The importance of the non-protein components of the diet in the plasma cholesterol response of rabbits to casein

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1. To characterize the hypercholesterolaemic effect of casein further, four groups of young male rabbits in two separate experiments were placed on cholesterol-free semi-purified diets for 12 weeks. The diets were similar in composition, with either casein or soya-bean-protein isolate providing the protein source (250 g/kg). In two of these diets the salt mix was reduced by 45% (normally 40 g/kg) and replaced by potassium bicarbonate.

2. Growth was unaffected by these alterations in dietary salts except for one group given the soya-bean–reduced-salts diet.

3. The mean concentrations of plasma cholesterol were significantly higher in all casein-fed groups as compared with their soya-bean-fed counterparts but the response was much greater in those given the casein–reduced-salts diet.

4. Contrary to expectations, analysis of the diets showed the zinc and copper concentrations of the casein diets to be less than those of the soya-bean diets. This was due to the greater concentrations of Cu (threefold) and Zn (twofold) in the soya-bean-protein isolate compared with casein.

5. The mean concentration of Zn in fur was significantly decreased in casein-fed rabbits and these animals also excreted less Zn but more Cu in their urine than those given the casein–reduced-salts diet.

6. The rabbits given the casein diet with the least salt mix showed the greatest degree of hypercholesterolaemia, suggesting an interaction between trace elements and the casein effect.

It has been clearly shown that dietary protein can influence cholesterol metabolism in rabbits (Carroll & Hamilton, 1975; Hermus, 1975; Kritchevsky, 1979). Hypercholesterolaemia and atherosclerosis can be evoked by feeding cholesterol-free, semi-purified diets containing casein as the protein component (Hermus, 1975; Roberts, 1981; Terpstra *et al.* 1982) and the observed hypercholesterolaemia has been attributed to the protein source (Huff *et al.* 1977; Huff & Carroll, 1980; Terpstra & Sanchez-Muniz, 1981). Huff & Carroll (1980) showed that the amino acid composition of dietary protein plays an important role in regulating serum cholesterol levels. Rabbits given semi-purified diets containing an amino acid mixture resembling the composition of casein also develop hypercholesterolaemia but not to the same extent as those given the intact protein. Studies by Terpstra & Sanchez-Muniz (1981) have confirmed the differential effect of dietary casein and soya-bean protein on serum cholesterol levels in rabbits, and have shown that soya-bean protein is able to reduce casein-induced hypercholesterolaemia.

However, other dietary components in combination with casein have been shown to influence plasma cholesterol levels in rabbits. The type and amount of fat (Lacombe & Nibbelink, 1980; Beynen & West, 1981) and carbohydrate (Carroll & Hamilton, 1975) modify the hypercholesterolaemic response of rabbits to casein (Wigand, 1959; Carroll, 1971). Polyunsaturated fatty acids have been shown to decrease the hypercholesterolaemic effect of casein (Carroll, 1971) and dietary cholesterol markedly elevates plasma cholesterol levels in casein-fed rabbits (Lacombe & Nibbelink, 1980).

Casein-containing diets not only influence cholesterol metabolism in rabbits but also reduce the animals' growth rate relative to chow-fed rabbits (Hermus, 1975). The effect that this may have on plasma cholesterol levels is unknown but it has been reported (Beynen &

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Table 1. *Composition (g/kg) of the semi-purified diets*

Diet...	Casein*	Casein†	Casein–reduced salts*	Casein–reduced salts†	Soya bean	Soya bean–reduced salts
Casein‡	270	292	270	292	—	—
Soya-bean-protein isolate‡	—	—	—	—	270	270
Dextrose	600	600	600	600	600	600
Celluflour	50	50	50	50	50	50
Molasses (500 ml/l)	20	20	20	20	20	20
Maize oil (ml)	20	10	20	10	20	20
Salt mix	40	40	22	22	40	22
Potassium bicarbonate	—	—	18	18	—	18

Soya-bean-protein isolate was obtained from Procol Products Pty Ltd, Sydney (Pro-col 90 Product of USA).
Dextrose monohydrate was obtained from Fielders Starches, Sydney.

Salt mix was obtained from ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio, USA or mixed as required, the composition being (g/kg): NaCl 193·7, K_2HPO_4 324·5, $CaHPO_4 . 2H_2O$ 62·4, $MgSO_4 . 7H_2O$ 102·7, $CaCO_3$ 301·9, Fe citrate 13·1, KI 0·81, $MnSO_4 . 4H_2O$ 0·35, $ZnCl_2$ 0·25, $CuSO_4 . 5H_2O$ 0·3.

Vitamins were obtained from the Sigma Chemical Co., St Louis, MO, USA.

Water-soluble vitamins (g/l): thiamin hydrochloride 0·05, pyridoxine monohydrochloride 0·05, D-biotin 0·02, myo-inositol 10, riboflavin 1, nicotinic acid 5, calcium pantothenoate 5·9, folic acid 0·05, choline chloride 100; dissolved in 50% (v/v) ethanol and 15 ml added/kg diet.

Fat-soluble vitamins (g/l maize oil): *trans*-retinol palmitate 10, DL-α-tocopherol acetate 11, menadione 3, dissolved in maize oil (to provide a source of essential fatty acids) and 10 ml added/kg diet.

* Casein obtained from ICN Pharmaceuticals Inc. (vitamin-free, purified, high nitrogen) (950 g protein/kg, proximate composition).

† Casein obtained from the Colac Dairying Co. Ltd, Victoria (colachydrochloric casein; edible extra grade) (920 g protein/kg, proximate composition, and 15 g fat/kg). The volume of maize oil was adjusted to account for the fat content of the casein product.

‡ Casein and soya-bean protein isolate were added in these amounts to provide 250 g protein/kg diet.

van Wanrooy-Stroeken, 1981) that weight gain can be improved by the addition of potassium bicarbonate to casein-containing diets. This addition would, however, alter the mineral balance of the diet which may also influence cholesterol metabolism.

As part of a study to explore casein-induced hypercholesterolaemia further, potassium bicarbonate was incorporated into the rabbits' diets. Since this involved altering the mineral mix of the diet and because of the importance of zinc and copper in cholesterol metabolism (Klevay, 1973; Allen & Klevay, 1978; Harvey & Allen, 1981; Lau & Klevay, 1982), we assessed the Zn and Cu concentrations of the diets. Contrary to the view that casein and soya-bean diets are similar in all respects except for the protein, the results reported here show that the casein diets contained significantly less Cu and Zn than the soya-bean diets and that this difference may contribute significantly to the hypercholesterolaemic effect of casein.

MATERIALS AND METHODS

Animals and diets

Young adult male rabbits, Castle Hill Laboratory White strain (University of Sydney, Castle Hill Animal House, NSW), about 6 weeks old and weighing between 1·5 and 2·0 kg, were housed individually in wire cages with mesh bottoms in an air-conditioned room with controlled temperature, 23–24°, and artificial lighting (12 h light–12 h dark cycle). On arrival the rabbits were maintained on rabbit chow (Purina rabbit and guinea-pig pellets, Allied Feeds, Rhodes, NSW) for up to 2 weeks. Two studies were carried out. Because of the unusual results obtained in the first, a second experiment was undertaken to obtain more information about the Cu and Zn status of the animals. In both experiments, animals were weight paired into four groups. Each group was then transferred to a low-fat, cholesterol-free, semi-purified pelleted diet, containing either casein or soya-bean-protein isolate as the protein source. In two of the diets 45% of the salt mix (Table 1) were substituted by potassium bicarbonate such that this salt provided 18 g/kg diet (the composition of the semi-purified diets is shown in Table 1). Numbers were initially equal in the groups of Expt 1 (*n* 7 for the standard salt diets, *n* 6 for the reduced-salts diet). However, two deaths in the soya-bean group, both unrelated to the treatment (one respiratory infection and one middle-ear infection), reduced numbers to five in that group. Food was restricted to 900 g/week per animal for all dietary groups. Deionized water was provided *ad lib.* and the rabbits were weighed weekly.

Sampling of tissues and chemical analyses

Plasma cholesterol was assayed fortnightly. Non-fasting animals were bled from the marginal ear vein into centrifuge tubes containing EDTA (0·4 mM, pH 7·4) and plasma separated by centrifugation (1170 *g*, 5 min). In the first experiment, total plasma cholesterol was extracted and saponified as described by Mann (1961) and analysed by the method of Zlatkis & Zak (1969). In the second experiment, plasma cholesterol was analysed by an automated enzymic method (Monotest Cholesterol, CHOD-PAP method; Boehringer, Mannheim, W. Germany).

At the end of the second experiment, animals were killed by anaesthetic overdose (Anathal; VR Laboratories Pty Ltd, Thornleigh, NSW). Fur was collected from the lumbar region and small samples (2–5 g) of liver removed for subsequent analysis.

Zn and Cu analyses of diets and tissues

Triplicate samples (1–5 g) of the diet and dietary components were ashed overnight in a muffle furnace (475°), the residue dissolved in 25 ml hydrochloric acid (3 M) and the Zn and Cu concentrations measured using an atomic absorption spectrophotometer (Varian Model AA-575) with appropriate standards. Samples of urine and plasma were diluted tenfold and the concentrations of Cu and Zn measured using the method of Meret & Henkin (1971). Fur, washed using the method of Mckenzie (1978), and freeze-dried liver were treated in the same manner as the diets.

RESULTS

Weight gain and plasma cholesterol

Mean gain in weight was similar between dietary groups except for one group (Expt 1) consuming the soya-bean–reduced-salts diet in which mean gain in weight was significantly greater than for all other groups (Table 2). Considerable variation in mean concentration of plasma total cholesterol was seen over the 12-week period in all groups (Table 3). To normalize the variance between groups, the values were expressed as the change in cholesterol concentration from the initial value. Those animals given the casein diets either showed no change or a slight increase in mean concentration of plasma cholesterol by 12 weeks, whereas those given the soya-bean diet decreased slightly and those given the soya-bean–reduced-salts diet decreased somewhat more from their initial levels. Analysis of variance of the values after 12 weeks on the diets indicated that there was a significant effect of protein on plasma cholesterol response but not of salt alone (Table 3). There was,

Table 2. *Effect of diet composition*† *on mean body-weights (kg) of rabbits*

(Mean values with their standard errors)

Diet	Expt no.	*n*	Period on diet (weeks) 0 Mean	0 SE	6 Mean	6 SE	12 Mean	12 SE	Mean wt gain (g/d) Mean	SE
Casein	1	7	2·01	0·05	2·37	0·04	2·48	0·09	4·89	0·88
	2	6	2·09	0·10	2·49	0·09	2·48	0·14	4·73	0·38
Soya-bean	1	5	2·08	0·04	2·41	0·09	2·60	0·14	5·91	1·52
	2	6	2·00	0·09	2·32	0·09	2·50	0·10	5·92	0·08
Casein–reduced-salts	1	6	1·86	0·10	2·21	0·05	2·31	0·11	5·59	1·24
	2	6	1·60	0·06	2·20	0·05	2·18	0·15	6·90	0·05
Soya-bean–reduced-salts	1	6	1·82	0·10	2·37	0·07	2·64	0·10	0·58*	1·25
	2	6	1·57	0·04	1·78	0·06	2·09	0·08	6·21	0·56

Mean value was significantly different from that for all other groups (Students' *t* test): $*P < 0.05$.

† For details, see Table 1 and p. 89.

however, a significant effect of the protein–salt interaction on the plasma cholesterol response. This is clear from the results where it can be seen that in both experiments casein, in the presence of reduced salts, produced a greater response than casein alone and that soya-bean in the presence of reduced salts produced a larger decrease than soya-bean alone.

Zn and Cu analyses of diets and tissues

The Cu and Zn concentrations of the soya-bean-containing diets were higher than those of the casein diets (Table 4), and the reduced-salts diets contained correspondingly less of these minerals than the standard-salts diets. The difference in the Zn and Cu contents of the soya-bean and casein diets was due to the differing amounts in the protein isolates, with the soya-bean-protein isolate containing about twice the concentration of Zn and three times that of Cu compared with casein (Table 5).

The mean concentrations of Zn and Cu in liver did not vary among the dietary groups, nor did the mean concentration of plasma Zn (Table 6). However, the mean concentration of Zn in fur was significantly decreased in casein-fed animals when compared with soya-bean-fed animals and was further decreased in those groups given the reduced-salts diets.

When urine volume was taken into account, Zn excretion was five times higher in those given the reduced-salts diets compared with the control groups and Cu excretion was lower in the group given casein reduced-salts.

DISCUSSION

These results suggest that mineral differences together with the type of dietary protein have a significant effect on plasma cholesterol levels in rabbits and that growth rate was unimportant in this response.

West *et al.* (1982) have suggested that differences in growth rates between rabbits given diets containing casein and soya-bean-protein may contribute to the observed differences in plasma cholesterol levels. The similar growth rates between the casein dietary groups and the significant differences in plasma cholesterol responses would seem to exclude any possibility

Table 3. *Effect of diet composition† on plasma cholesterol concentration (mg/l) of rabbits*

(Mean value with 95% confidence intervals. Results are expressed as absolute values for week 0 and change from week 0 at week 6 and week 12)

		Period on diet (weeks)					
		0		6		12	
Diet	*n*	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
Expt 1							
Casein	7	535	150	139	580	11	350
Soya-bean	5	358	180	−64	150	−156	210
Casein–reduced-salts	6	596	270	645	760	607	200
Soya-bean–reduced-salts	6	634	250	−393	210	−320	70
Expt 2							
Casein	6	489	550	473	450	535	750
Soya-bean	6	547	380	−56	220	−151	210
Casein–reduced-salts	6	993	460	321	770	1895	990
Soya-bean–reduced-salts	6	1096	230	−399	280	−548	400

Analysis of variance of response at week 12

Source of variation	Sum of squares	df	Mean square	Variance ratio
Expt 1‡				
Protein	18050	1	18050	14**
Salt	3651	1	3651	NS
Protein-salt	8529	1	8529	6·6*
Within subclasses	25863	20	1293	
Expt 2				
Protein	146808	1	146808	35**
Salt	13932	1	13932	NS
Protein-salt	46323	1	46323	10·9**
Within subclasses	84502	20	4225	

NS, not significant.
* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.
† For details, see Table 1 and p. 89.
‡ Adjusted for unequal sample size (Snedecor & Cochran, 1968).

Table 4. *Total zinc and copper contents (mg/kg) of the diets* given to rabbits*

(Results are expressed as the mean of triplicate analyses from two batches of each diet)

Diet...	Expt	Casein	Soya bean	Casein–reduced-salts	Soya-bean–reduced-salts
Zn	1	11·63	25·34	11·29	24·80
	2	13·59	20·73	11·88	20·78
Cu	1	3·97	5·73	2·55	5·20
	2	3·69	4·63	3·00	4·35

* For details, see Table 1 and p. 89.

Table 5. *Total zinc and copper contents (mg/kg) of the dietary components**

(Results are expressed as the mean of triplicate analyses)

	Salt mix	Molasses	Celluflour	Casein	Soya-bean-protein
Zn	58·92	9·27	1·68	27·42	62·29
Cu	83·79	3·32	0·59	1·58	5·22

* For details, see Table 1.

Table 6. *Expt 2. Effect of diet composition§ on the total zinc and copper contents of rabbit tissues*

(Mean values with their standard errors)

	Plasma (μg/ml)		Fur (μg/g)		Liver (μg/g wet wt)		Urine (μg/24 h)	
Diet	Mean	SE	Mean	SE	Mean	SE	Mean	SE
				Zinc				
Casein	0·9	0·10	197	1·8	38	4·5	13·2**	0·89
Soya-bean	1·0	0·09	241†	3·9	31	5·3	17·0**	0·61
Casein–reduced-salts	0·8	0·09	116	30·8	31	0·5	82·5	2·62
Soya-bean–reduced-salts	0·8	0·10	96	7·1	33	1·7	77·2	3·63
				Copper				
Casein	0·8	0·11	10	0·9	4·5	0·5	53·9‡	4·4
Soya-bean	0·9	0·06	11	1·1	3·1	0·9	35·7	1·8
Casein–reduced-salts	1·4	0·14	16	2·6	4·4	0·3	36·3	1·16
Soya-bean–reduced-salts	0·6	0·06	22	2·4	4·3	0·6	46·8	1·5

Mean value was significantly lower than that for reduced salts, dietary counterpart (Student's *t* test): ** $P < 0·01$.
Mean value was significantly different from that for other dietary groups within the same tissue (Student's *t* test): † $P < 0·05$.
Mean value was significantly different from that for corresponding reduced-salts diet (Student's *t* test): ‡ $P < 0·01$.

of a link between weight gain and plasma cholesterol in the present study. The substitution of a portion of the salt mix in the diets by potassium bicarbonate did not result in any apparent deficiency of essential elements required for adequate growth and did not affect the growth rate of the casein-fed rabbits. However the substitution of potassium bicarbonate in the soya-bean-containing diet significantly improved growth rate in one group (Expt 1) but not the other (Expt 2).

Variations were seen in the degree of induced hypercholesterolaemia amongst the animals given the different diets (Table 3). Rabbits developed hypercholesterolaemia when given the casein–reduced-salts diet, while rabbits given the casein diet maintained normal or only slightly elevated levels of plasma cholesterol, which were slightly lowered in those animals given either of the soya-bean-containing diets. The lack of a marked hypercholesterolaemic response in the casein-fed animals and the enhanced hypercholesterolaemic response seen when the salt mix was replaced by potassium bicarbonate suggests that potassium bicarbonate may have an independent effect on plasma cholesterol. Beynen & van Wanrooy-Stroeken (1981) have suggested that dietary bicarbonate may serve as a buffer, counteracting the observed aciduria induced by dietary casein and perhaps correcting a potential disturbance of acid–base equilibrium which in some way influences plasma cholesterol homeostasis. This effect, if any, was not observed in those animals given the soya-bean–reduced-salts diet compared with those given the soya-bean diet.

A second possibility concerning the effect of potassium bicarbonate substitution on plasma cholesterol levels relates to the altered mineral balance of the semi-purified diets. Klevay (1973) initially proposed that the ratio, zinc:copper can influence the degree of dietary-induced hypercholesterolaemia in rats. Surprisingly, the casein-containing diets used in the present study had lower concentrations of Cu and Zn than the soya-bean-containing diets (Table 4). Analysis of the dietary protein component of the diets showed the amount of Cu and Zn to be lower in casein than in soya-bean-protein isolate. Thus the reduction of total salt mix resulted in at least a gradation in the Cu and Zn contents of the diets, with soya-bean > soya-bean–reduced-salts > casein > casein–reduced-salts. It is evident, therefore, that the reduced-salts diet with the least Cu and Zn was the most hypercholesterolaemic (Table 3), and that an apparent inverse relation exists between the plasma cholesterol level at 12 weeks and the Cu and Zn concentrations of the diets. The quantity of the Zn and Cu in the various diets was sufficient in all cases to prevent overt signs of deficiency in the 12-week period of the study and all animals maintained or gained weight over this period. Plasma and liver concentrations of Zn and Cu were generally unaffected but fur Zn was reduced in the casein-fed animals and further reduced in animals given the reduced-salts diets. At the same time, the excretion of Zn was highest in those given the reduced-salts diets indicating some disturbance of trace element metabolism.

Huff & Carroll (1980) have suggested that non-protein constituents of the protein preparations may be partially responsible for the induced hypercholesterolaemia of casein-fed animals. The results obtained from the analysis of Cu and Zn concentrations in the semi-purified diets indicate that the amounts and dietary Cu:Zn values may be important in casein-induced hypercholesterolaemia in rabbits. The mechanism for such an interaction may involve the key enzymes of lipoprotein metabolism, lipoprotein lipase (LPL, *EC* 3.1.1.34) and lecithin cholesterol acyl transferase (LCAT, *EC* 2.3.1.43). In Cu-deficient rats, it has been suggested (Lau & Klevay, 1982) that the observed hypercholesterolaemia may be the result of depressed LPL activity, and the decrease in LCAT activity seen in Cu-deficient rats given a casein-containing diet (Harvey & Allen, 1981) may be due to changes in lipoprotein metabolism. The possible effects of altering the dietary mineral balance on plasma cholesterol levels need to be investigated further.

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