Effects of whey protein, casein, soya-bean and sunflower proteins on the serum, tissue and faecal steroids in rats*

By C. SAUTIER, K. DIENG, C. FLAMENT, C. DOUCET, J. P. SUQUET and D. LEMONNIER

Unité de Recherches sur la Nutrition et l'Alimentation, U.1. INSERM, Hôpital Bichat, 170 Bd. Ney, 75877 Paris Cédex 18, France

(*Received 18 May 1982 – Accepted 6 December 1982*)

1. Four groups of rats were fed for 49 d on one of four semi-purified diets, without added cholesterol and containing 230 g/kg of the following isolated proteins: casein, whey, soya-bean or sunflower.

2. Whey, soya-bean and sunflower proteins, when compared with casein, decreased the level of serum high-density-lipoprotein (HDL)-cholesterol. These low cholesterol levels were accompanied by an increase in the daily faecal excretion of neutral sterols and bile acids in the case of soya-bean protein, and by a decrease in the liver cholesterol content, when rats were fed on whey protein.

3. Considering the amino acid composition of the four purified proteins, we observed that serum total and HDL-cholesterol levels had a significant positive correlation with tyrosine and glutamic acid, and a negative correlation with cystine and alanine.

4. The present study showed that the hypocholesterolaemic effect of dietary proteins was not related to their animal or vegetable origin.

The hypocholesterolaemic effect of dietary soya-bean protein compared with animal protein was observed in healthy subjects with normal cholesterolaemia (Hodges *et al.* 1967; Carroll, Giovanetti *et al.* 1978), as well as in hypercholesterolaemic patients (Sirtori *et al.* 1975).

In animals, recent studies were made using semi-purified diets containing purified proteins (more than 800 g protein/kg purified protein), rich in sucrose and without cholesterol. In these conditions, the hypocholesterolaemic effect of soya-bean protein compared with casein was confirmed in the rat (Nagata *et al.* 1980; Pathirana *et al.* 1980; Nagata *et al.* 1981). The mechanism has not been elucidated, though two types of hypothesis were proposed: the action of non-protein substances accompanying these proteins or the action of their amino acid composition, or both, on cholesterol metabolism.

Purified vegetable proteins and isolated soya-bean protein may have contained fibre which adsorbed bile acids (Oakenfull & Fenwick, 1978) and decreased cholesterol absorption in the rat (Kritchevsky *et al.* 1974); however, several different types of fibre added to a semi-purified diet failed to prevent the hypercholesterolaemic effect of casein (Carroll, Hamilton *et al.* 1978). In the rat, saponaria saponins increased excretion of faecal steroids (Sautier *et al.* 1979) and decreased serum cholesterol levels (Oakenfull *et al.* 1979). Potter *et al.* (1979) attributed the hypocholesterolaemic effect of whole or hydrolysed soya-bean protein to the presence of saponins. However, the quantity of saponins remaining in very purified soya-bean isolates (3 g/kg) seems insufficient to modify cholesterolaemia. Feeding an amino acid mixture simulating soya-bean protein induced hypocholesterolaemia to the same extent as the protein in the rat (Yadav & Liener, 1977; Nagata *et al.* 1980), but to a lower extent in the rabbit (Huff *et al.* 1977; Huff & Carroll, 1980*b*). In the rabbit the average of atheromata was decreased by arginine and increased by lysine when added to a casein diet (Czarnecki & Kritchevsky, 1979). However, in the rat, the addition of lysine

* This work was supported in part by a grant from the U.E.R. Xavier-bichat, Université Paris VII, and presented in part at the Congress *Lipoproteins and Coronary Atherosclerosis*, Lugano, October 1981.

Table 1. *Composition of the diets*

Ingredients (g/kg)	Diets			
	Casein	Whey protein	Soya-bean protein	Sunflower protein
Sucrose	543	508	531	526
Lactose	0	43	0	0
Isolated proteins (230 g protein)	275	310	287	292
Maize oil	82	82	82	82
Cellulose	58	58	58	58
Salt mixture*	35	35	35	35
Vitamin mixture†	7	7	7	7

* Mineral mixture (mg/kg diet): KH_2PO_4 13615, $CaCO_3$ 13349, NaCl 4875·5, $MgSO_4$ 2005·5, $Fe(SO_4)_3$ 945, $MnSO_4 . H_2O$ 140·35, $Zn(CO_3)_2$ 49, KI 27·3, $ZnSO_4 . H_2O$ 19·18, $CrK(SO_4)_2 . 12H_2O$ 14, $CuSO_4$ 1·60, $CoCl_2$ 0·805, $Na_2SO_3 . 5H_2O$ 0·28.

† Vitamin mixture (mg/kg diet): choline 2000, vitamin E (Roche Rovimix E25) 1200, inositol 500, *p*-aminobenzoic acid 300, nicotinic acid 50, calcium pantothenate 50, retinol-cholecalciferol (Roche Rovimix A-D_3) 20, riboflavin 15, thiamin 15, pyridoxin 10, menadione 10, folic acid 2, biotin 1.

to that diet either decreased cholesterolaemia (Jarowsky & Pytelewski, 1975) or was without effect (Nagata *et al.* 1981), suggesting species differences. Huff & Carroll (1980*b*) could not modify cholesterolaemia in the rabbit by adding various amino acids to isolated soya-bean protein or to casein, but these authors have observed, from the amino acid composition of a large number of dietary proteins used in their laboratory, that cholesterolaemia in the rabbit was related positively to their total content in isoleucine, leucine, threonine, tyrosine and serine and negatively to the sum of arginine, glycine and glutamic acid.

Therefore the amino acid composition of dietary proteins could play a part in the regulation of cholesterolaemia and this has to be thoroughly studied using very purified proteins of known amino acid composition. The aim of the present work was to investigate the effects of four dietary proteins of animal (casein, whey) and vegetable (soya bean, sunflower) origin on the serum, tissue and faecal steroids in male rats. Our results show that the observed changes were not attributable to the origin of the proteins; they are discussed in relation to the proportions of some amino acids.

METHODS

Animals and diets

Thirty-two male Wistar rats, weighing 140 g, were given a commercial diet for 15 d before the start of the experiment. When they weighed $266 \pm 1·8$ g, they were randomized into four groups of eight rats each, transferred to wire-mesh cages and maintained at $22 \pm 2°$ with alternating 12 h periods of light (08·00–20·00 hours) and dark. They were fed on one of the following four semi-purified diets: casein, whey protein, soya-bean protein or sunflower protein (Table 1).

The chemical compositions of the isolated proteins were determined and are given in Table 2. Diets were given *ad lib.* Food consumption and body-weight were recorded once weekly. The rats were isolated into individual metabolic cages for faeces collection on the 44th and 45th days. After 49 d, they were fasted for 12 h, lightly anaesthetized with urethane, and killed by blood withdrawal through the abdominal aorta.

Table 2. *Compositions of isolated proteins*

Amino acids* (mmol/mol)	Casein†	Whey protein‡	Soya-bean protein§	Sunflower protein‖
Aspartic acid	68·5	99·6	111·7	122·1
Threonine	44·7	67·2	41·5	43·0
Serine	69·4	66·2	70·0	59·9
Glutamic acid	182·7	160·7	155·3	164·2
Proline	122·7	68·4	66·6	53·4
Glycine	30·4	31·5	70·1	89·7
Alanine	41·7	73·6	66·3	68·9
Cystine	3·0	23·9	14·5	17·0
Methionine	22·5	22·8	12·8	20·6
Valine	58·3	61·6	40·9	47·4
Isoleucine	39·3	56·1	36·6	36·9
Leucine	90·4	100·0	81·6	69·2
Tyrosine	41·0	19·6	30·0	24·3
Phenylalanine	40·4	23·1	43·5	47·0
Histidine	30·4	15·2	29·9	32·1
Lysine	82·5	87·2	70·0	34·4
Arginine	23·1	14·3	49·4	61·3
Tryptophan	9·0	9·1	8·6	8·6
	Other ingredients¶ (g/kg)			
Isolated proteins	837	785	801	787
(nitrogen factor)	(6·25)	(6·39)	(5·7)	(5·3)
Moisture	58	27	11	13
Fat	10·5	36	37	63
Ash	42	22	34	11
Fibre	0	0	2·5	48

* Chemical analysis using a Technicon amino acid analyser, except for tryptophan which was analysed microbiologically.

† Casein M90; Bel Industries, Paris.

‡ Whey protein S70; Bel Industries, Paris.

§ Soya-bean protein 610; Purina Protein Europe, Brussels.

‖ Sunflower protein TDP 51; Institut National de la Recherche Agronomique, Nantes.

¶ Determined in our laboratory. Fibres were measured by the method of Van Soest & McQueen (1973).

Chemical analysis

Blood serum was obtained by centrifugation. Serum total cholesterol and triglycerides were enzymically measured in an automatic analyser (Aba 100; Abbott). Serum high-density-lipoprotein (HDL)-cholesterol was measured in the supernatant fraction obtained after sodium phosphotungstate–magnesium chloride (Boehringer reagent) precipitation of the other lipoproteins by the method of Grove (1979).

Liver and aorta lipids were extracted by the method of Folch *et al.* (1957) and cholesterol determined by the method of Abell *et al.* (1952). Faeces were dried, weighed, powdered and then extracted with ethanol (960 ml/l) as previously described (Sautier *et al.* 1979). Faecal 3-β-hydroxy neutral sterols were determined by an enzymic method (Röschlau *et al.* 1974) using cholesterol esterase (*EC* 3.1.1.13) and cholesterol oxidase (*EC* 1.1.3.6) in an automatic analyser and 3-α-hydroxy bile acids by enzymic reaction (Weber *et al.* 1972).

Statistical analysis

The one-way statistical analysis of variance was performed. Significance was determined using Duncan's multiple range test with a Programma Olivetti 652 (Olivetti, London) (Snedecor & Cochran, 1967).

Table 3. *Energy intake, body-weight, cholesterol content of liver and aorta, after 49 d, in rats fed on the four experimental diets*

(Mean values with their standard errors for eight rats/dietary group)

					Liver				Aorta	
	Energy intake (kJ/rat per d)		Body-wt (g)		Cholesterol (μmol/g)		Total cholesterol (μmol)		Cholesterol (μmol/g)	
Diet	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Casein	347·4	7·96	432	8·9	7·2	$0·37^{ab}$	81·5	$3·57^{a}$	5·1	0·54
Whey protein	325·6	5·96	420	9·9	6·7	$0·25^{a}$	68·7	$2·37^{b}$	4·0	0·17
Soya-bean protein	325·1	7·24	424	12·1	7·9	$0·27^{b}$	82·6	$2·29^{a}$	4·0	0·22
Sunflower protein	339·7	6·00	411	9·4	7·7	$0·16^{ab}$	81·9	$3·47^{a}$	4·8	0·30

Values in the same vertical column not sharing common superscript letters were significantly different: $P < 0·05$.

Table 4. *Serum concentrations of cholesterol and triglycerides in rats given isolated proteins*

(Mean values with their standard errors)

	Serum concentration (mmol/l)					
	Cholesterol		HDL cholesterol		Triglycerides	
Diet	Mean	SE	Mean	SE	Mean	SE
Casein	3·07	$0·174^{a}$	1·64	$0·058^{A}$	1·05	0·158
Whey protein	2·27	$0·137^{b}$	1·19	$0·064^{B}$	0·82	0·101
Soya-bean protein	2·53	$0·206^{ab}$	1·28	$0·092^{B}$	1·00	0·073
Sunflower protein	2·60	$0·135^{ab}$	1·32	$0·067^{B}$	0·91	0·123

Values in the same vertical column not sharing common superscript letters were significantly different: ^{a,b} $P < 0·05$, ^{A,B} $P < 0·01$.

RESULTS

Daily energy intake and body-weight after 49 d were similar for the rats fed on the four different diets (Table 3). No statistical differences were found for liver, thymus, adrenal, kidney, spleen and adipose-tissue weights. Compared with the casein and vegetable-protein diets, the whey-protein diet decreased the liver cholesterol content. The aorta cholesterol levels were unchanged in the four groups of rats. Serum cholesterol levels were higher in rats fed on the casein diet compared with those fed on the whey-protein diet as shown in Table 4. An increase in the level of HDL-cholesterol was observed in the group of rats fed on casein compared to the three other groups. Serum triglycerides levels were similar in all the groups.

There was no difference between the groups in the daily faecal excretion of dry matter and lipids (Table 5). Neutral sterol excretion was higher in rats fed on the soya-bean-protein diet than in those fed on the other three diets. Compared with the casein and sunflower-protein diets, the soya-bean-protein diet increased the faecal bile acid excretion; the level of excretion, observed when whey protein was given, was intermediary and not different from the other three groups.

Comparing the amino acid concentration of the proteins with the serum total and

Table 5. *Daily faecal excretion at day 45 in rats fed on the four experimental diets*
(Mean values with their standard errors)

	Faecal excretion (/rat per d)							
	Dry wt (g)		Lipids (mg)		Neutral sterols (μmol)		Bile acids (μmol)	
Diet	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Casein	1·33	0·083	170·5	15·86	21·98	$1·659^{a}$	18·19	$1·804^{a}$
Whey protein	1·58	0·099	171·0	20·83	20·68	$0·542^{A}$	22·61	$1·085^{ab}$
Soya-bean protein	1·48	0·067	160·1	6·75	28·85	$2·084^{B}$	24·71	$1·811^{b}$
Sunflower protein	1·48	0·137	146·5	13·81	19·46	$1·855^{A}$	18·08	$1·655^{a}$

Values in the same vertical column not sharing common superscript letters were significantly different: ^{a,b} $P < 0·05$, ^{A,B} $P < 0·01$.

HDL-cholesterol levels we observed positive correlations with tyrosine and glutamic acid and negative correlations with cystine and alanine concentrations. These correlations were significant at $P < 0·01$ for the serum total cholesterol level and $P < 0·001$ for the HDL-cholesterol level. However, when the casein was not considered, all these relations were not significantly different.

DISCUSSION

The serum cholesterol level increased when rats were fed on casein compared with those fed on whey protein. As expected, cholesterolaemia was higher with the casein diet compared with the vegetable-protein diets. Thus casein differed from the other three proteins. The mechanisms which result in the low serum cholesterol levels observed with these three dietary proteins might be different. Indeed, although serum total and HDL-cholesterol levels were identical in the groups fed on whey, soya-bean and sunflower proteins, neutral sterols excretion was increased only in those fed on soya-bean protein. On the contrary, liver cholesterol content was significantly lower with the whey-protein diet than with the other three protein diets. These results could be interpreted as a decrease in the renewal of liver cholesterol; it has been observed, in the case of diets differing by the nature of the proteins, that the liver cholesterol concentration and synthesis measured by specific activity changed in the same direction (Huff & Carroll, 1980*a*). In our study, calculated cholesterol ingestion was small and ranged from 0·4 to 3·4 mg/d depending on the diet. Huff & Carroll (1980*a*) showed that in the rabbit 19 mg cholesterol added to a diet did not modify the quantity of absorbed cholesterol; therefore it appears that the differences in the cholesterol contents of our diets were not high enough to have an effect on liver metabolism. Our whey-protein diet contained 43 g lactose/kg diet where the lactose replaced sucrose. This quantity seemed too small to interfere with steroid metabolism, since it has been demonstrated that lactose concentrations of 300 g/kg were needed to modify neutral and acid steroids in the rat (Wells *et al.* 1960; Ahrens *et al.* 1968). Recently, Wostmann & Bruckner-Kardoss (1980) showed that, with 100 g lactose/kg diet, the only observed effect was a small change in the secondary excretion of bile acids. These authors even observed an increase in serum cholesterol levels induced by lactose in the gerbil.

The increment of faecal steroids that we observed in rats fed on the soya-bean-protein diet, compared with those fed on the other three diets, agrees with our previous study (Sautier *et al.* 1979) and with the results of Nagata *et al.* (1981). We calculated that the

daily intake of 3-β-hydroxy phytosterols was approximately 14 mg/d in the four groups of rats. This was almost entirely attributable to maize oil. The amount of these sterols contained in the other dietary components could be considered as negligible. We can calculate that the endogenous neutral sterols excretion amounted to 14 mg/d in rats fed on dietary soya-bean protein and 6–8 mg/d in rats fed on the other three diets. These amounts were not very different from those obtained by Nagata *et al.* (1981) who reported values of 8·4 mg/d in rats fed on a soya-bean-protein diet and 4·8 mg/d in rats fed on a casein diet for the sum cholesterol + coprostanol. Thus, in both experiments, the soya-bean-protein diet, compared with the casein diet, increased approximately twofold the endogenous neutral sterols excretion.

Kiribuchi *et al.* (1981) have recently described the presence, in the unsaponifiable fraction of soya bean, of triterpene alcohols which showed a synergistic hypocholesterolaemic effect with phytosterols and increased sterol excretion in rats. Thus their presence could explain the increase of faecal steroids excretion in rats fed on dietary soya-bean protein compared with those fed on the other three diets. However, the isolated sunflower protein had no effect on steroid excretion, although it contained slightly more phytosterols and also the highest level of fibre.

The difference in the amino acid compositions of the four purified dietary proteins used, led us to suggest that the effect on serum total and HDL-cholesterol concentrations in the rat could be related to the ratio (tyrosine + glutamic acid):(cystine + alanine). Bazzano (1969) has already shown that glutamic acid, when added to the diet, was hypercholesterolaemic in the rat but had no action in the rabbit and was hypocholesterolaemic in the gerbil. By contrast, Huff & Carroll (1980*b*) observed an inverse relationship between the glutamic acid content of the dietary proteins and the serum cholesterol concentration. Therefore, it is not surprising that we found amino acid correlations which are different from those obtained by Czarnecki & Kritchevsky (1979) and Huff & Carroll (1980*b*) in the rabbit. Moreover, in the rat, hypercholesterolaemia was accompanied by an increase in HDL level, while intermediate-density lipoprotein and very-low-density lipoprotein fractions were increased in the rabbit (Roberts *et al.* 1979). The relationship between dietary proteins and the variations of serum lipoprotein composition (chiefly of their protein fractions) are currently being studied in our laboratory. The use of purified proteins, with known amino acid compositions, allowed the maintenance of physiological conditions during digestion and absorption, where peptidic chains could play a role. Sirtori (1981) suggested that peptide components with hormone-like activity could modify cholesterol metabolism.

In conclusion, our results show that whey protein, compared with casein, decreased serum total and HDL-cholesterol levels without modifying faecal steroids excretion. Thus , the hypocholesterolaemic effect of dietary proteins was not always related to their vegetable origin. Sunflower and soya-bean protein, compared with casein, lowered the serum HDL-cholesterol level. An increase in faecal steroids excretion was observed only in rats fed on a soya-bean protein diet.

Further studies are needed to elucidate the role of the amino acid composition and structure of dietary proteins on the composition of serum lipoproteins and the metabolism of cholesterol.

REFERENCES

Abell, L. L., Levy, B. B., Brodie, B. B. & Kendall, F. E. (1952). *J. biol. Chem.* **195**, 357.
Ahrens, R. A., Wells, S. S., Adams, Y. L., Taylor, R. P. & Kelley, D. L. (1968). *J. Nutr.* **95**, 303.
Bazzano, G. (1969). *Proc. Soc. exp. Biol. Med.* **131**, 1463.
Carroll, K. K., Giovanetti, P. M., Huff, M. W., Moase, O., Roberts, D. C. K. & Wolfe, B. M. (1978). *Am. J. clin. Nutr.* **31**, 1312.
Carroll, K. K., Hamilton, R. M. G., Huff, M. W. & Falconer, A. D. (1978). *J. Clin. Nutr.* **31**, S.203.

Czarnecki, S. K. & Kritchevsky, D. (1979). *J. Am. Oil Chem. Soc.* **56**, 388A.
Folch, J., Lees, M. & Stanley, G. M. S. (1957). *J. biol. Chem.* **226**, 497.
Grove, T. H. (1979). *Clin Chem.* **25**, 560.
Hodges, R. E., Krehl, W. A., Stone, D. B. & Lopez, A. (1967). *J. clin. Nutr.* **20**, 198.
Huff, M. W. & Carroll, K. K. (1980*a*). *J. Lipid Res.* **21**, 546.
Huff, M. W. & Carroll, K. K. (1980*b*). *J. Nutr.* **110**, 1676.
Huff, M. W., Hamilton, R. M. G. & Carroll, K. K. (1977). *Atherosclerosis* **28**, 187.
Jarowsky, C. I. & Pytelewski, R. (1975). *J. Pharm. Sci.* **64**, 690.
Kiribuchi, M., Miura, K., Tokuda, S. & Kaneda, T. (1981). *XII Int. Congress of Nutrition*, San Diego, USA. Abstr., p. 98.
Kritchevsky, D., Tepper, R. P. & Story, J. A. (1974). *Nutr. Rep int.* **9**, 301.
Nagata, Y., Imizumi, K. & Sugano, M. (1980). *Br. J. Nutr.* **44**, 113.
Nagata, Y., Tanaka, K. & Sugano, M. (1981). *Br. J. Nutr.* **45**, 233.
Oakenfull, D. G. & Fenwick, D. E. (1978). *Br. J. Nutr.* **40**, 299.
Oakenfull, D. G., Fenwick, D. E., Hood, R. L., Topping, D. L., Illman, R. L. & Storer, G. B. (1979). *Br. J. Nutr.* **42**, 209.
Pathirana, C., Gibney, M. J. & Taylor, T. G. (1980). *Atherosclerosis* **36**, 595.
Potter, J. D., Topping, D. L. & Oakenfull, D. G. (1979). *Lancet* **i**, 223.
Roberts, D. C. K., Huff, M. W. & Carroll, K. K. (1979). *Lipids* **14**, 566.
Röschlau, P., Bernt, E. & Gruber, W. (1974). *Z. Klin. Chem. Klin. Biochem.* **12**, 403.
Sautier, C., Doucet, C., Flament, C. & Lemonnier, D. (1979). *Atherosclerosis* **34**, 233.
Sirtori, C. R. (1981). *Lipoproteins and Coronary Atherosclerosis*, Lugano. Abstr., p. 89.
Sirtori, C. R., Agradi, E., Conti, F., Mantero, O. & Gatti, E. (1975). *Lancet* **i**, 275.
Snedecor, G. W. & Cochran, W. G. (1967). *Statistical Methods*, 6th ed., p. 274. Ames, Iowa: Iowa State University Press.
Van Soest, P. J. & McQueen, R. W. (1973). *Proc. Nutr. Soc.* **32**, 123.
Weber, A. M., Chartrand, L., Doyon, G., Gordon, S. & Roy, C. C. (1972). *Clinica chim. Acta* **39**, 524.
Wells, W., Anderson, S. & Quanma, R. (1960. *J. Nutr.* **71**, 405.
Wostmann, B. S. & Bruckner-Kardoss, E. (1980). *J. Nutr.* **110**, 82.
Yadav, N. R. & Liener, I. E. (1977). *Nutr. Rep. int.* **16**, 385.

Printed in Great Britain