

Reduction of Casein-induced Hypercholesterolaemia and Atherosclerosis in Rabbits and Rats by Dietary Glycine, Arginine and Alanine

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Summary

We have studied the effect of amino acid supplementation on the hypercholesterolaemia and atherosclerosis caused by cholesterol-free casein-containing diets in rabbits, and on the hypercholesterolaemia caused in rats by diets containing casein plus added cholesterol.

Five groups of rabbits each received for 48 weeks semi-purified diets containing casein as a protein source. In one group the animals received additional arginine and alanine, and in three other groups the diets were supplemented with arginine, alanine and three different levels of glycine. These supplementations were chosen so as to bring the amino acid composition of the diets closer to that of commercial rabbit pellets.

Average body weights at the end of the experiment were 3400 g in the casein group and 3800–3900 g in the four amino-acid-supplemented groups. Average serum cholesterol levels over the last 10 weeks were 15.7 mmol/l for the casein group, 12.0 for the casein-arginine-alanine group and 8.5, 8.8 and 8.1 for the three groups receiving arginine, alanine and increasing amounts of glycine. Serum cholesterol concentrations in the combined glycine-receiving groups were significantly lower than in the unsupplemented casein group. At the end of the experiment aortas were dissected from all rabbits and the sudanophilic surface area was

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quantitated. The extent of aortic sudanophilia was less in the amino-acid-supplemented groups than in the casein group. There was a strongly significant correlation between the proportion of aortic surface covered by sudanophilic plaques and the serum cholesterol concentration per animal. The effect of dietary glycine was not limited to rabbits: when we fed rats semi-purified diets containing cholesterol, and casein, casein plus glycine, or soy isolate, we obtained mean serum cholesterol levels of 10.4 mmol/l in the casein group, 4.9 in the casein-plus-glycine group and 3.5 in the soy group.

We conclude that casein-induced hypercholesterolaemia and its sequelae are partly relieved by addition of glycine to the diet, while supplementation of casein-based rations with arginine and/or alanine may be necessary for rabbits. However, the beneficial effects of these amino acids could just as well be explained by the presence of an excess of other amino acids in casein (amino acid imbalance) as by an absolute shortage of glycine, arginine or alanine.

Key words: *Casein – Dietary amino acids – Dietary cholesterol – Rabbits – Semi-purified diets – Soy protein – Zucker rats*

Introduction

In 1909 Ignatowski [1] reported that the feeding of animal foodstuffs to rabbits caused severe atherosclerosis, and he suggested that this was due to the protein part of such foodstuffs. This suggestion was later dismissed when it was discovered that the atherogenic effect of foods such as egg yolk could be mimicked by the addition of pure cholesterol to the diet.

Recent experiments, however, leave no doubt that certain dietary proteins, such as casein from cow's milk, can cause hypercholesterolaemia and atheromatous plaques in animals, independent of cholesterol and saturated fat in the diet (for reviews see [2] and [3]). It has thus become of interest to pinpoint the components of such proteins responsible for this effect. In preliminary experiments [4] we found that fortification of semi-purified diets containing casein with glycine reduced its hypercholesterolaemic effect. The proportion of glycine in casein is very low. We now report on a long-term study where the effect of glycine, alanine and arginine in the diet on serum cholesterol concentrations and aortic lesions in rabbits has been quantified. We have also extended these studies to rats, where we found a similar protective effect of dietary glycine against casein-plus-cholesterol induced hypercholesterolaemia. Arginine and alanine were included because, like glycine, their proportion is low in casein compared with soy protein or commercial rabbit pellets.

Some of these results have been presented earlier in a preliminary form [5].

Methods

Rabbit experiment

The male New Zealand white rabbits were obtained at the age of 8–10 weeks from the Broekman Institute, 5708 EG Helmond, The Netherlands. They were maintained at the Institute for Animal Nutrition Research–ILOB (Wageningen). The rabbits were housed in cages constructed of galvanised steel, including the wire screen floors, and were situated in a room with adequate ventilation (5–15 changes of air each hour) and controlled illumination (15 h/day). The temperature of the room was maintained between 16 and 18°C and the humidity was controlled (65%). Food and water were supplied ad libitum at all times. On arrival, the animals were maintained for 2 weeks on a diet of commercial rabbit pellets (Cunicon 1, Trouw & Co. N.V., 3881 LB Putten, The Netherlands), which contained (as g/100 g feed) crude protein, 19.0% (digestible, 15.6%); crude fat, 4.3%; crude fibre, 9.4%; other carbohydrates, 49.3%; ash, 7.0%; and water, 11.0%. The rabbits were then transferred gradually to a semi-purified diet containing casein. After two weeks they were allocated to the various diet groups on the basis of their serum cholesterol levels and body weights. Each group initially consisted of 17 animals, except for a group receiving commercial laboratory pellets, which had only 8 rabbits. The test

TABLE 1
COMPOSITION OF THE BASAL CASEIN-CONTAINING SEMI-PURIFIED RABBIT DIET

| Component | Amount (g/100 g) |
|--------------------------|------------------|
| Casein ^a | 20.8 |
| Corn starch ^b | 35.6 |
| Sawdust ^c | 21.1 |
| Coconut oil ^d | 13.8 |
| Corn oil | 0.5 |
| Salts ^e | 6.1 |
| Vitamins ^f | 1.2 |
| Minerals ^g | 1.0 |

^a Acid casein, DMV BV, 5466 BA Veghel: crude protein, 86.5%; lactose, 0.5–1.0%; ash, 2.5%.

^b Maisamyl, Honig Merkartikelen BV, 1541 KA Koog aan de Zaan: crude protein, 1.2%; fat, 0.4%; dry matter, 88%.

^c Woody meal, clean S.P.F., sterilised by radiation, Broekman Institute BV, 6708 EG Helmond.

^d Refined coconut oil, Type "Cose", Croklaan BV, 1520 AA Wormerveer.

^e KHCO₃, 1.81 g; CaHPO₄·2 H₂O, 2.9 g; NaCl, 0.85 g; MgCO₃, 0.3 g; and MgO, 0.2 g.

^f Containing Thiamin, 6 mg; riboflavin, 2.25 mg; niacin, 15.2 mg; Ca-pantothenate, 5.6 mg; choline chloride, 200 mg; inositol, 100 mg; folic acid, 0.85 mg; biotin, 0.06 mg; pyridoxine hydrochloride, 2.25 mg; PABA, 50 mg; vitamin B₁₂, 1.5 µg; *dl*- α -tocopheryl acetate, 5 mg; vitamin K₃, 0.4 mg; vitamin A, 1500 i.u.; vitamin D₃, 300 i.u.; and vitamin C, 40 mg.

^g Containing Na citrate·2 H₂O, 153.5 mg; FeSO₄·7 H₂O, 90 mg; MnO₂, 14 mg; KAl(SO₄)₂·12 H₂O, 10 mg; ZnSO₄·7 H₂O, 20 mg; KBr, 2 mg; NiSO₄·6 H₂O, 0.85 mg; CuSO₄·5 H₂O, 10 mg; CoSO₄·7 H₂O, 0.5 mg; Na₂MoO₄·2 H₂O, 0.5 mg; KI, 0.5 mg; As₂O₃, 0.02 mg; NaF, 0.85 mg; and Na₂B₄O₇·10 H₂O, 0.5 mg.

TABLE 2

THE AMOUNTS OF AMINO ACID ADDED PER 100 g OF TEST DIET, AND THE FINAL CONCENTRATIONS OF ARGININE, ALANINE AND GLYCINE IN THE COMPLETE DIETS

All diets contained 20.8 g of casein per 100 g (Table 1).

| Group | n ^a | Additions | Final concentrations (g/100 g of feed) | | |
|-------|----------------|---|--|-----------|---------|
| | | | L-Arginine | L-Alanine | Glycine |
| II | 13 | None | 0.72 | 0.61 | 0.35 |
| III | 12 | Arginine (0.35%) and alanine (0.54%) | 1.07 | 1.15 | 0.35 |
| IV | 15 | Arginine (0.35%), alanine (0.54%) and glycine (0.65%) | 1.07 | 1.15 | 1.00 |
| V | 15 | Arginine (0.35%), alanine (0.54%) and glycine (1.40%) | 1.07 | 1.15 | 1.75 |
| VI | 15 | Arginine (0.35%), alanine (0.54%) and glycine (3.15%) | 1.07 | 1.15 | 3.50 |

^a Number of animals alive at the end of the experiment.

diets were provided as a wet mash and were fed for 48 weeks; 14 blood samples were taken over this period, in the manner described before [6]. The animals were killed at the end of the experiment, and heart and aorta were immediately removed as a whole and cut longitudinally from the iliac bifurcation to the heart. The heart and aorta were flattened between glass plates, fixed and stained with Sudan III and Sudan IV as described [7], washed and photographed. Both the sudanophilic and the total inner aortic surface were measured on 30 × 40 cm photographs using an MOP-AM 02 microcomputer-assisted digitizer (Kontron, Mijdrecht, The Netherlands).

Serum cholesterol was measured in a rigidly standardised laboratory, as described [8].

The composition of the basal semi-purified casein-containing diet is given in Table 1. L-Arginine, L-alanine and glycine (Welding & Co., D 2000 Hamburg, F.R.G.) were added to the casein diet at the expense of saw dust. The final levels of these amino acids are shown in Table 2.

Rat experiment

The animals were female Zucker rats [9] of the lean phenotype, and were raised and maintained at the Centre for Small Animals of the Agricultural University. They had been weaned onto commercial rat pellets 3 weeks after birth. They were initially housed individually in stainless steel wire cages, in a room with a constant temperature of 21°C, a relative humidity of 65%, a day length of 12 h and adequate ventilation (12–15 changes of air each hour). From the 14th week on they were housed in groups of 5–6 animals in stainless steel wire cages, under the same conditions as described above.

At the start of the experiment the animals were 4–5 weeks old. They were randomized into 4 diet groups: casein ($n = 24$), casein plus glycine ($n = 17$), soy protein ($n = 17$) and control laboratory rat feed ($n = 9$), and were kept on these diets for 27 weeks. Food and water were provided ad libitum.

The rat feed, type RMH-B, was from Hope Farms (Woerden, The Netherlands). The semi-purified diets were identical to the casein diet described above for the rabbits except for the following:

(1) To each diet, cholesterol (Van Schuppen Chemie, Veenendaal, The Netherlands) was added to a final concentration of 1.2 g/100 g by dissolving it in molten coconut oil.

(2) Corn oil was omitted from the diets.

(3) To prepare the casein-plus-glycine diet, 2 g of sawdust in the casein diet was replaced by 2 g of glycine (Welding & Co.).

(4) The soy diet contained 20 g of soy protein isolate/100 g (Promine F, Central Soya Company, Chicago), and no casein. NaCl was reduced to 0.20 g/100 g, and 0.2 g of DL-methionine/100 g was added.

Blood was obtained by orbital puncture after a 16-h fast. The cholesterol concentration was determined enzymatically (CHOD-PAP kit, Boehringer-Mannheim, F.R.G.).

Results

Weight gain and mortality in rabbits

The initial weight of the rabbits at the start of the test period was 2160 g on average. Weight gains after 44 weeks of test period were on average 1260 g for the casein diet, 1670 for the casein plus arginine and alanine diet, 1670, 1750 and 1740 for the three glycine-fortified diet groups, and 2580 for the control group. Out of the 93 animals, 16 died during the experiment; in 12 cases the diagnosis was chronic lung disease, as described before [7]. No relationship between diet group and disease or mortality was apparent.

Total serum cholesterol concentrations in rabbits

Figure 1 shows the time-course of total serum cholesterol concentrations per diet group. The initial level, at week 0, is already elevated because all animals had been exposed to the hypercholesterolaemic casein diet for a short period prior to randomization. On the commercial control diet, levels were normalised within about 8 weeks. Serum cholesterol concentrations continued to rise on each of the semi-purified casein diets, but the final levels in the glycine-supplemented groups were only about half that of the unsupplemented casein group (Table 3). There was a large individual variation in the response of different animals to the diets, as noted by other authors [10]. As a consequence, the differences between the separate diet groups were not statistically significant. However, when the data of the three glycine-supplemented diet groups were pooled the difference between the serum cholesterol concentrations of these groups and of the unfortified casein group was highly significant ($P < 0.01$).

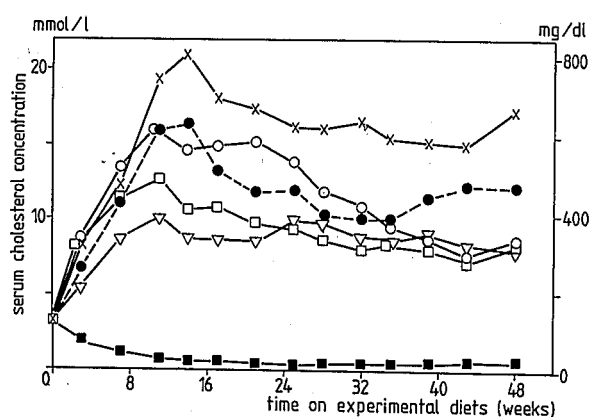


Fig. 1. Mean concentration of serum cholesterol in rabbits fed semi-purified cholesterol-free diets containing casein and various amino acid supplementations. \times — \times , 21% casein (13 rabbits); \bullet — \bullet , 21% casein supplemented with 0.35% L-arginine and 0.54% L-alanine (12 rabbits); ∇ — ∇ , 21% casein with 0.35% arginine, 0.54% alanine and 0.65% glycine (15 rabbits); \circ — \circ , 21% casein with 0.35% arginine, 0.54% alanine and 1.40% glycine (15 rabbits); \square — \square , 21% casein with 0.35% arginine, 0.54% alanine and 3.15% glycine (15 rabbits); \blacksquare — \blacksquare , commercial diet (7 rabbits). Results are given for those animals alive at the end of the experiment.

TABLE 3

CONCENTRATION OF SERUM CHOLESTEROL IN RABBITS FED SEMI-PURIFIED CHOLESTEROL-FREE DIETS CONTAINING CASEIN AND VARIOUS AMINO ACID SUPPLEMENTATIONS

Values represent the averages of the last three bleedings, at weeks 39, 43 and 48. SD's are not given because the data were not normally distributed.

| Group | n | Added amino acids | Serum cholesterol concentration | |
|----------|----|---------------------------------------|---------------------------------|----------|
| | | | Mean | Range |
| (mmol/l) | | | | |
| II | 13 | None | 15.7 * | 3.4-46.7 |
| III | 12 | Arginine and alanine | 12.0 | 1.3-36.7 |
| IV | 15 | Arginine, alanine and glycine (0.65%) | 8.5 | 1.4-30.3 |
| V | 15 | Arginine, alanine and glycine (1.40%) | 8.8 | 1.4-27.3 |
| VI | 15 | Arginine, alanine and glycine (3.15%) | 8.1 | 2.7-14.8 |

* Significantly different from group IV, and from combined groups IV-VI, by non-parametric two-tailed Wilcoxon rank sum test.

This was true both if serum cholesterol was expressed as the mean of all measurements obtained during the experiment and if only the last three measurements were averaged.

Extent of aortic atherosclerosis

After 48 weeks on the experimental diets the animals were killed, and the heart and aorta were dissected, and stained for lipid. Figure 2 shows the proportion of aortic inner surface covered by lipid-rich plaques as a function of the time-averaged serum cholesterol concentration per animal. The animals fed laboratory chow (indicated by full squares) showed absolutely no evidence of macroscopic atherosclerosis. Of the animals receiving semi-purified diets with casein as sole protein source, all animals but one were affected, and several quite severely, with less than half of the aortic surface remaining free from lipid-staining plaques. The effects of amino acid supplementation on hypercholesterolaemia and atherosclerosis showed large variations between rabbits. In 10 out of the 57 amino-acid-supplemented animals, average cholesterol concentrations over the duration of the experiment were less than 5 mmol/l, and plaques were absent. 22 of the 57 amino-acid-supplemented animals had less than 5% of their aortic surface covered by plaques, as compared with 2 out of the 13 animals in the unsupplemented casein group. Thus the majority of the supplemented animals also had serious atherosclerosis. The Pearson linear correlation coefficient between extent of atherosclerosis and the logarithm of time-averaged serum cholesterol concentration for the 45 animals supplemented with glycine, alanine and arginine was $r = 0.65$ (95% confidence limits: $r = 0.44 - 0.79$). For all animals on semi-purified diets combined, the correlation between the proportion of aortic surface covered by plaques and the logarithm of time-averaged

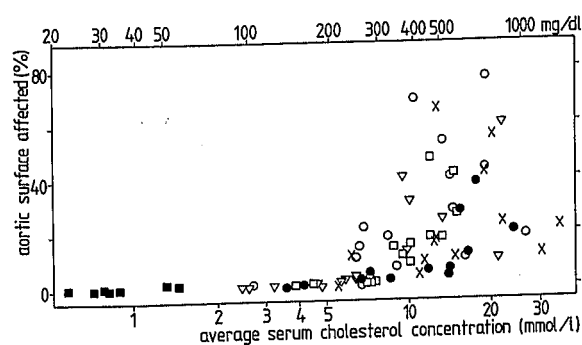


Fig. 2. Proportion of aortic surface covered by sudanophilic plaques as a function of mean serum cholesterol concentration over the duration of the experiment. The animals had been fed semi-purified cholesterol-free diets containing casein and various amino acid supplementations. X, 21% casein (13 rabbits); ●, 21% casein supplemented with 0.35% L-arginine and 0.54% L-alanine (12 rabbits); ▽, 21% casein with 0.35% arginine, 0.54% alanine and 0.65% glycine (15 rabbits); ○, 21% casein with 0.35% arginine, 0.54% alanine and 1.40% glycine (15 rabbits); □, 21% casein with 0.35% arginine, 0.54% alanine and 3.15% glycine (15 rabbits); ■, commercial diet (7 rabbits). Results are given for those animals alive at the end of the experiment.

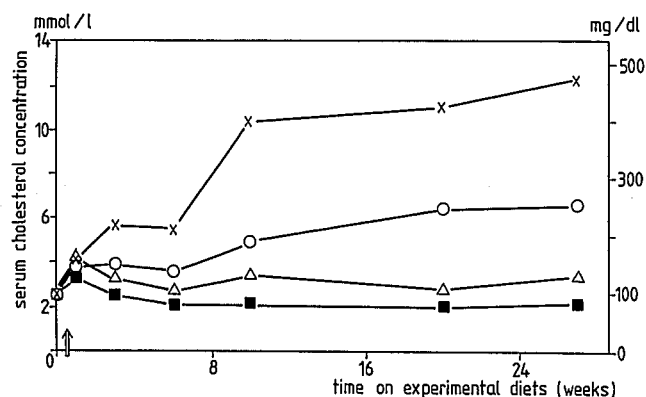


Fig. 3. Mean concentration of serum cholesterol in lean female Zucker rats fed semi-purified diets containing 1.2% cholesterol and different proteins. \times — \times , 20% casein (24 rats); \circ — \circ , 20% casein with 2% glycine (17 rats); \triangle — \triangle , 20% soy isolate protein (17 rats); \blacksquare — \blacksquare , commercial diet (9 rats). The arrow indicates when the test diets were started.

cholesterol level was $r = 0.56$ ($n = 67$; 95% confidence levels for r , 0.37–0.70). Within each diet group, the proportion of aortic surface covered by lipid plaques was also correlated with individual average serum cholesterol concentrations, with correlation coefficients ranging from 0.40 to 0.76.

Effect of glycine supplementation in rats

The mean weight of the rats at the start of the test period was 61 g. Mean weight gain over the first 11 weeks of the test period was 104, 110, 107 and 119 g for the casein, casein-plus-glycine, soy protein and commercial feed group, respectively. None of the animals became seriously ill or died during the study.

Figure 3 shows the time course of average total serum cholesterol levels in the rats. The unsupplemented casein-cholesterol diet caused serious hypercholesterolaemia; the final level at 27 weeks was 12.3 ± 6.2 mmol/l (mean \pm SD). In the group where glycine was added to the casein diet the mean final level of serum cholesterol was 6.6 ± 3.1 mmol/l. The difference between this group and the casein group was already highly significant at the third week ($P < 0.01$, two-tailed Wilcoxon rank sum test) and remained so until the end of the experiment.

The semi-purified soy protein/cholesterol diet caused a slight but significant elevation of total cholesterol concentrations compared with the standard laboratory pellets (final concentrations, 3.4 ± 0.7 and 2.2 ± 0.3 , respectively).

Discussion

Hypercholesterolaemia and atherosclerosis in rabbits

The hypercholesterolaemic and atherogenic effects of animal proteins in rabbits have been known for over 70 years [1], but the mechanism of these effects is still obscure.

In some experiments, non-protein components of the dietary protein preparations have probably played a role. Thus the atherosclerosis observed by Ignatowski [1] in rabbits fed animal products such as meat, eggs or milk was probably due to the cholesterol contained in these foodstuffs, while on the other hand soy protein preparations may contain saponins that protect against hypercholesterolaemia [11]. However, the amino acid composition of the diet itself must have some influence because the effects of different dietary proteins can be partly mimicked by the corresponding mixtures of amino acids [3,12-14]. Our observations confirm and extend this conclusion: if addition of small amounts of one or a few amino acids has such a large influence on cholesterol concentrations in serum then non-protein contaminants in the proteins used are not likely to be of major importance. Our data also support the suggestion that one of the causes of the atherosclerosis seen in rabbits on cholesterol-free semi-purified casein diets is an amino acid imbalance, in interaction with other defects of these diets. When the absolute amount of casein in semi-purified diets is increased, hypercholesterolaemia is aggravated [12,16]. Thus, lipid metabolism appears to be affected by the relative proportions rather than by the absolute amounts of the various amino acids in casein-based rations.

Several investigators have tried to define which amino acids are most crucial in relieving casein-induced hypercholesterolaemia in rabbits [3]. Kritchevsky suggested that the lysine:arginine ratio is important, and showed that addition of arginine to casein diets could reduce hypercholesterolaemia and atherosclerosis [15]. In the present study, addition of arginine plus alanine caused some reduction of cholesterol levels, but a still larger effect was obtained if glycine was added on top of the fortification with alanine and arginine. This specific effect of glycine agrees with our earlier observations [4]. The amount of additional glycine required was rather small, because higher doses of glycine did not depress cholesterol levels any further (Table 3 and Fig. 1).

Even in the groups supplemented with all three amino acids, cholesterol levels remained above control levels. Thus, a shortage of arginine plus alanine plus glycine relative to other amino acids accounts for only a part of the hyperlipaemia induced by semi-purified casein diets. This confirms observations of Kritchevsky et al. [15,17,18] who showed that the hypercholesterolaemia and atherosclerosis induced by cholesterol-free semi-purified diets is due to several components and/or combinations of components in these diets.

In order to relate the extent of hypercholesterolaemia with the extent of atherosclerosis in a large number of rabbits we had to condense the data into two numerical values for each rabbit. The cholesterol concentration was averaged per animal over the duration of the experiment, and the extent of atherosclerosis was quantified by measuring the total surface area of lipid-staining tissue on the inside of the aorta. Of course, information is lost as a result of such a procedure. Transitory increases in the concentration of serum cholesterol, which are averaged out, and the distribution of cholesterol between the various lipoprotein fractions are both almost certainly critical in the development of the disease. No discrimination is made between plaques of similar area but with different height and morphology. Thus one would not expect a perfect relationship between our parameters for cholesterol and

for atherosclerosis even if plaque formation was determined completely by serum lipoproteins. Inspection of Fig. 1 suggests that a high concentration of cholesterol in serum is a necessary but not a sufficient condition for the development of sudanophilia. Other factors besides hypercholesterolaemia appear to determine to what extent atherosclerosis will actually develop. These factors could be the concentration of cholesterol in certain lipoprotein fractions and/or at certain points in time, they could be genetic, or they could be random events not controlled for in our design. Although care was taken to ensure that the treatments of animals varied only in their diet, complete equality of all experimental conditions at every point in time cannot be guaranteed.

Hypercholesterolaemia in rats

The experiment with rats was performed because rabbits are herbivores, and animal products could conceivably have species-specific effects in rabbits not found in omnivores, such as man. Rats, on the other hand, are omnivorous, and their cholesterol metabolism is somewhat different from that of rabbits.

Opposite effects of casein and soy protein on serum cholesterol levels in rats have been found by several experimenters (reviewed in Terpstra et al. [3]). We showed earlier that casein-induced hypercholesterolaemia in Zucker rats is partly prevented if part of the dietary casein is replaced by gelatin and fish protein [19]. This replacement increased the proportion of glycine, arginine and alanine in the diet. The present results suggest that out of these three, an adequate proportion of glycine is crucial, because addition of pure glycine alone reduced cholesterol concentrations by on average about 50%.

In a study of Aust et al. [20] partial replacement of casein by gelatin caused lower serum triglyceride levels in rats. Cholesterol concentrations, however, were not affected. This is probably explained by the low level of dietary cholesterol and/or the high level of polyunsaturated fatty acids in the diets used by Aust et al. [20]. Casein-induced hypercholesterolaemia is usually observed only on atherogenic diets that are low in polyunsaturated fat and/or high in cholesterol [3]. For that reason we added 1.2% cholesterol to the rat rations. The excess serum cholesterol in the rats was carried mainly by very low density lipoproteins with an abnormally high cholesterol content (Vroomen and Katan, unpublished observations). One might speculate that the amino acid composition of the casein diet caused an increased concentration of very low density lipoproteins, and that these particles contain excess cholesterol only if large quantities of cholesterol are added to the diet. This would explain why Aust et al. [20] observed changes in serum triglycerides (for which normal VLDL is the main vehicle) but not in serum cholesterol.

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