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The phosphorylation state of casein and the species-dependency of its hypercholesterolaemic effect

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1. The present study concerns the question whether the hypercholesterolaemic effect of casein, a phosphorylated protein, is modified in species differing both in glycine–taurine conjugation of bile acids and in intestinal alkaline phosphatase (*EC* 3.1.3.1).

2. Since these two variables are entirely different in rabbits and rats, identical (cholesterol-free) semi-purified diets containing either casein or soya-bean protein were given to both species.

3. In rabbits casein, as compared with soya-bean protein, did not affect calcium absorption but immediately increased phosphate absorption and decreased faecal excretion of bile acids. These effects preceded the accumulation of apo B-cholesterol in serum, which indicates a cause-and-effect relation.

4. In contrast, none of these casein-specific effects were observed in rats.

5. These results suggest that the hypercholesterolaemic potential of casein is expressed mainly in species (like the rabbit) with a low activity of intestinal phosphatase and with a high glycine conjugation of bile acids. This might explain why species (like rat and man) are rather insensitive to dietary casein.

Substitution of casein for soya-bean protein in cholesterol-free, semi-purified diets induces a severe hypercholesterolaemia in the rabbit (for review, see Terpstra *et al.* (1983)). In contrast, other species like rat (Nagata *et al.* 1980; Beynen *et al.* 1983; Terpstra *et al.* 1983) and man (Van Raaij *et al.* 1981; Grundy & Abrams, 1983; Sacks *et al.* 1983) appear to be rather insensitive in this respect. The mechanisms underlying this species-dependent response to dietary casein are not known. However, it has been suggested (Van der Meer, 1983) that casein might induce (species-dependent) hypercholesterolaemia because it is a phosphorylated protein (about 40% of its serine residues are esterified with phosphate). The hypothesis is that, in the small intestinal lumen, bile acids can bind to insoluble calcium phosphate, and that this binding is inhibited by dietary casein and phosphopeptides derived from it. Thus, dietary casein would increase the free concentration of bile acids and consequently stimulate the reabsorption of steroids from the gut. Eventually, this may cause an increase in serum cholesterol concentration because of the well-known suppression of hepatic apo B/E receptors (Brown & Goldstein, 1983). It was suggested that the species-dependency of the casein response might be due to differences either in conjugation of bile acids with glycine or taurine, or in activity of intestinal alkaline phosphatase (*EC* 3.1.3.1). This enzyme can dephosphorylate casein (Lorient & Linden, 1976) and, consequently, might prevent the accumulation of phosphopeptides in the intestinal lumen. In addition, it was found that, in vitro, glycine-conjugated dihydroxy bile acids, but not their taurine-conjugated counterparts, bind to insoluble calcium phosphate (Van der Meer & De Vries, 1985). Thus intestinal alkaline phosphatase and glycine–taurine conjugation of bile acids might modulate the interaction of casein with the enterohepatic cycle of bile acids.

In the present study the in vivo relevance of this hypothesis was tested by studying the casein response in rabbits and rats, using the same, cholesterol-free diets. These two species were chosen because they differ entirely in activity of intestinal alkaline phosphatase (McComb *et al.* 1979) and in glycine–taurine conjugation of bile acids (see, for example,

Coleman *et al.* 1979; La Font *et al.* 1985). Since the hypothesis purports that casein-induced hypercholesterolaemia is the consequence of the primary intestinal interaction of casein with calcium phosphate and bile acids, the time-course of these features was studied in both species in order to reveal possible cause-and-effect relations. In rabbits, casein-specific effects on phosphate absorption and on faecal excretion of bile acids preceded hypercholesterolaemia. In contrast, none of these casein-specific effects was observed in rats. These results indicate that species with a high activity of intestinal alkaline phosphatase and with a significant taurine conjugation of bile acids are protected against casein-induced hypercholesterolaemia. Some preliminary results of the present study have been presented at a Dutch symposium (Van der Meer *et al.* 1985*c*).

METHODS

Experimental animals and diets

Eighteen male New Zealand White rabbits (Broekman Institute, Someren, The Netherlands) and twelve male Wistar rats from a random-bred colony (Small Animal Center, Agricultural University, Wageningen) were housed individually in cages located in rooms with a 12 h light–12 h dark cycle and with constant humidity (60%) and temperature (18° for rabbits and 21° for rats). Before the experimental period all animals were fed on the isolated soya-bean-protein-containing diet (Purina Protein 500 E; Ralston Purina Co., St Louis, MO, USA) for 2 weeks. From day 0 of the experimental period twelve rabbits and six rats were fed on the semi-purified diet containing casein (acid casein; DMV, Veghel, The Netherlands) for 4 weeks. During the experimental period the other six rabbits and six rats were maintained on the soya-bean-protein diet. The number of rabbits in the casein group was twice that in the soya-bean-protein group to allow for the variability of the casein response in this species (West *et al.* 1982). The compositions of the (cholesterol-free) diets (g/kg diet) were: casein 210 (or soya-bean protein 208, methionine 2), maize starch 170, glucose 210, coconut fat 90, soya-bean oil 10, sawdust 178 (in the soya-bean diet 180), molasses 50, $CaHPO_4.2H_2O$ 29, NaCl 8 (in the soya-bean diet 6), $MgCO_3$ 3, MgO 2, $KHCO_3$ 18, vitamin mix 12, mineral mix 10. The compositions of the vitamin and mineral mixtures have been described elsewhere (West *et al.* 1982). These diets were prepared and pelleted by the Institute for Animal Nutrition Research (ILOB-TNO, Wageningen) and were stored at 4° during the course of the study. Rabbits were fed on the pelleted diets on a restricted basis (70 g/d). Rats were fed on the same diets *ad lib.*; the pelleted diets were offered in ground form, so that individual food intakes could be measured accurately. Drinking water was also provided *ad lib.*

Animal weights were recorded weekly. At the time points indicated in Fig. 1 (p. 470), food intake was measured and faeces were collected quantitatively for periods of at least 24 h.

Analytical methods

With the animals in the non-fasting state, rabbit blood was taken from a marginal ear vein, whereas rat blood was obtained by orbital puncture under light diethyl-ether anaesthesia. Blood samples were allowed to clot at room temperature for 1–2 h and subsequently serum was obtained after centrifugation (10000 ***g*** for 2 min). Simultaneously, total cholesterol and high-density-lipoprotein (HDL)-cholesterol were measured enzymically using the Monotest and HDL-cholesterol kits (Boehringer Mannheim, FRG). With this latter method apo B-containing lipoproteins are precipitated (Burnstein & Scholnick, 1973). Since in our experiments the contribution of chylomicrons to serum cholesterol is negligible (R. Van der Meer, unpublished observations), the precipitated cholesterol almost

Table 1. *Body-weight and food intake of rabbits and rats fed for 4 weeks on semi-purified diets containing either casein or soya-bean protein*

(Mean values with their standard errors)

Species	Dietary protein	No. of animals	Body-wt (g) Initial Mean	Initial SE	Final Mean	Final SE	Food intake (g/d) Mean	Food intake SE
Rabbit	Casein	12	2102	44	2523	43	70	0
	Soya-bean protein	6	2082	57	2474	47	70	0
Rat	Casein	6	199	8	270	13	18·3	0·6
	Soya-bean protein	6	197	8	267	11	19·2	0·5

exclusively represents cholesterol from the very-low and low-density lipoproteins; for convenience this cholesterol is defined as apo B-cholesterol.

Faecal bile acid excretion was measured as described elsewhere (Van der Meer *et al.* 1985*a*). Briefly, freeze-dried faeces were extracted with a *t*-butanol–water (1:1, v/v) mixture and subsequently bile acids were assayed enzymically (Turley & Dietschy, 1978). The results of this rapid extraction and assay procedure have been validated by comparison with the established extraction and gas–liquid chromatographic procedures for faecal bile acids (Grundy *et al.* 1965).

After wet ashing of diets and faeces, calcium was measured in an atomic absorption spectrophotometer (Perkin-Elmer 503) and phosphate was determined as described by Fiske & Subbarow (1925). The mean Ca and phosphate contents of the diets were 218 (SD 9) and 233 (SD 7) μmol/g respectively, without any significant difference between the casein and soya-bean-protein diets. Net absorption of Ca and phosphate was calculated as the difference between the daily dietary intake and faecal excretion of Ca and phosphate. In all analyses appropriate reference pools and standards were measured simultaneously.

Values are given as means with their standard errors. After analysis of variance, differences between means were tested using Student's *t* test.

RESULTS

Food consumption and body-weight

Values for food consumption and initial and final body-weights of the two groups of rabbits and rats are presented in Table 1. All rabbits consumed their food within 8 h; in rats the type of dietary protein did not affect food intake. Neither did the type of dietary protein influence final body-weight in both species. During the experimental period of 4 weeks mean weight gains of rabbits and rats were 411 (SE 12) and 71 (SE 4) g respectively and no significant dietary effects were observed.

Ca and phosphate absorption

In Fig. 1 the time-course of the effects of casein and soya-bean protein on net Ca and phosphate absorption is given. In rabbits, casein did not affect Ca absorption (Fig. 1(*a*)) but stimulated phosphate absorption almost instantaneously (Fig. 1(*b*)). In the rabbits kept on the soya-bean-protein diet, Ca and phosphate absorption remained essentially constant.

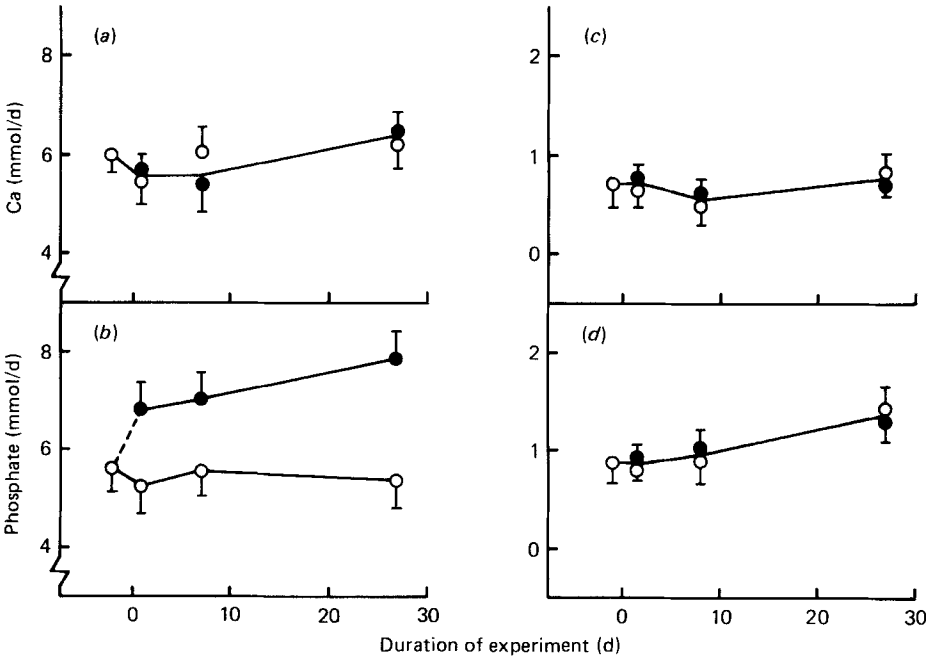

Fig. 1. Time-course of the effects of casein (●—●) and soya-bean protein (○—○) on net absorption of calcium (*a*, *c*) and of phosphate (*b*, *d*) in rabbits (*a*, *b*) and rats (*c*, *d*), fed on the same cholesterol-free, semi-purified diets. Points are means, with their standard errors represented by vertical bars. Different curves indicate a significant ($P < 0{\cdot}05$) effect of the type of dietary protein.

Because of their smaller body-weight, the magnitude of mineral absorption is smaller in rats than in rabbits. Notwithstanding that, net absorption could be accurately measured in rats and showed that also in this species casein did not affect Ca absorption (Fig. 1(*c*)). Neither did casein affect phosphate absorption (Fig. 1(*d*)), which is in remarkable contrast to its effect in rabbits.

Faecal excretion of bile acids and serum cholesterol

The time-course of the differential effects of casein and soya-bean protein on the daily excretion of faecal bile acids and on apo B-cholesterol and HDL-cholesterol is presented in Fig. 2. As shown in Fig. 2(*a*), casein in rabbits immediately inhibited the faecal excretion of bile acids and this effect persisted throughout the experimental period. For some unknown reason soya-bean protein tended to decrease faecal bile acids excretion on day 1, but it should be noted that throughout the experimental period this excretion remained essentially constant. As regards serum cholesterol, casein gradually increased apo B-cholesterol (Fig. 2(*b*)), whereas HDL-cholesterol was not affected and remained essentially constant (Fig. 2(*c*)).

Completely different results were observed in rats fed on the same, cholesterol-free diets. In this species, casein did not inhibit the faecal excretion of bile acids (Fig. 2(*d*)) and serum cholesterol was not affected either (Fig. 2(*e*) and (*f*)).

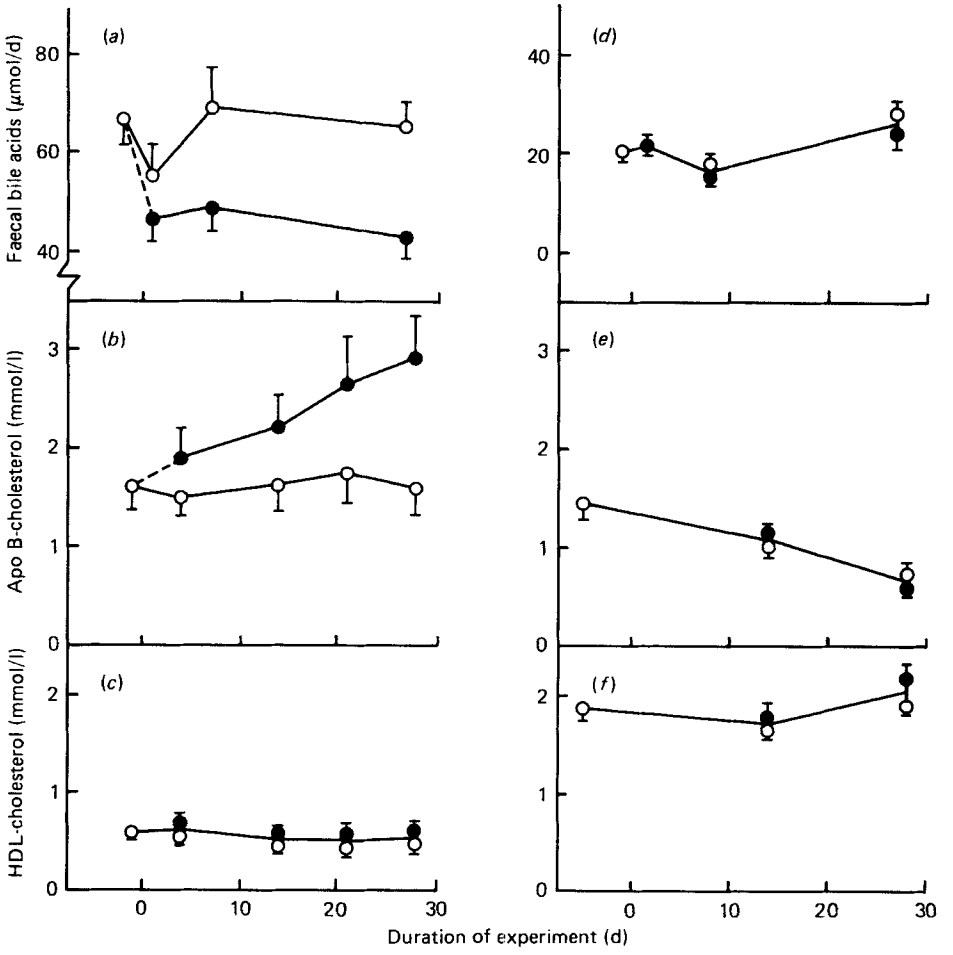

Fig. 2. Time-course of the effects of casein (●—●) and soya-bean protein (○—○) on the faecal excretion of bile acids (*a*, *d*), serum apo B-cholesterol (*b*, *e*) and serum high-density-lipoprotein (HDL)-cholesterol (*c*, *f*) in rabbits (*a–c*) and rats (*d–f*), fed on the same cholesterol-free, semi-purified diets. Points are means, with their standard errors represented by vertical bars. Different curves indicate a significant ($P < 0.05$) effect of the type of dietary protein.

DISCUSSION

Since in the present study both species were fed on the same diets it is evident that the differences observed between rabbits and rats are genuine and are not confounded by species-dependent differences in dietary background. By giving rabbits and humans the same human diets, Van Raaij *et al.* (1981) established that the differentiation in the hypercholesterolaemic effect of casein between these two species is genuine. Thus, specific species-dependent differences in biochemical variables are required to explain this intriguing effect of dietary casein. For this reason, a discussion of the putative effects of intestinal alkaline phosphatase and of glycine–taurine conjugation of bile acids may be appropriate.

In rabbits, the activity of intestinal alkaline phosphatase is low (less than 100 IU/g protein, McComb *et al.* 1979) which may preserve the high degree of phosphorylation of casein and its peptides in the small intestinal lumen. In vitro it has been observed (Reynolds *et al.* 1982; Van der Meer *et al.* 1985*c*) that the phosphoserine residues in casein exchange

with the inorganic phosphate of insoluble calcium phosphate and thus increase the free concentration (activity) of phosphate. In vivo this increased activity of phosphate may explain the casein-induced increase in phosphate absorption (Fig. 1(*b*)). This anion-exchanging property of insoluble calcium phosphate does not necessarily affect the activity of Ca. Therefore, it is not surprising that casein does not stimulate net Ca absorption (Fig. 1(*a*)). In rabbits, bile acids are almost exclusively glycine-conjugated and dihydroxylated (Coleman *et al.* 1979). In vitro these bile acids bind to insoluble calcium phosphate (Van der Meer & De Vries, 1985) and casein inhibits this binding because of its phosphorylation state (Van der Meer *et al.* 1985*c*). This would render, in vivo, more bile acids available for absorption. The casein-induced inhibition of the faecal excretion of bile acids (Fig. 2(*a*)) is in accordance with this suggestion. It should be stressed that, almost instantaneously, the intestinal effects of casein on phosphate absorption and bile acids excretion reach a new steady state, whereas apo B-cholesterol continues to increase (compare Figs. 1(*b*) and 2(*a*) with Fig. 2(*b*)), which indicates a possible cause-and-effect relation. An analogous relation between faecal bile acids excretion and total serum cholesterol has recently also been observed in female rabbits (Kuyvenhoven *et al.* 1986). As amply discussed elsewhere (Beynen *et al.* 1986), the accumulation of apo B-cholesterol in rabbit serum (Fig. 2(*b*)) is then the ultimate result of a down-regulation of hepatic apo B/E-receptors by the reabsorbed bile acids, a mechanism which has been elegantly described by Brown & Goldstein (1983).

Since faecal bile acids excretion is affected neither in rats (Fig. 2(*d*)) nor in humans (Grundy & Abrams, 1983), it is likely that hypercholesterolaemia is absent because the primary, intestinal effects of casein do not occur in these species. Ample evidence is available that rats, fed on cholesterol-free diets, conjugate their bile acids almost exclusively with taurine (see, for example, La Font *et al.* 1985). Since these bile acids do not bind to calcium phosphate (Van der Meer & De Vries, 1985), this could explain why casein does not disturb the enterohepatic cycle in this species. However, neither does casein affect phosphate absorption (Fig. 1(*d*)), which suggests that the intestinal concentration of phosphopeptides is low. This is consistent with our model, because rats do have a high activity (about 700 IU/g protein) of intestinal alkaline phosphatase (McComb *et al.* 1979). In humans, only about 35% of bile acids are glycine-conjugated and dihydroxylated (Hardison & Grundy, 1983; Leiss *et al.* 1984). Also, the activity of intestinal phosphatase is fairly high in humans (about 350 IU/g protein, Keane *et al.* 1983). This activity may prevent the accumulation of phosphopeptides in the small intestine, because dietary casein seems not to affect phosphate absorption in humans (Schuette & Linkswiler, 1982).

Our model, while obviously giving a highly simplified view of sterol metabolism, provides at least some molecular explanation of the differential effects of casein in rabbits, rats and humans. It indicates that the hypercholesterolaemic potential of casein will mainly be expressed in species (like the rabbit) with a low intestinal phosphatase activity and with a high glycine conjugation of bile acids. It may also explain why hypercholesterolaemia in rabbits is aggravated by an increased dietary amount of casein (Terpstra *et al.* 1981) and inhibited by an increased dietary amount of Ca (Van der Meer *et al.* 1985*b*). Whereas dietary casein may decrease the number of binding sites for bile acids on intestinal calcium phosphate, dietary Ca may increase the intestinal amount of calcium phosphate and thus stimulate the faecal excretion of bile acids. Recently, Samman & Roberts (1984, 1987) stressed that protein–mineral interaction is involved in casein-induced hypercholesterolaemia. Whether their hypocholesterolaemic effect of dietary zinc is compatible with our phosphorylation hypothesis requires further investigation. However, it may be of relevance in this regard that Zn binds to phosphoserine residues in casein with very high affinity (Harzer & Kauer, 1982).

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