of nephrocalcinosis in rats fed the high level of casein amino acids. At the low dietary nitrogen concentration, amino acid mixtures simulating either casein or whey protein produced similar degrees of nephrocalcinosis. Thus, the differential effect of casein and whey protein on nephrocalcinosis may not lie in their different amino acid patterns. The anti-nephrocalcinogenic effect of increasing nitrogen intake was clearly seen with both the intact proteins and the amino acid mixtures. It appears that the effect of type of protein on nephrocalcinogenesis may involve protein structure or non-protein components rather than amino acids per se in the protein, whereas the effect of amount of protein is dependent on amino acids in the protein.

Intact whey protein versus casein in the diet lowered urinary phosphorus concentrations and raised urinary calcium and magnesium concentrations. Whey protein elevated urinary pH values. Low urinary pH values, low urinary phosphorus, high magnesium and low calcium concentrations protect against nephrocalcinosis (Ritskes-Hoitinga and Beynen 1992). Thus, the lower urinary phosphorus concentrations in rats fed whey protein may be responsible for the observed anti-nephrocalcinogenic effect of whey protein. This is substantiated by the fact that in individual rats urinary phosphorus concentrations were positively correlated with both kidney calcium concentrations and nephrocalcinosis scores. The nephrocalcinosis inhibiting effect of low urinary phosphorus concentrations in rats fed whey protein apparently was stronger than the nephrocalcinogenic effects of raised urinary calcium concentrations and pH values.

The lower urinary phosphorus concentrations in rats fed intact whey protein cannot be explained by a lower efficiency of absorption of phosphorus but rather

by a lower phosphorus intake when compared with rats fed casein. However, comparison of the differences in phosphorus intakes (Table 4) and urinary phosphorus concentrations (Fig. 2) between the dietary groups suggests that in rats fed whey protein urinary phosphorus concentrations are not lowered proportionally to phosphorus intake. Thus, there may be an additional cause for the relatively low urinary phosphorus concentrations in rats fed whey protein. The anti-nephrocalcinogenic effect of high intake of whey protein amino acids versus casein amino acids was not associated with a lowering of urinary phosphorus concentrations. This effect may rather be explained by a lowering of urinary pH.

Hypercalciuria promotes nephrocalcinogenesis because it enhances the risk for precipitation of calcium phosphates in the tubuli (Ritskes-Hoitinga and Beynen 1992). In this study however, nephrocalcinosis in individual rats was negatively correlated with urinary calcium concentrations. Furthermore, whey protein induced higher urinary calcium concentrations than casein but dampened nephrocalcinogenesis. Thus, urinary phosphorus concentrations may be more important in the development of nephrocalcinosis than urinary calcium concentrations. Urinary phosphorus may also be more important than urinary pH. Although acidification of urine is associated with inhibition of nephrocalcinosis (Kootstra et al. 1990), urinary pH in individual rats was not significantly correlated with kidney calcium concentration ($r = 0.064$, $n = 96$) and nephrocalcinosis scores ($R = -0.068$, $n = 96$).

Whey protein in the diet produced higher urinary calcium and magnesium concentrations than casein. The higher urinary calcium concentrations in rats fed whey protein occurred while calcium absorption was not increased. Absolute calcium excretion in urine was significantly raised in rats fed whey protein (data not shown). Thus , the slightly lower urinary volumes after whey protein feeding are not the sole cause of the raised urinary calcium concentrations. Whey protein stimulated magnesium absorption when compared with casein, and this explains the higher urinary magnesium concentrations in rats fed whey protein.

Increasing intakes of nitrogen, either in the form of the intact proteins or amino acid mixtures, significantly reduced the degree of nephrocalcinosis. This effect can be explained by a combination of changes in urinary composition. Extra nitrogen in the diet generally lowered urinary pH and lowered urinary phosphorus concentrations. Such effects are expected to inhibit nephrocalcinogenesis (Ritskes-Hoitinga and Beynen 1992). The lowering of urinary phosphorus concentration after increasing nitrogen intake is not explained by a lowering of phosphorus absorption. On the contrary, extra nitrogen in the diet raised the efficiency of phosphorus absorption. The fall of urinary phosphorus concentration may be explained by the rise in urinary volume seen after increasing nitrogen intake. The associated lowering of urinary pH may be caused by enhanced protein catabolism, as evidenced by the observed raised urinary and plasma urea concentrations, which is accompanied by raised urinary excretion of sulphate (Whiting and Draper 1980) causing urinary pH to drop.

Increased nitrogen intake raised urinary calcium concentrations but lowered those of magnesium. The efficiency of calcium absorption was not enhanced after increased intake of either protein or amino acid mixture, while absolute excretion of calcium in urine was significantly elevated (results not shown). Thus, in rats fed high-protein diets the kidney may excrete more calcium than in rats fed low-protein diets (Whiting and Draper 1980), but it is difficult to see how a steady state is reached. High nitrogen intake raised the efficiency of magnesium absorption, which has been shown earlier (Sterck et al. 1992), and it significantly stimulated the absolute excretion of magnesium in urine (data not shown). Thus, the lowered urinary magnesium concentrations in rats fed the high-nitrogen diets are most likely due to the raised urinary volume.

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Chapter 8

Nephrocalcinosis in Female Analbuminemic and Sprague-Dawley Rats Fed Diets Containing either Soybean Protein or Casein

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ABSTRACT. Nephrocalcinosis is a common disorder in female laboratory rats. We have tested the hypotheses that female Nagase analbuminemic rats (NA) are less sensitive to nephrocalcinogenesis than Sprague-Dawley (SD) rats and that soybean protein in diet is less nephrocalcinogenic than casein. The severity of nephrocalcinosis in NA rats was found to be consistently lower than that in SD rats, irrespective of the dietary protein source. It is suggested that this relates to the lower urinary pH value, higher urinary magnesium and slightly lower urinary phosphorus concentrations in NA rats when fed the diet with soybean protein. After feeding the casein diet, the lower degree of nephrocalcinosis in NA rats may be explained by lower group mean urinary phosphorus concentrations. Dietary casein versus soybean protein caused increased kidney calcium concentrations in both strains; this was probably due to the raised urinary phosphorus concentrations.

INTRODUCTION

Nephrocalcinosis is a deposition of calcium phosphates in the corticomedullary junction of the kidney, which is frequently found in female laboratory rats fed commercial, natural-ingredient diets (1). Severe nephrocalcinosis impairs kidney function as is evidenced by increased urinary albumin excretion and increased plasma urea concentrations (2, 3). The Nagase analbuminemic (NA) rat is a mutant Sprague-Dawley (SD) rat characterized by an almost absolute absence of plasma albumin (4). NA rats have low kidney calcium concentrations (5). In one-year-old, female NA rats fed a commercial diet, kidney calcinosis could not be detected whereas this disorder was apparent in their SD counterparts (6). Thus, NA rats may be relatively insensitive to nephrocalcinogenesis.

The composition of the diet is an important determinant of nephrocalcinosis in laboratory rats (7). Low-magnesium (8) and high-phosphorus diets (9) promote nephrocalcinogenesis, whereas high-protein diets (10) inhibit this process. There is suggestive evidence that the type of protein in the diet also affects the degree

of nephrocalcinosis. Soybean protein versus casein tended to dampen the development of nephrocalcinosis (11).

In the present study, two questions were addressed. First, are NA rats less sensitive to nephrocalcinogenic diets than are SD rats ? Secondly, is soybean protein in the diet less nephrocalcinogenic than is casein ? We used high-phosphorus, purified diets containing either soybean protein or casein. The diets were isonitrogenous and carefully balanced for minerals in the protein preparations. Weanling female rats were used because they are more sensitive to nephrocalcinogenesis than males (12).

MATERIALS AND METHODS

The experimental protocol was approved by the animal ethical committee of the State University, Utrecht.

Diets. Purified diets containing either soybean protein or casein were used (Table 1). The diets were isonitrogenous and balanced for residual fat, cholesterol, calcium, phosphorus and magnesium in the protein preparations. The analysed composition of the diets (Table 2) agreed well with the calculated composition. The diets were in pelleted form and stored at 4°C until feeding. Feed and demineralized water were provided ad libitum.

Animals and housing. Female 3-wk old rats of the Sprague-Dawley (SD/Hsd-Ola, Harlan-CPB, Zeist, The Netherlands) and Nagase analbuminemic strain (bred from rats kindly supplied by Dr. S. Nagase, Tokyo, Japan) were used. All animals were fed the purified diet containing soybean protein (Table 1) and demineralized water for two weeks. Then on day 0 of the experiment, the rats were divided into 2 groups of 6 rats each per strain. Within each strain the groups were stratified body weight and randomly assigned to one of the two diets. The rats were housed three in a wire-topped polycarbonate cage (37.5 x 22.5 x 15.0cm) with

TABLE 1. *Ingredient composition of the experimental diets*

Ingredient	Soybean protein	Casein
	g/100 g diet	
Soy isolate[1]	17.87	-
Methionine	0.15	-
Casein[2]	-	17.07
Soybean oil	2.05	3.00
Coconut fat	9.00	8.71
Cholesterol	0.004	-
Glucose	60.976	60.62
$CaCO_3$	1.37	1.47
$NaH_2PO_4.2H_2O$	2.31	2.83
$MgCO_3$	0.20	0.23
Constant components[3]	6.07	6.07

1 Analysed composition (g/100 g): nitrogen, 13.43; fat, 5.3; cholesterol, 0.0001; calcium, 0.28; magnesium, 0.07; phosphorus, 0.79.

2 Analysed composition (g/100 g): nitrogen, 14.06; fat, 1.7; cholesterol, 0.026; calcium, 0.07; magnesium, 0.01; phosphorus, 0.22.

3 Constant components consisted of the following (g/100 g diet): cellulose, 3.0; KCl, 0.1; $KHCO_3$, 0.77; mineral premix, 1.0; vitamin premix, 1.2. The composition of the mineral and vitamin premix has been described(9).

sawdust as bedding. During the last week of the experiment (d 15-21), the rats were housed individually in metabolic cages (20 x 12 x 10 cm). The animal room had controlled temperature (20-24°C), relative humidity (40-45%), and alternating 12-hour periods of light (light, 06:00-18:00 h). The experiment lasted 21 days.

The rats were weighed weekly and feed intake of the last week (d 15-21) was recorded.

Collection of samples. During the period between d 17 and d 20, feces and urine were collected daily from each rat. The cages and tubes for collecting urine and feces had been washed with phosphorus-free soap, 0.1 N HCl and distilled water. On d 21, the rats were anesthetized with diazepam (2.5 mg/kg, administered intraperitoneally) and fentanyl-fluanisone (0.5 ml/kg, administered intramuscularly) and exsanguinated by aortic puncture into EDTA containing tubes. Kidneys were removed and weighed. The right kidney was frozen at -20°C for chemical analyses and the left kidney was fixed in 10% (v/v) formalin solution. Blood was centrifuged to collect plasma. Plasma samples were stored at -20°C until analysis.

Chemical analyses. Nitrogen in protein preparations and diets was determined by the Kjeldahl method. Crude fat was determined by extraction according to the Soxhlet method. Plasma total protein concentrations were measured colorimetrically using a commercially available kit (Bio-Rad Lab, Munich, Germany). Albumin in plasma was determined as described (2). Calcium and magnesium in protein preparations, diets, urine and feces were analysed by atomic absorption spectroscopy as described (9). Phosphorus in protein preparations, diets, feces and urine were analysed using a commercial kit (MA-KIT Phosphate, Roche, Basel, Switzerland). Creatinine in plasma and urine were measured with kits (MA-kit creatinine) purchased from F. Hoffmann-La Roche Co. Ltd., Diagnostica, Basel, Switzerland.

Histological analyses. Left kidney for histological examination were processed as described (9). Sections stained with Von Kossa's method were graded in random order and blind on a scale from 0 (no calcium phosphate deposits) to 3 (severe calcinosis).

Statistical analyses. The data were subjected to two-way analysis of variance (ANOVA) with strain of rats (NA rats versus SD rats) and type of dietary protein

TABLE 2. Analysed composition of the experimental diets

Component	Soybean protein	Casein
	g/100 g diet	
Dry matter	91.3	91.5
Nitrogen	2.47	2.44
Fat	11.0	11.7
Cholesterol	0.003	0.004
Calcium	0.70	0.71
Magnesium	0.09	0.09
Phosphorus	0.69	0.75

(casein versus soybean protein) as main effects. The probability of a type I error < 0.05 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; a P value of < 0.025 was used to take into account the increased probability of a type I error due to multiple comparisons. Statistical analysis was carried out with the SPSS/PC+ program (13).

RESULTS

Characteristics of NA rats. Although the NA rats were almost completely deficient in plasma albumin, their plasma total protein concentrations did not differ from that of SD rats (Table 3). When compared with SD rats, the NA rats had a lower body and kidney weight.

Feed intake (Table 3) and thus also the intakes of calcium and magnesium (Table 5) were similar in NA and SD rats. Kidney dry weight was lower in NA than SD rats (Table 4). NA rats had lower group means of calcium and phosphorus

TABLE 3. Growth performance, kidney weight and plasma albumin concentrations of SD and NA rats fed the experimental diets[a,b]

	SD rats		NA rats			
	Soybean protein	Casein	Soybean protein	Casein	Pooled SEM	ANOVA[c]
Body weight, g						
d 0	109	108	90[s]	81[s]	6.35	S
d 21	181	187	157[s]	153[s]	11.27	S
Feed intake, g/d (d 19-21)	13.1	14.1	13.0	13.1	1.76	
Kidney weight, g/100 g body weight (d 21)	0.40	0.55[P]	0.37	0.43[P,s]	0.06	S,P
Plasma total protein, g/L (d 21)	62	66	62	63	1.67	
Plasma albumin, g/L (d 21)	22	22	0.06[s]	0.06[s]	1.00	S

[a] Values are means for six rats, except for the group of NA rats fed diet containing casein, which consisted of four rats.

[b] Group comparisons ($P < 0.025$): s = significant strain difference (NA versus SD rats); p = significant effect of dietary protein type within strains (casein versus soybean protein).

[c] ANOVA significance ($p < 0.05$): S = significant strain effect (NA versus SD rats); P = significant effect of dietary protein type (casein versus soybean protein); significant effect of interactions: PxS.

TABLE 4. Kidney mineral concentrations and nephrocalcinosis scores in SD and NA rats fed the experimental diets for 21 d[a,b]

	SD rats		NA rats			
	Soy protein	Casein	Soy protein	Casein	Pooled SEM	ANOVA[c]
Kidney dry weight, mg	182	264P	135S	167	14.26	S,P
Kidney minerals, % of dry weight						
Calcium	1.5	5.5 P	0.2S	2.8	0.87	S,P
Magnesium	0.10	0.18P	0.10	0.13	0.06	P
Phosphorus	1.72	3.15P	1.67	2.42	0.33	P
Nephrocalcinosis						
Incidence, positive/total number of rats						
	5/6	6/6	3/6	4/4		
Score, on a scale of 0-3[d]						
	1.6	2.6	0.8	2.4P		

[a] Values are means for six rats, except for the group of NA rats fed diet containing casein, which consisted of four rats.

[b] Group comparisons ($P < 0.025$): s = significant strain difference (NA versus SD rats); p = significant effect of dietary protein type within strains (casein versus soybean protein).

[c] ANOVA significance ($p < 0.05$): S = significant strain effect (NA versus SD rats); P = significant effect of dietary protein type (casein versus soybean protein); significant effect of interactions: PxS.

[d] Group mean nephrocalcinosis scores are given; Mann-Whitney U test was used to evaluate scores for significant differences.

concentrations in kidney and lower nephrocalcinosis scores (Table 4). Creatinine clearance was similar in NA and SD rats, the values on the soybean protein and casein diets being 0.63 ± 0.11 and 0.60 ± 0.10; and 0.60 ± 0.14 and 0.50 ± 0.10 ml/min. 100g body weight, respectively (mean ± SE). Urinary excretion of calcium was higher in NA than SD rats. Urinary excretion of magnesium and phosphorus were also higher in NA rats, but this was seen in the rats fed soybean protein diet only (Table 5). Urinary pH was similar in NA and SD rats (Table 5).

Response to dietary proteins. There were two NA rats fed the casein diet that died of unknown cause. Table 3 shows that the type of dietary protein did not significantly affect body weight and feed intake of both strains. Casein versus soybean protein significantly increased phosphorus intake in both strains (Table 5). Casein versus soybean protein in the diet raised kidney weight of both strains, this effect being somewhat greater in SD rats.

Replacement of soybean protein in the diet by casein caused an increase in mineral concentrations in the kidney, this increase being most pronounced in SD rats. Nephrocalcinosis scores and kidney dry weight were also more markedly raised in SD than NA rats after feeding the casein diet.

Casein versus soybean protein significantly lowered urinary pH value in the two strains (Table 5). Casein significantly stimulated urinary calcium and magnesium excretion in SD rats but not in NA rats. Dietary protein type did not significantly influence calcium and magnesium absorption. In both SD and NA rats fed casein diets, urinary phosphorus excretion was significantly higher than in their counterparts fed soybean protein. Casein raised phosphorus absorption in the two rat strains.

DISCUSSION

The degree of nephrocalcinosis as assessed chemically by measuring kidney calcium concentration was consistently lower in NA rats than SD rats. Group mean

TABLE 5. Calcium, magesium and phosphorus balance, urine volume and urinary pH (d18-20) in SD and NA rats fed the experimental diets[a,b]

	SD rats		NA rats			
	Soy protein	Casein	Soy protein	Casein	Pooled SEM	ANOVA[c]
Calcium						
Intake, mg/d	92.0	99.8	90.9	98.6	4.12	
Feces, mg/d	30.2	26.4	27.4	30.6	3.68	
Urine, mg/d	0.88	1.42	2.26[s]	2.64[s]	0.26	S
Urine, mg/L	99.3	200.0	164.9[s]	201.3	24.39	P
Apparent absorption, % of intake	67.3	73.4	69.5	69.6	3.40	
Magnesium						
Intake, mg/d	11.7	12.2	11.6	12.1	0.52	
Feces, mg/d	3.4	2.9	3.1	3.9	0.16	
Urine, mg/d	0.78	2.88[p]	2.39[s]	2.63	0.43	P,PxS
Urine, mg/L	90.1	340.4[p]	166.1[s]	187.6[s]	30.52	P,PxS
Apparent absorption, % of intake	70.7	75.9	73.1	68.1	3.46	
Phosphorus						
Intake, mg/d	86.1	105.9[p]	85.4	104.7[p]	4.15	P
Feces, mg/d	29.6	26.8	24.5	22.0	2.11	S
Urine, mg/d	21.6	38.5[p]	28.0[s]	40.2[p]	2.27	P
Urine, mg/mL	2.41	4.68[p]	2.16	3.07	0.12	S,P
Apparent absorption, % of intake	65.7	74.5[p]	70.9	78.9	2.22	S,P
Urine (d 18-20)						
Volume, ml/d	8.5	8.0	13.4	12.9[s]	4.66	S
pH	8.1	6.6[p]	7.4	6.5[p]	0.19	P

[a] Values are means for six rats, except for the group of NA rats fed diet containing casein, which consisted of four rats.

[b] Group comparisons ($P < 0.025$): s = significant strain difference (NA versus SD rats); p = significant effect of dietary protein type within strains (casein versus soybean protein).

[c] ANOVA significance ($p < 0.05$): S = significant strain effect (NA versus SD rats); P = significant effect of dietary protein type (casein versus soybean protein); significant effect of interactions: PxS.

histological nephrocalcinosis scores were also lower in NA rats. The mineral balance data may provide clues as to the relative insensitivity of NA rats to the development of nephrocalcinosis.

In nephrocalcinosis, the primary lesion is intratubular deposition of calcium phosphates (14). Thus, it is likely that high calcium and high phosphorus concentrations in urine point to favouring of nephrocalcinogenesis. Indeed, feeding high-calcium and high-phosphorus diets produces nephrocalcinosis, which is accompanied by raised urinary calcium and phosphorus concentrations (2, 8, 9). High urinary magnesium concentrations may inhibit nephrocalcinogenesis because *in vitro* studies (15) have shown that magnesium inhibits precipitation of calcium phosphates. High-magnesium diets prevent nephrocalcinogenesis and raise urinary magnesium concentrations (3, 8). A low urinary pH may also antagonize nephrocalcinogenesis because *in-vitro* studies (16) have shown that precipitation of calcium phosphate does not occur under acidic conditions. Supplementation of a high-phosphorus diet with the urine acidifier ammonium chloride instead of an equimolar amount of ammonium carbonate indeed lowered urinary pH and prevented nephrocalcinosis (17).

The nephrocalcinogenic effect of casein versus soybean protein in the two strains is probably due to increased urinary phosphorus concentrations. Apparently, the raised urinary magnesium concentrations and lower urinary pH could not counteract this influence. Casein versus soybean protein stimulated apparent phosphorus absorption, which explains the increase in urinary phosphorus concentration. In addition, the casein diet contained more phosphorus than did the diet containing soybean protein (Table 2) so that phosphorus intake was higher in rats fed the casein diet (Table 5). This also contributed to the higher urinary phosphorus concentrations after feeding casein. Clearly, balancing the diets for phosphorus concentrations was not fully successful.

When fed the diet with soybean protein, NA rats had higher urinary calcium

and magnesium concentrations, lower urinary pH values and slightly lower urinary phosphorus concentrations than SD rats fed the same diet. Thus, it seems that the higher urinary magnesium concentration, lower urinary phosphorus concentration and lower urinary pH value in NA rats explain their relative insensitivity to nephrocalcinogenesis when fed the diet with soybean protein. After feeding the casein diet, NA rats also had less severe nephrocalcinosis than SD rats. When fed the casein diet, NA rats had lower group mean urinary phosphorus concentrations than SD rats. This might explain the lower degree of nephrocalcinosis in NA versus SD rats fed the casein diet. The different urinary mineral concentrations in NA rats and SD rats are caused by a combination of the higher urinary volume in NA rats and different retention of minerals. NA rats absorbed phosphorus more efficiently than SD rats, which by itself will cause increased urinary phosphorus concentrations, but the high urinary volume in NA rats overrode this effect. One-year old female NA rats are free of nephrocalcinosis (5, 6), indicating that either their urinary composition is non-nephrocalcinogenic continuously or that protection against nephrocalcinosis in the first few weeks after weaning determines its absence in adulthood.

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GENERAL DISCUSSION

PART I

DIETARY PROTEIN AND CHOLESTEROL METABOLISM

The type of dietary protein influences cholesterol metabolism in both experimental animals and man. The general dogma is that animal proteins produce higher plasma cholesterol concentrations than plant proteins (1). However, this is an oversimplification because among animal proteins there can be marked differences in their cholesterolemic effects. Even among different fish proteins the cholesterolemic activity can differ markedly (Chapter 1). One of the objectives of this thesis was to compare in female rats the cholesterolemic responses of various animal proteins, including fish proteins.

Chapter 2 shows that cod meal produces lower plasma and liver cholesterol concentrations than casein, and that an increment of protein intake amplifies this protein type effect. Plaice meal in the diet had a less cholesterolemic effect than whiting meal. Because the isonitrogenous diets used were balanced for cholesterol and fat in the fish proteins, the protein-dependent cholesterolemic responses can be attributed to the amino acid composition of the proteins.

Chapter 3 shows that milk-whey protein produces lower plasma and liver cholesterol concentrations than casein. An increment of protein level in the diet caused a greater difference between the effects of whey protein and casein. The cholesterol lowering effect of whey protein was associated with a decrease in very-low-density lipoprotein cholesterol. The effects of whey protein versus casein could not be reproduced by amino acid mixtures simulating these proteins. Thus, the effect of whey protein versus casein may relate to protein structure or unknown non-protein components.

Analbuminemic rats are more sensitive to the hypercholesterolemic action of casein versus soybean protein in the diet than Sprague-Dawley rats (Chapter 4). It is suggested that this relates to a lack of inhibition of *de novo* cholesterol

synthesis after casein feeding in the analbuminemic rats. The marked cholesterolemic response to casein in analbuminemic rats makes this rat strain an interesting model in cholesterol metabolism research (Chapter 4).

PART II
DIETARY PROTEINS AND NEPHROCALCINOSIS

Nephrocalcinosis refers to the deposition of calcium phosphates in the corticomedullary junction of kidney (2). Both type and amount of protein in the diet had been shown to affect the development of nephrocalcinosis (3-5). However, the number of proteins studied was limited and the underlying mechanisms were unknown. Thus, the purpose of the second part of this thesis is to characterize the effects of dietary proteins on nephrocalcinogenesis in female rats.

In nephrocalcinosis, the primary lesion probably is an intratubular deposition of calcium phosphates (2). Thus, it is likely that high calcium and high phosphorus concentrations in the lumen of the proximal tubule enhance the risk for nephrocalcinosis. The feeding of high-calcium and high-phosphorus diets produces raised urinary calcium and phosphorus concentrations (6). In *in vitro* studies, magnesium inhibits precipitation of calcium phosphates (9). High-magnesium diets prevent nephrocalcinogenesis and raise urinary magnesium concentrations (8, 9). A low urinary pH, as induced by the feeding of ammonium chloride inhibits nephrocalcinogenesis (10). Thus, it was hypothesized that dietary proteins influence nephrocalcinosis by changing either urinary pH, urinary calcium, magnesium or phosphorus concentrations, or by a combination of these measures.

Chapter 6 reports the nephrocalcinogenic effects of different amounts of dietary cod meal, soybean protein and casein. The diets used were balanced for

calcium, magnesium and phosphorus in the protein preparations. An increment of casein or soybean protein concentration in the diet lowered kidney calcium concentrations. Cod meal produced lower kidney calcium concentrations than either soybean protein or casein. Increasing intakes of cod meal did not further lower the degree of nephrocalcinosis. There were no common changes in urinary pH or urinary calcium, magnesium or phosphorus concentrations that may explain the nephrocalcinogenic effects of amount and type of protein in the diet.

Replacement of casein by whey protein effectively reduced the severity of nephrocalcinosis (Chapter 7). This protein type effect was not mimicked by amino acid mixtures corresponding to the intact proteins. Increased protein intake reduced nephrocalcinosis, and so did increased intake of the amino acid mixtures. Whey protein probably inhibited nephrocalcinogenesis by lowering urinary phosphorus concentrations. Increased intakes of nitrogen were accompanied by increased urinary concentrations of magnesium and decreased urinary pH values, which may explain the anti-nephrocalcinogenic effect of high-protein intake.

Analbuminemic rats were found to have lower kidney calcium concentrations and lower degrees of histologically-determined nephrocalcinosis when compared with Sprague-Dawley rats (Chapter 8). This probably relates to the lower urinary pH, higher urinary magnesium and lower urinary phosphorus concentrations in analbuminemic rats. Replacement of casein by soybean protein lowered the severity of nephrocalcinosis in the two strains of rats. This is most likely due to a decrease in urinary phosphorus concentrations.

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Summary

PART I. DIETARY PROTEIN AND CHOLESTEROL METABOLISM

The type and amount of animal proteins in the diet of female rats was found to influence plasma and liver cholesterol concentrations. Cod meal produced lower plasma and liver cholesterol concentrations than casein, and an increment protein intake amplified this protein type effect. Plaice meal in the diet had a less cholesterolemic effects than whiting meal. Milk-whey protein produced lower plasma and liver cholesterol concentrations than casein. An increment of protein level in the diet caused a greater difference between the effects of whey protein and casein. The cholesterol lowering effect of whey protein may result from inhibition of cholesterol synthesis. Because the diets used were balanced for cholesterol and fat in the protein preparations, the protein-dependent cholesterolemic responses can not be attributed to non-protein components of the proteins. However, the hypocholesterolemic effect of whey protein versus casein could not be reproduced by amino acid mixtures simulating these proteins.

Analbuminemic rats are more sensitive to the hypercholesterolemic action of casein versus soybean protein in the diet than Sprague-Dawley rats. The marked cholesterolemic response to casein in the analbuminemic rat makes this rat strain an interesting model in cholesterol metabolism research.

PART II. DIETARY PROTEIN AND NEPHROCALCINOSIS

With diets balanced for calcium, magnesium and phosphorus, an increment of casein or soybean protein intake lowered kidney calcium concentrations in female rats. Cod meal produced lower kidney calcium concentrations than either

soybean protein or casein. Increasing intakes of cod meal did not further lower the degree of nephrocalcinosis. Replacement of casein by whey protein effectively reduced the severity of nephrocalcinosis. Whey protein probably inhibited nephrocalcinogenesis by lowering urinary phosphorus concentrations. Increased intakes of either the intact proteins or corresponding amino acid mixtures reduced nephrocalcinosis. Increased intakes of nitrogen were accompanied by increased urinary concentrations of magnesium and decreased urinary pH values, which may explain the anti-nephrocalcinogenic effect of high-protein intake. Analbuminemic rats had lower kidney calcium concentrations and lower degrees of nephrocalcinosis than Sprague-Dawley rats. This may relate to the lower urinary pH, higher urinary magnesium and lower urinary phosphorus concentrations in analbuminemic rats. Replacement of casein by soybean protein lowered the severity of nephrocalcinosis in the two strains of rats.

Samenvatting

DEEL I. VOEDINGSEIWIT EN CHOLESTEROLSTOFWISSELING

In dit onderzoek werd gevonden dat de soort en hoeveelheid dierlijk eiwit in het voer de cholesterolconcentraties van plasma en lever beïnvloeden bij vrouwelijke ratten. Kabeljauwmeel als eiwitbron induceerde lagere plasma- en levercholesterolconcentraties dan caseïne; een verhoogde eiwitconcentratie in het voer versterkte dit effect. Scholmeel reduceerde de plasmacholesterolconcentratie vergeleken met wijtingmeel. Wei-eiwit produceerde lagere plasma- en levercholesterolconcentraties dan caseïne; dit effect werd versterkt door verhoging van de eiwitconcentratie in het voer. Het cholesterolverlagende effect van wei-eiwit is mogelijk het gevolg van remming van de cholesterolsynthese. Het is aannemelijk dat de cholesterolreaktie aan het eiwit in de eiwitpreparaten kan worden toegeschreven, mede omdat de voeders gebalanceerd waren voor het cholesterol en vet in de eiwitpreparaten. Het cholesterolverlagende effect van wei-eiwit versus caseïne kon niet worden gereproduceerd door aminozuurmengsels met eenzelfde samenstelling als deze eiwitten.

Albumine-deficiënte ratten (Nagase analbuminemic rats) waren gevoeliger dan Sprague-Dawley ratten voor de cholesterolverhogende werking van caseïne versus soja-eiwit. De albumine-deficiënte rattenstam lijkt een interessant model voor onderzoek betreffende de cholesterolstofwisseling.

DEEL II. VOEDINGSEIWIT EN NIERVERKALKING

Een verhoogde opname van caseïne of soja-eiwit met voeders, die gebalanceerd waren voor calcium, magnesium en fosfor, veroorzaakte een afname van de calciumgehaltes in de nieren van vrouwelijke ratten. Kabeljauwmeel als eiwitbron

produceerde minder nierverkalking dan soja-eiwit of caseïne, maar hogere concentraties van kabeljauwmeel lieten geen verdere afname van de mate van nierverkalking zien. Vervanging van caseïne door wei-eiwit resulteerde in een duidelijke vermindering van de ernst van nierverkalking; dit is waarschijnlijk het gevolg van een verlaging van de fosforconcentratie in de urine. Een verhoogde opname van zowel de intacte eiwitten als van de corresponderende aminozuurmengsels verminderde de nierverkalking. Een verhoogde stikstofopname ging gepaard met verhoogde magnesiumgehaltes en een verlaagde pH van de urine, hetgeen mogelijk verklaart waarom een verhoogde eiwitopname nierverkalking tegengaat. Albumine-deficiënte ratten (Nagase analbuminemic rats) hadden lagere calciumgehaltes in de nier dan Sprague-Dawley ratten. De lagere pH, de hogere magnesiumconcentratie en de lagere fosforconcentratie in de urine van albumine-deficiënte ratten zouden voor de minder ernstige nierverkalking verantwoordelijk kunnen zijn. Vervanging van caseïne door soja-eiwit induceerde minder nierverkalking in beide rattenstammen.

Curriculum vitae

The author was born on September 29, 1953 in Datong City, Shanxi Province, The People's Republic of China. He graduated in 1977 with a B.Sc. degree majoring in public health, from the Faculty of Preventive Medicine, Shanxi Medical College, and then worked as an assistant teacher in the Department of Environmental Hygiene for four years. During the period 1982-1984, he carried out work as requirement for the M.Sc degree in the Department of Nutrition and Food Hygiene, Shanxi Medical College, and subsequently worked in this Department as a lecturer, and this position has been kept until now. In October 1988, he started his research for the Ph.D. degree on the influence of dietary proteins on cholesterol metabolism and nephrocalcinogenesis. This research was carried out under the supervision of Professor Anton C. Beynen in the Department of Laboratory Animal Science, Utrecht University, The Netherlands.

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