Effects of soya-bean protein and casein on serum cholesterol levels in rats

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1. The effect of the soya-bean protein isolate and casein, both given 200 g/kg diet for 3–4 weeks, on serum cholesterol was compared in male rats.

2. Soya-bean protein exerted a hypocholesteraemic effect only in a cholesterol-free low-fat (10 g maize oil/kg) diet, when the lowering action appeared independent of the strain of the rat or the feeding pattern. The results obtained with diets containing cholesterol or higher levels of fats or both showed no definite pattern of response.

3. Although the decrease in serum cholesterol appeared greater in α-lipoproteins than in β-lipoproteins, the proportion of the former to total cholesterol remained almost unchanged. The concentration of serum apo A-I was significantly lower in rats given the vegetable protein.

4. Rats given soya-bean protein excreted significantly more neutral sterols.

5. The serum amino acid pattern did not reflect the difference in dietary protein. Addition of cholesterol to the diets modified the serum aminogarm, the decrease in threonine being most marked in both protein groups.

6. This study shows that the hypocholesteraemic action of soya-bean protein is easily modified by the type of diet.

Recently, the effects of dietary protein on hypercholesterolaemia and atherosclerosis have received much attention. Carroll and co-workers (Carroll & Hamilton, 1975; Hamilton & Carroll, 1976; Huff *et al.* 1977) have carried out studies on the effects of animal or vegetable protein on plasma cholesterol levels in rabbits; animal proteins were more cholesteraemic and atherogenic than vegetable proteins. Comparative studies with casein and soya-bean protein have also been performed in rabbit (Belton & Truswell, 1978; Fumagalli *et al.* 1978) and in various other animals such as rat (Yadav & Liener, 1977), chicken (Hevia & Visek, 1979) and swine (Kim *et al.* 1978). In man also vegetable protein appears to be less cholesteraemic than animal protein (Carroll *et al.* 1978; Noseda *et al.* 1979; Sirtori *et al.* 1977, 1979), although Anderson *et al.* (1971) have shown that substitution of animal proteins by vegetable proteins has no significant effects on serum cholesterol levels in healthy adult males.

The mechanism of action by which plant protein exerts its hypocholesteraemic effect is not clear. One possible source of the difference between casein and soya-bean protein might be in their relative amino acid composition. Huff *et al.* (1977) using a low-fat cholesterol-free diet found that in rabbits when the proteins were partially hydrolyzed the differences in cholesteraemia persisted, but that this was not necessarily the situation when component amino acids were fed. In contrast, Yadav & Liener (1977) using an amino-acid-mixture diet high in cholesterol demonstrated a significant difference in the serum cholesterol level of rats. Kritchevsky *et al.* (1978) and Kritchevsky (1979) have paid attention to the difference in the ratio, arginine : lysine; in soya-bean protein it is twice that of casein. There is also a possibility that the actual level of these two amino acids seems to be the determining factor (Kritchevsky, 1979).

It has been suggested for some time that the reduction of plasma cholesterol by vegetable

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proteins is not due entirely to the protein itself but due partly to non-protein materials present in the preparations. Potter *et al.* (1979) have suggested that the hypocholesteraemic action of whole soya-bean protein or protein hydrolysate is attributable, not to the amino acid composition, but to the presence of saponins as previously presumed by Oakenfull & Fenwick (1978).

The present study was undertaken to investigate the effect of casein or the soya-bean protein isolate on serum levels of cholesterol in male rats. Since in many of the preceding studies rather crude soya-bean protein preparations (in some instances no details of preparation were given) were used, the soya-bean isolate which has the same protein content as that of casein (more than 900 g/kg) was used. The fasting serum amino acid profile was also determined in order to elucidate the difference in hypocholesteraemic activity between animal and vegetable proteins.

METHODS

Materials

Casein (vitamin-free; ICN Pharmaceuticals Inc., Cleveland Ohio, USA) and the soya-bean protein isolate (Fujipro R; Fuji Oil Co., Osaka) were used. Both proteins contained 60 g moisture/kg and the nitrogen content (g/kg dry matter) was 14·5 and 14·4 for casein and soya-bean protein respectively.

Animals and diets

Male Wistar rats (Kyudo Co., Kumamoto) were used throughout. In one experiment Sprague Dawley (Japan Cler Co., Osaka) and Donryu (Japan Rat Co., Saitama) rats were also used. The animals were housed individually in an air-conditioned room (22–25°, light period 06.00–18.00 hours and were given commercial pellets (Type NMF; Oriental Yeast Co., Tokyo) at least for 1 week before initiation of experiments. The composition of the basal diet (Sugano *et al.* 1977) was (g/kg): protein 200, mineral mixture 40, vitamin mixture (water soluble) 10, choline chloride 1·5, cellulose powder 20, and sucrose to 1 kg. Fat was added at the expense of sucrose. Vitamin and mineral mixtures (Oriental Yeast Co., Tokyo) were according to Harper (1959). The diet contained (/kg): retinyl palmitate 4000 μg, cholecalciferol 50 μg, DL-α-tocopheryl acetate 100 mg. The high-cholesterol diet contained (g/kg): 10 cholesterol, 2·5 sodium cholate (both added at the expense of sucrose). The present studies were composed of eight experiments in which different levels and kinds of dietary fats were used (g/kg): Expts 1–3 maize oil 50, Expts 4, 7, 8 maize oil 10, Expt 5 hydrogenated coconut oil 150, Expt 6 lard 100. The effects of feeding pattern and of the strain of rats were also examined. Rats were given the diets for 3–4 weeks, fasted overnight (18.00–09.00 hours) and killed by decapitation.

Lipid analysis

Serum and liver lipids were extracted according to the method of Folch *et al.* (1957) and analyzed for cholesterol (Sperry & Webb, 1950), triglyceride (Fletcher, 1968) and phospholipid (Gomori, 1942). Serum β-lipoproteins were precipitated with mepesulfate (Florsheim & Gonzales, 1960) and the concentration of cholesterol in the supernatant fraction (α-lipoproteins) was determined, and β-lipoprotein cholesterol was calculated by difference. The concentration of serum apo A-I was determined by a modification of the rocket immunoelectrophoretic method of Laurell (1966) as described previously (Imaizumi *et al.* 1978).

Faeces were collected for 2 d at the end of Expt 7 and lyophilized. From the unsaponifiable fraction, sterols were precipitated with digitonin. The digitonide was dissolved in dimethyl sulphoxide and sterol was extracted with hexane. Gas–liquid chromatographic

analysis of faecal neutral sterols was carried out as previously reported (Sugano *et al.* 1977) using 5α-cholestane as a calibration standard (Miettinen *et al.* 1965).

Amino acid analysis

Serum free amino acids were determined using an amino acid autoanalyzer (JEOL JLC-6AH, Japan Electron Optical Lab., Tokyo) as described previously (Sugano *et al.* 1978).

RESULTS

Serum and liver lipid concentration

Table 1 summarizes results obtained for weight gain, food intake, liver weight and the concentration of serum and liver lipids. There were no statistically-significant differences in weight gain between rats given different proteins in each experiment. In general, food intake was greater and liver weight was lower in the rats given soya-bean protein than in those given casein.

In rats given diets containing maize oil at 50 g/kg, the concentration of serum cholesterol was virtually the same for both dietary protein groups, regardless of the difference in the method of feeding (Expts 1–3). However, when cholesterol was included in the diet, the response of serum cholesterol showed no definite pattern. No consistent effects of different proteins on serum triglyceride and phospholipid and hepatic lipids could be observed.

When the dietary fat level was reduced to 10 g/kg (Expt 4), soya-bean protein produced a significantly lower serum cholesterol than casein. However, this difference disappeared when cholesterol was added to the diet.

The effect of different types of dietary fat was examined in Expts 5 and 6. When feeding a diet containing hydrogenated coconut oil or lard at 150 and 100 g/kg respectively, there was no difference in the concentration of serum cholesterol between the two dietary protein groups.

These experiments showed that the effect of dietary protein on serum cholesterol of the rat was influenced by the amount of dietary fat, and soya-bean protein had a cholesterol-lowering effect only when a low-fat diet was fed.

Soya-bean protein, in a low-fat diet, produced a significantly lower serum cholesterol level than casein regardless of the difference in the method of feeding (*ad lib.* or paired-feeding; Expts 7–1 and 7–2). In Expt 8, the effect of dietary protein was studied with different strains of rats of the same age and again soya-bean protein produced a cholesterol-lowering effect in Wistar rats. With Sprague Dawley or Donryu rats, the response resembled that found in Wistar rats, but the difference between the two dietary proteins was significant only in Donryu rats. In the experiments with a low-maize-oil diet, hepatic cholesterol tended to decrease when soya-bean protein was fed and the differences were often statistically significant.

As Table 2 shows the low level of serum cholesterol of rats given soya-bean protein was mainly due to a decrease in α-lipoprotein cholesterol, the decrease in β-lipoprotein cholesterol was relatively small. Similar results were obtained when β-lipoproteins were precipitated with heparin and manganese (Warnick & Albers, 1978). However, the proportion of α-lipoprotein cholesterol to total cholesterol was apparently the same between two dietary protein groups. The concentration of serum apo A-I was significantly lower in rats given soya-bean protein.

Faecal excretion of neutral sterols

Table 3 shows that rats given soya-bean protein, when compared with the animals given casein, excreted significantly greater amounts of neutral sterols of endogenous origin, accompanied by the increase in faecal weight. The conversion of cholesterol to coprostanol was not influenced by the type of dietary protein.

Table 1. *Body-weight gain, liver weight and serum and liver lipids of rats given soya-bean protein or casein*

(Mean values with their standard errors for six rats/dietary group except where indicated in parentheses)

Expt no.†	Dietary regimens	Body-wt gain (g)		Food intake (g/d)		Liver wt (g/kg body-wt)		Serum lipids (mg/l)				Liver lipids (mg/g)			
								Cholesterol		Triglyceride		Cholesterol		Triglyceride	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	50MO·S−	138	7	18·6	0·8	30·5	1·0	858	24	1390	91	3·7	0·2	23·9	2·5
	50MO·C−	134	5	16·6	0·8	33·3	0·9	837	62	1550	90	3·5	0·2	21·5	1·8
	50MO·S+	124	6	17·9	0·3	39·8	1·0	1780	198	731	49	63·1	2·8	53·5	8·7
	50MO·C+	129	6	16·5	1·1	45·2	1·9	1800	217	1190	120	57·2	0·2	45·6	3·8
2	50MO·S−	92	2	15·8	0·3	36·1	1·3	684	60	1230	140	2·4	0·1	17·4	0·7
	50MO·C−	111	8	15·7	0·3	38·7	1·8	828	66	1470	90	2·7	0·2	19·5	1·2
	50MO·S+	85	2	15·5	0·3	43·2	0·7	2600	172**	946	85	55·1	3·5	37·4	4·3
	50MO·C+	95	2	14·8	0·7	44·1	0·8	1530	165	1070	58	43·9	4·4	28·6	2·2
3	50MO·S−	119	3	19·0	0·5	29·8	0·7	914	63	1150	65*	3·8	0·1	23·3	2·7
	50MO·C−	120	4	18·0	0·3	33·1	1·6	982	44	1600	167	3·8	0·3	23·3	2·3
	50MO·S+	124	4	20·0	0·7	41·9	1·5	1920	162	948	164	75·3	8·3	69·7	8·8
	50MO·C+	125	2	18·6	0·6	45·4	1·8	2600	434	853	74	76·5	9·6	59·4	4·5
4	10MO·S−	118	5	17·9	0·5	28·8	0·8	764	38*	1270	127	3·4	0·2	20·2	1·1
	10MO·C−	127	3	17·5	0·4	33·2	0·8	934	64	1300	122	3·9	0·2	23·1	0·7
	10MO·S+	124	4	19·5	0·6***	37·8	1·5	1570	164	1020	73	36·5	1·6	32·2	3·4
	10MO·C+	103	6	15·7	0·5	38·9	1·5	2000	445	871	82	37·9	1·5	33·7	3·2
5	150HCNO·S−	106	6	17·1	0·5	30·0	0·9	683	69	1470	250	3·1	0·1	23·5	2·3
	150HCNO·C−	115	6	15·9	0·5	31·3	0·6	625	106	996	130	3·1	0·1	23·2	1·1
	150HCNO·S+	102	5	16·9	0·6	41·8	1·1	1570	221	830	124	69·2	3·5	54·3	3·6**
	150HCNO·C+	97	3	15·3	0·3	44·9	1·4	2330	294	483	167	62·8	5·1	37·7	2·6
6	100LA·S−	157	7	19·3	0·5	29·9	0·6	682	43	1650	154	3·3	0·1	20·9	1·4
	100LA·C−	162	9	17·5	0·6	30·9	0·7	768	30	1600	183	3·6	0·3	19·1	0·7
	100LA·S+	140	4	18·5	0·2	45·4	0·4	1820	178	888	39***	108	3·6	73·3	8·6
	100LA·C+	132	13	16·5	0·7	49·6	1·2	1530	240	1130	74	103	5·1	59·0	6·1

7–1	10MO·S−	153	6	20·0	0·9	29·4	1·2	756	48*	1840	138	3·2	0·1	18·3	0·7
	10MO·C−	148	5	18·2	0·3	31·3	0·3	927	39	1630	137	3·4	0·2	17·5	1·1
7–2	10MO·S−	129	3	18·5	0·1	31·9	1·7	788	31*	1820	154	2·9	0·2**	17·9	0·9
	10MO·C−	132	5	18·3	0·3	32·5	1·8	960	50	1860	165	3·6	0·1	19·3	0·9
8–1	10MO·S−(5)	114	3	17·4	0·4	31·8	0·5**	594	21*	2330	245	3·2	0·1***	18·7	1·4
	10MO·C−(5)	111	5	16·7	0·4	35·1	0·6	754	46	2120	237	3·6	0·0	20·6	1·2
8–2	10MO·S−(5)	142	11	20·8	1·2	30·9	1·1	612	67	1200	184	3·0	0·0***	22·0	1·7
	10MO·C−(5)	143	7	20·0	0·6	32·1	1·2	714	63	1960	427	4·2	0·2	24·1	1·7
8–3	10MO·S−(5)	177	6	22·1	0·5	31·5	0·7	604	40*	953	85	4·3	0·2	31·9	4·3
	10MO·C−(5)	172	4	21·2	0·5	33·4	0·4	793	61	921	70	4·1	0·2	22·0	1·9

MO, maize oil; HCNO, hydrogenated coconut oil; LA, lard; S, soya-bean protein; C, casein; −, cholesterol-free; +, cholesterol added. 10, 50, 100, 150, fat content of diet (g/kg).

Values were significantly different from those for the corresponding casein group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ respectively.

† Male Wistar rats were used throughout except Expts 8–2 (Sprague Dawley) and 8–3 (Donryu). Initial body-weights were 154–164 g except in Expts 8–1 (120 g). Rats were fed diets *ad lib.*, except in Expts 2 and 7–2 (pair feeding) and 3 (pair-weight feeding), for 3 weeks except in Expts 6 and 7 (4 weeks).

Table 2. *Expt* 8. *Concentration of serum lipoprotein cholestrol (mg/l) and apo A-I (μg/ml) in rats given soya-bean protein or casein*

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

		Cholesterol					
		α-Lipoprotein		β-Lipoprotein		Apo A-I	
Rats	Dietary proteins	Mean	SE	Mean	SE	Mean	SE
Wistar	Soya-bean protein	414	22*	180	14	545	24**
	Casein	516	30	238	25	831	37
Sprague–Dawley	Soya-bean protein	407	46	205	21	532	43**
	Casein	484	55	208	29	873	62
Donryu	Soya-bean protein	453	31*	152	12	670	67*
	Casein	612	60	141	7	932	67

Values were significantly different from those for the corresponding casein group: * $P < 0.05$, ** $P < 0.001$ respectively.

Table 3. *Faecal excretion of endogenous neutral sterols in rats fed soya-bean protein or casein*

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

		Faeces† excreted (g/d)		Neutral sterols (mg/d)					
				Coprostanol		Cholesterol		Total	
Expt no.	Dietary proteins	Mean	SE	Mean	SE	Mean	SE	Mean	SE
7–1	Soya-bean protein	2·0	0·1*	2·50	0·26*	1·50	0·45	4·00	0·20**
	Casein	1·4	0·2	1·13	0·18	0·87	0·22	2·00	0·35
7–2	Soya-bean protein	2·0	0·1*	2·50	0·20**	1·20	0·11*	3·70	0·26*
	Casein	1·5	0·1	1·27	0·11	0·70	0·08	1·97	0·11

Values were significantly different from those for the corresponding casein group: * $P < 0.01$, ** $P < 0.001$ respectively.

† Weight of lyophilized faeces.

Serum aminogram

In order to elucidate the mechanism by which soya-bean protein produces the low serum cholesterol level, the serum amino acid pattern was analysed and typical examples of the results obtained are summarized in Fig. 1. The serum aminogram indicated virtually the same pattern in rats given animal or vegetable protein, irrespective of the amounts and types of dietary fat. The addition of cholesterol to the diet caused a characteristic change in the profile, but again the response was essentially the same in both dietary protein groups. Several amino acids, proline, histidine, methionine, arginine and threonine, tended to decrease; the decrease was particularly marked with threonine.

DISCUSSION

The cholesterol-lowering activity of soya-bean protein could not be attributed simply to the decrease in food intake and hence weight gain. Huff *et al.* (1977) and Yadav & Liener (1977), using rabbits and rats respectively, came to the same conclusion. Soya-bean protein exerted a hypocholesteraemic effect only when the diet contained a low level of maize oil, and liver cholesterol also tended to decrease under this dietary treatment. The low-maize-

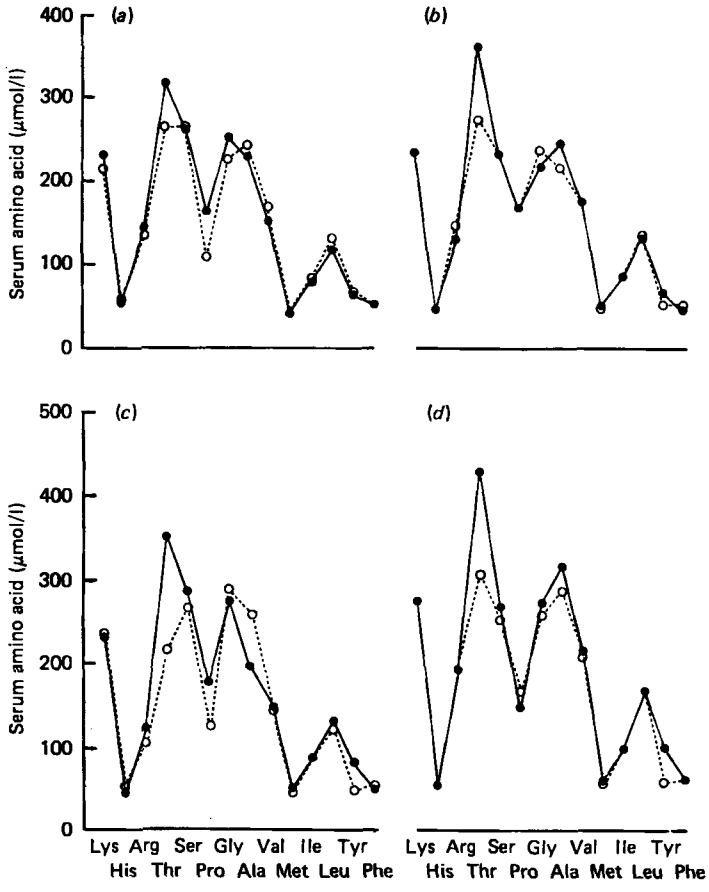

Fig. 1. The serum aminogram of rats given soya-bean protein or casein. Each point represents means of six rats. (*a*), Soya-bean protein (Expt 4); (*b*), casein (Expt 4); (*c*), soya-bean protein (Expt 1); (*d*), casein (Expt 1). ●——●, Cholesterol-free; ○ - - - - ○, cholesterol added. For details of experiments, see Table 1. Cholesterol feeding caused a significant decrease in the concentration of threonine ($P < 0.001$) and proline ($P < 0.05$) in (*c*) and threonine ($P < 0.05$) in (*b*).

oil diet supplied linoleic acid at a minimum of 1·3 % as energy and there was no sign of essential fatty acid deficiency as judged by fatty acid compositions of liver and serum lipids. With 50 g maize oil/kg diet, the hypocholesteraemic effect of soya-bean protein isolate was hardly noticeable. However, Carroll & Hamilton (1975) showed that plant proteins were less cholesteraemic than animal proteins in rabbits when a low-fat diet was ingested, but addition of maize oil to a casein diet largely prevented the increase in plasma cholesterol, thus making the difference small. When the fat source was replaced by hydrogenated coconut oil or lard no cholesterol-lowering action of soya-bean protein was observed. It is thus suggested that the regulatory effect of dietary proteins on serum cholesterol levels in rats is easily modified by the type and amount of dietary fat.

Since the first demonstration by Sirtori *et al.* (1977) on the cholesterol-lowering effect of soya-bean protein in hypercholesteraemic patients, there have been several human studies on this subject (Carroll *et al.* (1978); Noseda *et al.* (1979); Sirtori *et al.* (1979)). Sirtori *et al.* (1977) have found that the hypocholesteraemic effect of soya-bean protein is independent of the lipid composition of the diet. In addition, it appears that the hypocholesteraemic effect of the vegetable protein can be observed with the wide range of dietary fat levels (Carroll *et al.*

(1978); Noseda *et al.* (1979); Sirtori *et al.* (1979)). The basic reason for the apparent difference between human and the experimental animals is not clear.

Garlich *et al.* (1970) using diets consisting of an amino acid mixture demonstrated that glutamic acid had a hypocholesteraemic effect in humans. Huff *et al.* (1977) using a low-fat cholesterol-free diet provided evidence that rabbits given partially-hydrolysed soya-bean protein exhibited a greater cholesterol-lowering tendency than rabbits given the corresponding casein diet, but with an amino acid mixture equivalent to that of casein or soya-bean protein the difference was markedly neutralized. In contrast, Yadav & Liener (1977) have observed with rats given a high-cholesterol diet that the amino acid mixture simulating soya-bean protein unlike that of casein has a significant hypocholesteraemic activity. If amino acid composition is the factor responsible for producing the change in serum cholesterol of rats, then the cholesterol-lowering effect of soya-bean protein is presumably ascribed to absolute or relative excess of specific amino acid(s) so far as the growth of rats is not influenced as in the present study.

In connexion with this assumption, Kritchevsky (1979) has recently pointed out that the ratio, arginine : lysine might be the major factor responsible for causing a change in plasma cholesterol of rabbits given different dietary proteins. The addition of lysine to soya-bean protein enhanced the atherogenecity of the diet containing that preparation, but results with the arginine–casein mixture have not shown a definitive response (Kritchevsky, 1979). Huff *et al.* (1977) have examined the effects of feeding the mixture with different proportions of casein and soya-bean protein isolate in rabbits, no increase in plasma cholesterol was observed with a 50 : 50 (w/w) mixture of these proteins and with a 75 : 25 mixture the level was intermediate to that observed with casein or soya-bean protein alone. Arginine : lysine in the latter mixture was calculated to be 0·64 compared to that of 0·48 for casein. It is not known how this small difference in the ratio influences the plasma cholesterol level.

Our results for the serum aminogram did not give any information about the relationship between the concentration of cholesterol and the type of dietary protein. Dietary supplements of cholesterol markedly reduced some amino acids, especially threonine, indicating that specific amino acid(s) may be closely involved in the metabolism of cholesterol; more threonine may be required when the intake of cholesterol into the body is increased. This response appeared to be influenced by the type of dietary carbohydrate (Sugano *et al.* 1978). In view of these results, the addition of specific amino acid(s) to the diet seems to influence the response of serum cholesterol to different dietary proteins. In contrast with the results of Kritchevsky (1979), Jarowski and co-workers showed that dietary lysine lowered plasma cholesterol of rats (Jarowski & Pytelewski, 1975) and hyperlipidemic subjects (Raja & Jarowski, 1975).

Feeding soya-bean protein resulted in a decrease in intermediate-density (1·006–1·019)-lipoprotein cholesterol in rabbits (Carroll *et al.* 1977) or low-density-lipoprotein cholesterol in humans (Sirtori *et al.* 1977, 1979). In contrast, in rats there was a decrease in high-density-lipoprotein cholesterol after feeding soya-bean protein. This decrease accompanied the decreased level of apo A-I. In this context, differences between these studies in respect of experimental procedures together with a species-specific distribution of cholesterol in serum lipoproteins should be taken into account. The significance of the cholesterol-lowering potential of soya-bean protein in rats should not be underestimated. Fumagalli *et al.* (1978) reported that rabbits given soya-bean protein, in comparison with the animals given casein, excreted more neutral sterols in the faeces, whereas the excretion of bile acids remained unchanged. Our results (Table 3) were consistent with this observation. Feeding soya-bean protein to rabbits results in accelerated oxidation and turnover of labelled cholesterol than that observed with casein (Huff & Carroll, 1977).

On the other hand, there is evidence against the previously-mentioned considerations.

Oakenfull & Fenwick (1978) and Potter *et al.* (1979) ascribed the hypocholesteraemic action of vegetable protein to saponins remaining in the protein preparations. The soya-bean protein isolate used in the present experiment contained essentially no saponins (less than 4 g/kg). It is unlikely that these low levels of saponins influence serum cholesterol, since larger amounts of saponins are usually required to produce a significant hypocholesteraemic effect (Malinow *et al.* 1977). However, soya-bean protein isolate often contains approximately 100 g non-proteinous materials/kg; thus residual components should be considered. Indeed, gas–liquid chromatography revealed that faeces excreted by rats given soya-bean protein, but not casein, contained several unidentified peaks (presumably saponins) in its unsaponifiable fraction. No such peaks were detected when the digitonin precipitable fraction was applied. Further investigation of the mechanism involved in the cholesterol-lowering effect of soya-bean protein is currently in progress in our laboratory.

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